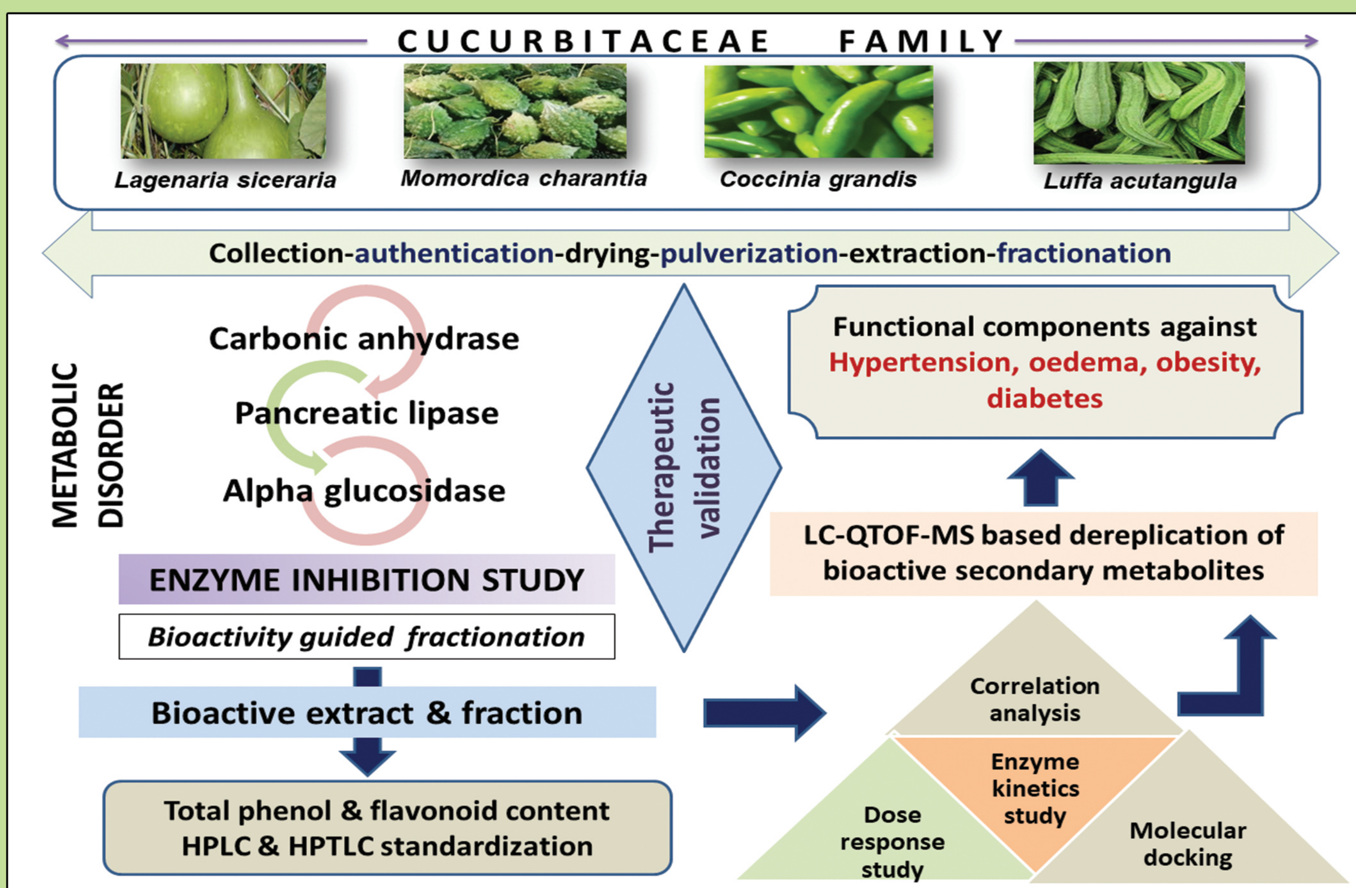
The development of functional foods generates a newer insight in the discovery of molecular targets in the management of hypertension, diabetes, obesity. The cucurbitaceae family contains large number of plants which can be consumed as food as well as medicine. In relation to their ethnomedicinal uses, *Lagenaria siceraria*, *Luffa acutangula*, *Momordica charantia* and *Coccinia grandis* were selected in order to perform standardisation and therapeutic evaluation based on their enzyme inhibiting potential of carbonic anhydrase, pancreatic lipase and alpha glucosidase linked with several metabolic disorders. The application of LC-QTOF-MS based dereplication strategy was found helpful in characterizing the functional components in order to explore the synergism among the bioactive phyto-constituents present in the plants of cucurbitaceae family. Thus the present work highlights on the carbonic anhydrase, pancreatic lipase and alpha glucosidase inhibitory effects of four plants of Cucurbitaceae family.



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Ph.D Thesis

Therapeutic evaluation and standardisation of some medicinal herbs of *Cucurbitaceae* family

Mr. Joydeb Chanda, M. Pharm

2019

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Thesis Submitted By

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3. Carbonic anhydrase inhibitory potential of *Lagenaria siceraria* Stand and identification of its bioactive compounds - An LC-MS/MS Approach in 64th ASMS Conference on Mass Spectrometry and Allied topics, San Antonio, Texas during June 5 - 9, 2016.

4. Exploration of carbonic anhydrase inhibitory activity of *Luffa acutangula* in International seminar on Promotion, Prevention and Pacification: Ayurvedic Landscape organized by J.B. Roy State Ayurvedic Medical College and Hospital, Kolkata during February 9-10, 2016.

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Certificate

This is to certify that the thesis entitled "Therapeutic evaluation and standardisation of some medicinal herbs of Cucurbitaceae family" submitted by Shri. Joydeb Chanda, who got his name registered on 25/11/2014 for the award of Ph.D (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of Prof. Pulok K. Mukherjee that neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

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Declaration

I hereby declare that my research work embodied in this Ph.D. thesis entitled **"Therapeutic evaluation and standardisation of some medicinal herbs of Cucurbitaceae family"** have been carried out by me in the School of Natural Product Studies, Dept. of Pharm. Tech., Jadavpur University, West Bengal, Kolkata, India under the direct supervision of Prof. Pulok K. Mukherjee, Director, School of Natural Product Studies, Dept. of Pharm. Tech., Jadavpur University, West Bengal, Kolkata, India. I also confirm that this work is original and has not been submitted partially or in full for any other degree or diploma to this or other University or Institute.

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(Joydeb Chanda)

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Abbreviations Used

Abb. form	Explanation	Abb. form	Explanation
[E]	Enzyme concentration	LOD	Limit of detection
4-CA	p-Coumaric acid	LDL	Low density lipoprotein
4-NPA	4-nitrophenyl acetate	LP	Lipoprotein lipase
4-NPA	4-nitro phenyl acetate	LQC	Low quality control
bCA II	Bovine carbonic anhydrase II	m/z	Mass to charge ratio
CA II	Carbonic anhydrase II	MQC	Medium quality control
CAI	Carbonic anhydrase inhibitor;	NCD	Non-communicable disease
CFA	Coniferyl alcohol	OD	Optical density
Da	Dalton	ODS	Octa decyl silane
DPPH	2,2-diphenyl-1-picrylhydrazyl.	PDA	Photodiode array detector
Ɛ	Molar extinction coefficient	PDB	Protein data bank
ES	Enzyme substrate	pNPC	p-nitrophenyl caprylate
FA	Ferulic acid	p-NPG	4-Nitrophenyl α-D-glucopyranoside
FFA	Free fatty acid	ppm	Parts per million
Fr	Fraction	RE	Rutin equivalent
GAE	Gallic acid equivalent	RSD	Relative standard deviation
HDL	High density lipoprotein	RT	Retention time
HPLC	High-performance liquid chromatography	S	Slope
HPTLC	High performance thin layer chromatography	TFC	Total flavonoid content
HQC	High quality control	TPC	Total phenol content
IC_{50}	Half maximal inhibitory concentration	U	Unit
ICH	International conference on Harmonization	UV	Ultraviolet
K_{app}	Apparent K_m;	v	Velocity
Ki	Inhibitor constant	VLDL	Very Low Density Lipoprotein
K_m	Michaelis constant;	V_{max}	Maximum velocity
LB Plot	Lineweaver Burk plot	W/A	Wilbur-Anderson
LC-QTOF-MS	Liquid chromatography-quadrupole-time of flight-mass spectrometry	WHO	World Health Organization
LOQ	Limit of quantification	σ	Standard deviation

DEDICATED TO

TO ALL THOSE KNOWN AND UNKNOWN SCIENTISTS WHO SPEND THEIR LIVES IN THE FOUR WALLS OF LABORATORY FOR THE BETTERMENT OF HUMANITY

Abstract

Lifestyle has a significant influence on physical and mental health of human being. The occurrence of several lifestyle-related/non-communicable diseases (NCDs) becoming a major public health challenge in developing countries due to their irregular and sedentary lifestyle. It has been noticed that the functional food ingredients offer significant therapeutic benefits by modulating the well-being of individuals either therapeutically or prophylactically. The development of functional foods generates newer insights into the discovery of molecular targets in the management of these diseases. The combination of diet and medicine also found a suitable treatment approach in hypertension, diabetes, obesity. It was found that plants belonging to cucurbitaceae family exhibits potential therapeutic benefit against these diseases and most of the biological activity of cucurbitaceous plants is attributable from the phenolic glycosides, flavonoids, terpenoids, and minerals, water-soluble polysaccharides, dietary fibers, etc present in it. The work presented in this thesis focuses on standardization, identification of bioactive compounds and elucidates probable enzymatic interaction mechanisms underlying the beneficial effects of some selected plants of cucurbitaceae family. The study was designed to screen four plants of cucurbitaceae family *viz. Lagenaria siceraia*, *Luffa acutangula, Momordica charantia* and *Coccinia grandis* based on their ethnopharmacological relevance found in the literature, against three enzymes *viz.* carbonic anhydrase, pancreatic lipase, α-glucosidase linked with hypertension, edema, obesity, cardiac hypertrophy etc. The study also focuses on the evaluation of enzyme kinetics behavior of all the related enzymes. Further, bioactivity guided fractionation coupled with chromatographic evaluation including was carried out for identification of active phytoconstituents present in the extract and fractions of the plants of cucurbitaceae family. The results of the study have been found useful for phytochemical and therapeutic validation of cucurbitaceae family plants against some metabolic disorders linked with the selected enzymes. Further isolation of chemical compounds followed by *in vivo* animal study will be able to validate the therapeutic claim of the cucurbitaceae plants as functional foods used in the treatment of non-communicable diseases and thus it can help to improve the quality of life.

Chapter 1

1. Cucurbitaceae: A Medicinally important plant family

1.1. Different genus of cucurbitaceae family
1.2. Geographical distribution
1.3. Morphological properties
1.4. Phytochemical profile
1.5. Pharmacological potential
1.6. Nutritional and Economical importance
1.7. Toxicity

1.1. Different genus of cucurbitaceae family

Cucurbitaceae is the largest family of summer vegetable crops, which includes approximately 125 genera and 960 species. Cucurbitaceae family comprises the major vegetable and edible fruit crops in the world. The various parts (fruit, seeds, stems, leaves) of the plants belonging to the cucurbitaceae family are very popular for their uses in culinary purposes from the ancient time. The uses of the plants of this family are also mentioned in Ayurvedic and folk medicine for their several therapeutic importances. The family Cucurbitaceae can be classified into two major subfamilies, Cucurbitoideae, and Zanonioideae based on their morphological, cytological and floral characteristics. Most of the edible varieties are originated from the subfamily Cucurbitoideae which can be further classified into 15 tribes and related genera. Specifically, there are four tribes *viz.* Benincaseae, Cucurbiteae, Momordiceae, Sicyoeae mainly produces edible cucurbitaceae plants in the Indian subcontinent (Renner et al., 2013). The members of the Cucurbiteae tribe produce economically valuable fruits, called gourds, which include crops like squashes (including pumpkins), luffas, and melons (including watermelons) whereas the Benincaseae tribe contains a genus called *Lagenaria*. A details list of different tribes and genera of Cucurbitaceae family has been described in Table 1.1. A lot of medicinal benefits of the family cucurbitaceae have been noticed, among which the most widely cultivated are Wax Gourd (*Benincasa hispida*), Apple Gourd (*Benincasa fistulosa*), Ivy Gourd (*Coccinia grandis*) Ridge gourd (*Luffa acutangula)*, Snake gourd (*Trichosanthes cucumerina*) Spine gourd (*Momordica dioica*), Bitter gourd (*Momordica charantia*). These plants are mainly belongs to the genus *Bennincasa*, *Coccinia* (wight & Arn.), *Luffa* L., *Cucurbita* L., *Cucumis* L., *Citrullus*., *Colocynthis* Mill, *Lagenaria* L., and *Momordica* L. (Dhiman et al., 2012).

1.2. Geographical distribution

The cucurbitaceae family is considered to be the most important plant family supplying edible products and useful fibers to humans for a long time. This family is predominantly tropical, having 90% of the species are found in three main areas; Africa and Madagascar, Central, and South America and Southeast Asia and Malaysia (Saboo et al., 2013; Avinash and Rai, 2017). Specifically, cucurbitaceae family is originated in India and other parts of Western Asia. As India is having a wide range of climatic zones

(mainly tropical and subtropical region), it is found very suitable for their cultivation. The Cucurbitaceous food plants are mostly cultivated in Uttar Pradesh, Uttarakhand, Madhya Pradesh, West Bengal, Gujrat, Bihar, Karnataka, Tamilnadu, Maharashtra (Renner et al., 2013). In addition, there are some other species of the cucurbitaceae family found in the foothills of the Himalaya Mountains, which cannot be cultivated due to their extreme bitterness as well as dormancy in seeds and delayed maturity. Although there are some other varieties of cucurbitaceae plants largely found in China and Middle East, cucumbers were most likely domesticated in Asia.

The plants of cucurbitaceae family grow vigorously in warm, loamy soil mostly enriched with nutrients and moisture. The plants are well grown in warmer temperature (between 75 and 95ºF) which helps in faster germination of cucurbitaceae seeds for maximum plant growth. The well-irrigated, fertilized land with a lot of vertical and horizontal growing rooms is required for the development of most cucurbitaceous plants. Cucurbitaceae plants need an immense amount of heat, long days of light and a lot of moisture to flourish. A fertile and well-drained soil containing clay, sand, and a significant amount of decomposed organic matter should be provided for the rapid growth of the plants. The growth of some cucrbitaceae family plants can be controlled by using some fence, rellis or other vertical structure which in climbing of the vine for their productive growth. It should be recommended to avoid potential frost encounter during its cultivation as this may lead to very detrimental to the cucurbitaceous crop (McCreight, 2016). The geographical distribution of the plants of cucurbitaceae family has been described in Table 1.1.

1.3. Morphological properties

The plants of cucurbitaceae family are mostly annual or perennial weak stem trailing or decumbent vines, usually climbing by means of tendrils and with plenty of juicy sap in the leaves and stems. The roots are taproot, branched get thickened due to storage of food and water. The stem is herbaceous, climbing by means of tendrils or trailing, rooting at nodes, angular. Leaves are alternate brood, usually simple but often deeply lobed or divided and palmately veined, reticulate, petiole long and hollow. Tendrils may be simple or branched arising in the axil or opposite to the leaf at the node (Dhiman et al., 2012). The floral characters of the species exhibit a wide range of inflorescence, variable flowers often solitary, large and showy or sometimes in racemes or cymes or in panides,

unisexual, male and female borne on the same plant (monoecious e.g. Luffa, Cucumis) or on different plants (dioecious e.g., Trichosanthes). The flowers are also regular, unisexual, rarely bisexual, smaller or large showy, white or yellow, epigymous. The male flowers are usually produced in much larger number, campanulate in nature. The calyx is divided into five sepals and fused, pointed, petaloid, campamulate, aestivation is imbricate. The corolla consists of five petals, fused or free (Luffa) often deeply five-lobed, valvate, imbricate, inserted on calyx tube when the free form of corolla may be campanulate or rotate. The androecium consists of 5 stamens, sometimes 3, free or combined to form a central column inserted on the calyx tube. The female flowers are fewer than the male flower. The fruits are soft, fleshy, and generally indehiscent and sometimes of enormous size (Saboo et al., 2013). Some selected plants of cucurbitacea family have been shown in Figure 1.1.

A. Bottle gourd

B. Ridge gourd

C. Bitter gourd

D. Ivy gourd

Figure 1.1. (A-D) Some selected food crops of cucurbitaceae family

1.4. Phytochemical profile

Most of the phytoconstituents present in the plant species can be classified into two groups, primary and secondary metabolites. The primary metabolites are found to have nutritional value whereas the secondary metabolites are responsible for their medicinal properties. It has been observed that, the plants belonging to the cucurbitaceae family are of tremendous medicinally important owing to the presence of a variety of phytoconstituents like phenolics, flavonoids, tannins, cardiac glycosides, carbohydrates, resins, saponins, carotenoids and phytosterols and most importantly triterpenoid cucurbitacins. The details of primary and secondary metabolites have been discussed in the subsequent sections.

1.4.1. Primary metabolites

1.4.1.1. Proteins

The plants of cucurbitaceae family composed of several amino acids and proteins with diverse pharmacological activities. Most of the proteins derived from the plants offer promising activity against some specific pathogen related to fungal infection. On the basis of their nature and biological activity, the anti-fungal proteins are classified as pathogen-related (PR) proteins, ribosomal inactivating proteins (RIP), vicilin like proteins and others (Yadav et al., 2013). PR proteins are classified into different sub-families (PR-1 to PR-5) depending on their amino acid sequences and enzymatic or biological activity. PR-1 is the most abundant protein. PR proteins exhibit strong antifungal and antimicrobial activities which make them essential for the proper development and function of a plant as a whole or in parts (Brederode et al., 1991). The role of RIPs involves in arresting the synthesis of foreign proteins by inactivating fungal ribosome by *N*-glycosidase activity. They are further classified into three sub-groups (Type1, Type 2, and Type 3) on the basis of their structural varieties (Yadav et al., 2013). The two major RIPs, *viz.* hispin, α-momorcharin reported to offer potential antifungal activity against various pathogenic fungal species. Another protein derivatives, charantin, viciline (isolated from *Momordica charantia*) showed anti-diabetic and insulinomimetic activities (Ng et. al., 2002; Wang et al., 2012). Luffangulin, a novel ribosome inactivating peptide was also reported from *Luffa acutangula* with some therapeutic benefits (Wang & Ng, 2002). In addition, several amino acids like methionine, arginine, and tryptophan were reported in plants of cucurbitaceae family.

1.4.1.2. Vitamins

Most of the vegetables, belonging to cucurbitaceae family are a good source of α, β, γ carotene, non-provitamin A carotenoids (*viz.* lutein, violaxanthin, and neoxanthin) (Hemmige et. al., 2017). In specific, *Cucurbita maxima* is a good source of vitamin A (Ragasa et al., 2005), whereas small amounts of vitamin A is present in *Momordica dioica*, *Cucumis sativus* and *Citrullus lanatus* species (Bawara et al., 2010; Abou-Zaid et al., 2001; Koushik et al., 2015). In addition, vitamin C, vitamin-B complex, ascorbic acid, thiamin, riboflavin, niacin, vitamin B-6, folate, DFE have been reported in several species of cucurbitaceae plants (Shah & Seth, 2010). The report has been found that *Tricosanthes cucumerina* (snake gourd), *Coccinia indica* (Ivy gourd) contains some amount of vitamin C (Adebooye, 2008). Some cucurbitaceae plants also contain a trace amount of vitamin K, vitamin E (Avinash and Rai, 2017; Sachin et al., 2018).

1.4.1.3. Minerals

The plants of cucurbitaceae family are a good source of minerals, calcium, iron, magnesium, phosphorus, potassium, sodium, zinc. In specific, *Momordica charantia* leaves are a good source of calcium, magnesium, potassium, phosphorus, and iron (Kumar et al., 2010), whereas *Cucurbita pepo*, contains phosphorus, potassium, magnesium, calcium, iron, zinc etc. The seeds and fruit pulps of *Cucurbita pepo* have been reported to possess high contents of K and Na. (Hashash et al., 2017). The mineral content of the cucurbitaceous crops is very beneficial for their nutritive and health benefits (Avinash and Rai, 2017).

1.4.1.4. Carbohydrates & dietary fibers

Cucurbitaceae crops are good sources of carbohydrates, dietary fibers as nutritional components (Avinash and Rai, 2017). For example, *Cucurbita pepo*, *Cucurbita maxima* seeds contain a high amount (6-10%) of carbohydrates [Assesment report on Cucurbita pepo]. *Cucurbita maxima* contain a significant amount of carbohydrates (Hashash et al., 2017; Saha et al., 2011). In fruit parts of *Citrullus lanatus* contains carbohydrates as major nutritional constituents (Koushik et al., 2015). Some carbohydrates are found in the seed of cucurbitaceae family (Elkhedir et al., 2015). It has been observed that, the fruits of cucurbitaceae plants are an important source of dietary fibers with several

therapeutic benefits against constipation, diabetes, obesity and some related disorders (Saboo et al., 2013).

1.4.1.5. Others

There are several monosaturated, unsaturated and polysaturated fatty acids (*viz.* linoleic acid, oleic acid, stearic acids, myristic acids etc.), fixed oils present in cucurbitaceae plants, particularly in its seed's part. *Cucurbita pepo* seed yields 50% oil (mostly linoleic and oleic acid) whereas the presence of fixed oil and free acids are also reported in *Momordica charantia* seeds (Kumar et al., 2010). *Citrullus colocynthis* seed contains a significant amount of palmitic, stearic and linoleic acid (Dhakad et al., 2017). The trace amount of volatile oils is also present in *Benincasa hispida* fruits (Al Snafi et al., 2013). In addition, some reducing sugar, resins, crude fibers, free acids, lycopene, carotenoids, lutein and zeaxanthin, sterols, tocopherol, are also found in cucurbitaceous plants as nutritional components (Avinash and Rai, 2017).

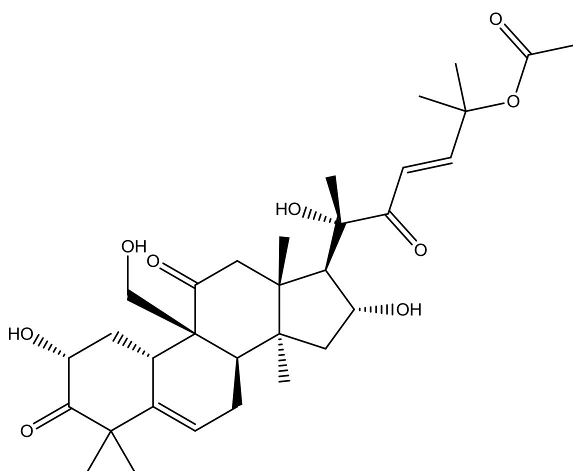
1.4.2. Secondary metabolites

1.4.2.1. Triterpenoids

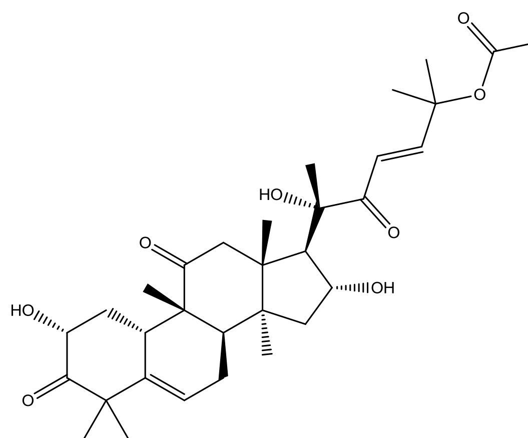
The presence of triterpenoid compounds, cucurbitacins are one of the major characteristics of cucurbitaceae family plants. Cucurbitacin consists of tetracyclic cucurbitane nucleus skeleton with a variety of oxygenation functionalities at different positions with diverse chemical categories. The cucurbitacins are present as non-glycosylated or glycosylated triterpenoids and divided into twelve categories, incorporating cucurbitacins A-T. The structural diversity of the cucurbitacin lies in several unsaturation as well as the presence of numerous keto-, hydroxy- and acetoxy-groups (Chen et al., 2005). The bitter principle of cucurbitaceae plants is observed from the predominance of the cucurbitacins as active constituents. The hydrophobic property of the cucurbitacin nucleus is a major regulating factor for their cytotoxic effects and it increases linearly with their hydrophobicity (Bartalis et al., 2005). In particular, cucurbitacin E and their glycosides are the most widely distributed chemical constituents in food plants of cucurbitaceae family (Dhiman et al., 2012). Cucurbitacins have been reported to possess anti-inflammatory, anti-angiogenic, immunomodulatory, cytotoxic, cytostatic and hepatoprotective properties (Attard and Cuschieri, 2004; Shyam et al., 2010) in both *in vitro* and *in vivo* model. Despite the potential therapeutic activities of

cucurbitacin E and cucurbitacin E glycoside, their chronic exposure is undesirable due to their extremely bitter and disagreeable taste as well as their toxicological effects found in experimental animals (Rupachandra et al., 2013). The different types of structurally diverse cucurbitacins are described as follows:

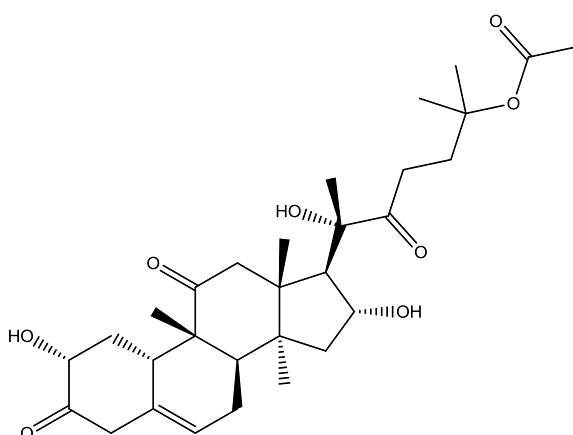
Cucurbitacin A **(1)** is a member of the class of compounds known as cucurbitacins. Cucurbitacins are polycyclic compounds containing the tetracyclic cucurbitane nucleus skeleton, 19-(10->9b)-abeo-10alanost-5-ene (also known as 9b-methyl-19-nor lanosta-5-ene), with a variety of oxygenation functionalities at different positions. Cucurbitacin A is insoluble in water and a very weakly acidic compound. Cucurbitacin A is only found in Cucumis species (cucumber) (Chen et al., 2005)



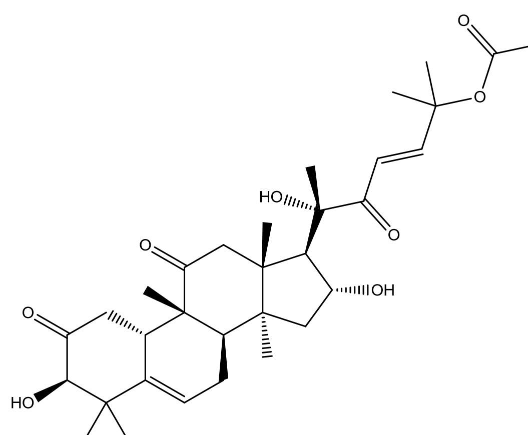
Cucurbitacin A (1)



Cucurbitacin B (2)

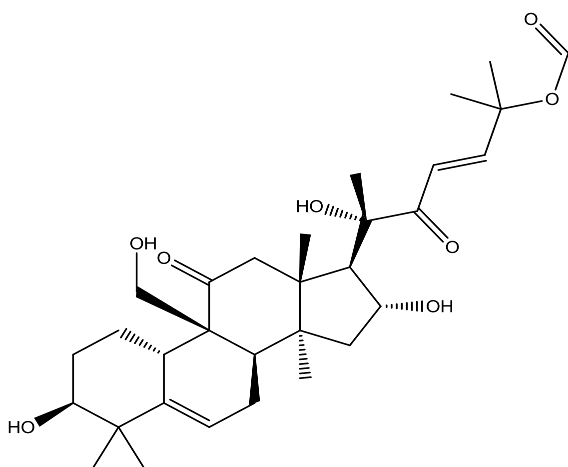


Dihydrocucurbitacin B (3)

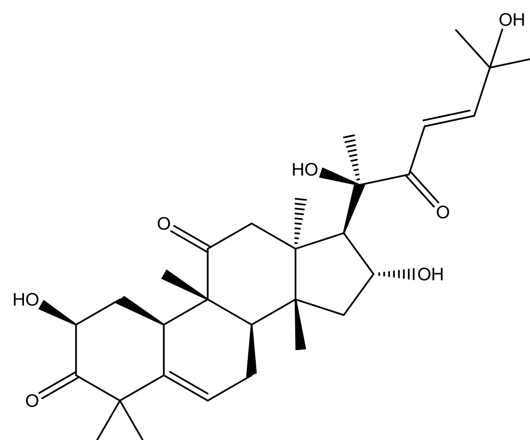


Isocucurbitacin B (4)

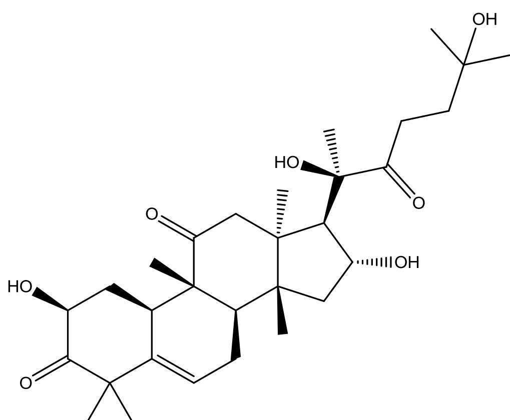
Cucurbitacin B **(2)** is widely present in cucurbitaceae family plants. Most of the cucurbitacin B occurs as dihydrocucurbitacin **(3)**, dihydroisocucurbitacin, isocucurbitacin B **(4)** and their glucosidic form. Cucurbitacin B, iso-cucurbitacin B are reported to possess potent antitumor activity. Cucurbitacin B has been shown to be effective against inflammation and chronic hepatitis (Miro 1995). Cucurbitacin C **(5)** is only identified in *Cucumis sativus* species. It is reported to have antimicrobial activity (Miro, 1995). Cucurbitacin D **(6)** is the most ubiquitously present in cucurbitaceae species, lacks the acetyl group at the 25-OH in its parent structure. Several congeners of cucurbitacin D *viz*. dihydrocucurbitacin D **(7)**, deoxycucurbitacin D **(8)**, epi-isocucurbitacin D have been isolated from plants, found to exhibit potential anti-proliferative and anti-tyrosinase activities.



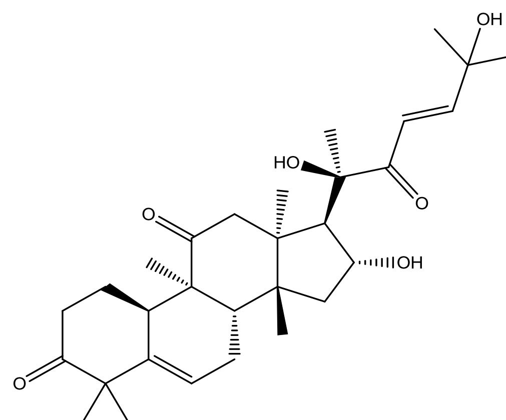
Cucurbitacin C (5)



Cucurbitacin D (6)

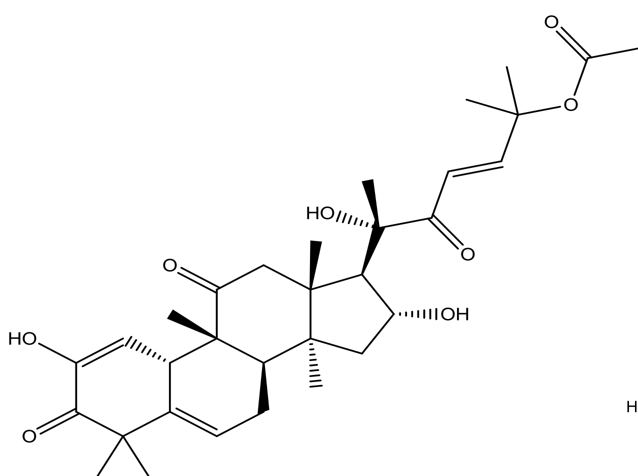


Dihydrocucurbitacin D (7)

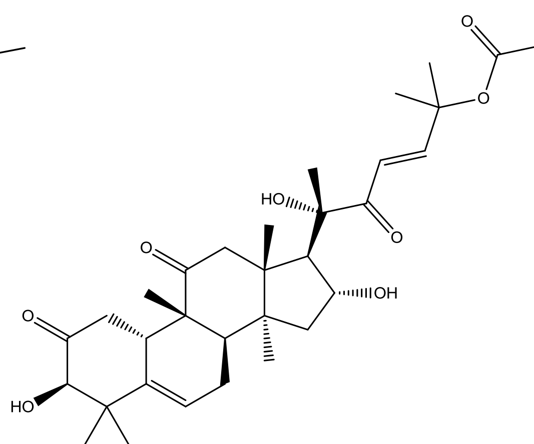


Deoxycucurbitacin D (8)

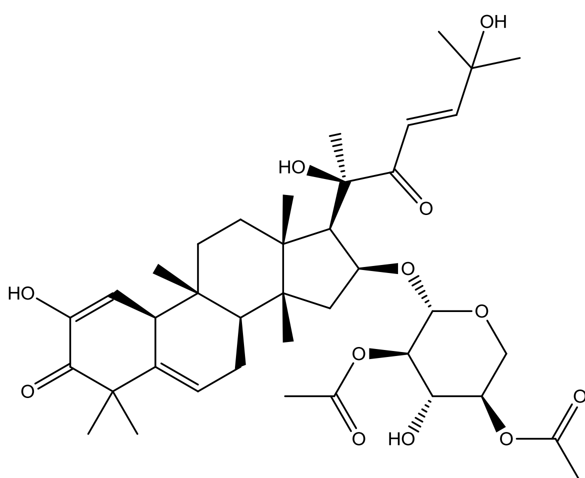
Cucurbitacin E **(9)**, a highly oxygenated triterpene) is considered to be the most important triterpenoids due to its wide range of therapeutic activity. It is the first cucurbitacin isolated from elaterium (thus named as α-elaterin) usually present as monoglucoside (Miro, 1995). Cucurbitcin E is also occurred as dihydrocucurbitacin E, dihydroisocucurbitacin E, isocucurbitacin E **(10)** and their glucosidic form. Cucurbitacin E and its glycosides possess neuroprotective (Arel-Dubeau et al., 2014), anti-inflammatory, antipyretic, antitumor (Abdelkhalek et al., 2017), antiallergic (Yoshikawa et al., 2007), anthelmintic, purgative activity (Miro, 1995).



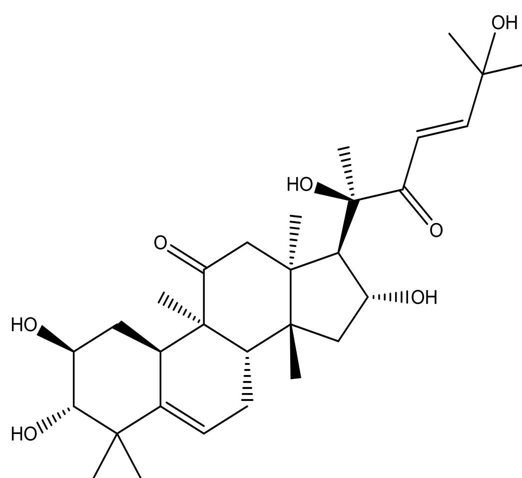
Cucurbitcin E (9)



Isocucurbitacin E (10)



Cucurbitacin I (12)



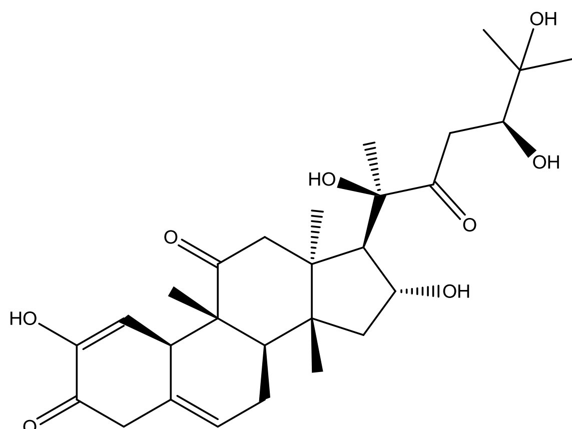
Cucurbitacin F (11)

Momordiciside A (13) **Morgoside V (14)**

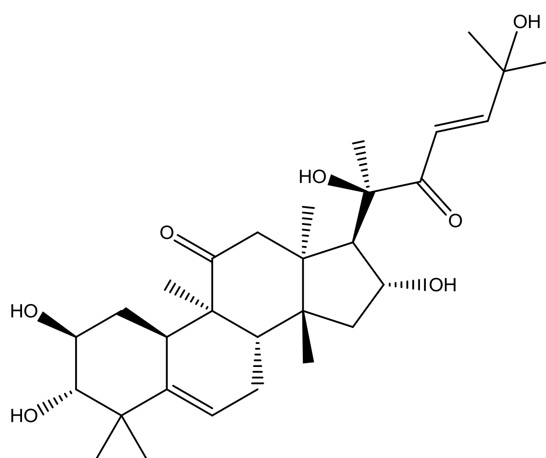
Cucurbitacin F **(11)** is well known in traditional Chinese medicines for their potential pharmacological benefits. It widely occurs as dihydrocucrbitacin F, hexanorcucurbitacin F, oxocucurbitacin F and their glycosidic form (Chen et al., 2005). Some isolated derivatives of cucurbitacin F, *viz.* 23, dihydro and 15-oxo-cucurbitacin F (3), and 15-oxo-23,24-dihydrocucurbitacin F have been reported to possess anti-HIV activity (Konoshima et al., 1994).

Spinosides A (15) **Brydioside A (16)**

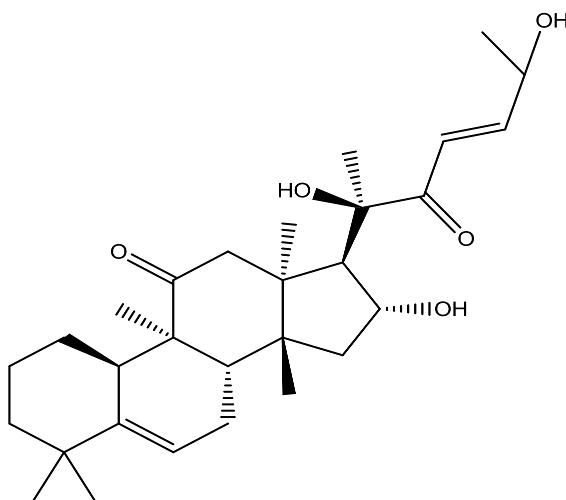
Cucurbitacin I **(12)** is known as (elatericin B), exists as its congener *viz.* hexanorcucurbitacin, 23,24-dihydrocucurbitacin I (Cucurbitacin L), khekadaengosides D, deoxycucrbitacin glycoside (Spinosides A) **(15)** with potential antitumor activity. Several glycosides of Cucurbitacin L (brydioside A) **(16)**, and bryoamaride etc. also isolated from the plants of Cucurbitaceae family.



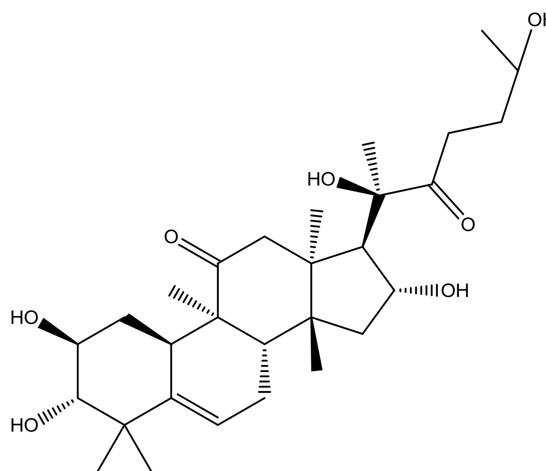
Cucurbitacin J (17)



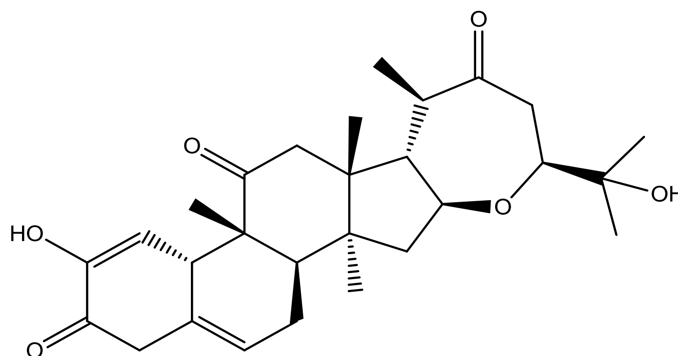
Cucurbitacin O (18)



Cucurbitacin P (19)



Cucurbitacin Q (20)

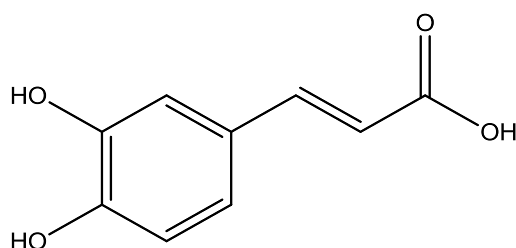


Cucurbitacin S (21)

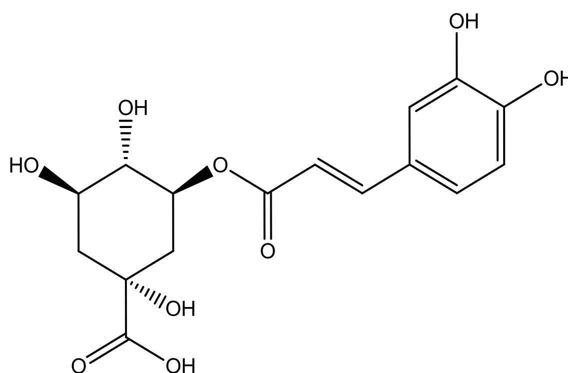
Others Cucurbitacins including J **(17)**, K, O **(18)**, P **(19)**, Q **(20)**, R, S **(21)**, T, and their glycosides have been reported but they are not very therapeutically important. Apart from that, there are a few specific cucurbitacins and cucurbitane glycosides are present in the genus *Momordica*, *viz.* momordicosides A–E **(13)**, momordicines, neomorgoside etc. This class of compounds exhibits potential antimicrobial activity (Miro, 1995) Some sweet cucurbitane glycosides, viz. mogrosides **(14)**, oxomogroside, and their glycosides are reported from the plants of Cucurbitaceae family (Chen et al., 2005).

1.4.2.2. Polyphenols

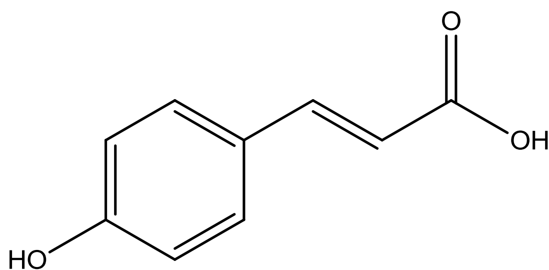
Polyphenols are a class of natural compounds which possesses antioxidant activity by scavenging free-radicals in our body. Phenolic compounds including simple phenols (eg. hydroquinone) and phenolic acids, hydroxycinnamic acid derivatives and flavonoids are considered to be bioactive substances occuring widely in cucurbitaceous plants (Oksana et al., 2012). In has been observed that several hydroxycinnamic acids (mostly derived from caffeic acid) **(22)**, chlorogenic acid **(23)**, *p*-coumaric acid **(24)**, gallic acid, *p*-hydroxybenzoic acid, vanillic acid **(25)**, sinapic acid, ferulic acid etc and their esters and glycosides are present in cucurbitaceae plants, particularly in their fruits. The occurance of flavonoid compounds *viz.* catechins **(26)**, proanthocyanins, anthocyanidins, flavanones **(27)**, isoflavones **(28)**, dihydroflavonols, flavonols, flavan-3-ols **(29)** and their glycosides are also noticed in the plants of this family. Most of these compounds are present as aglycones and their glycosides which can be readily hydrolyzed.



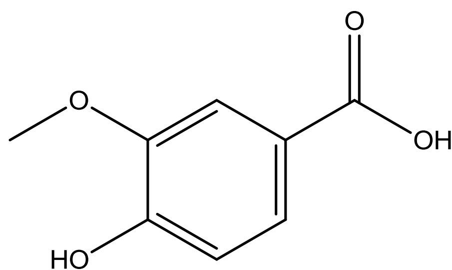
Caffeic acid (22)



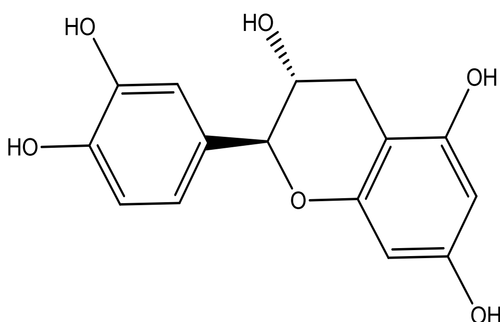
Chlorogenic acid (23)



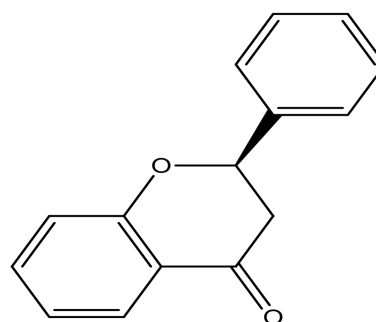
***p*-coumaric acid (24)**



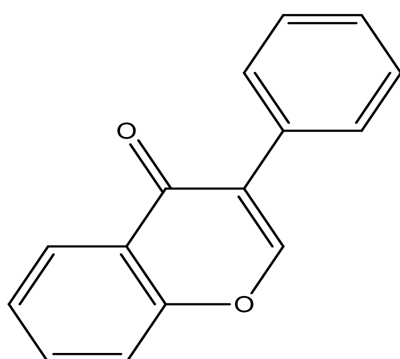
Vanillic acid (25)



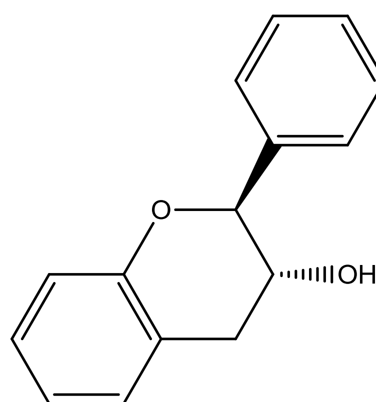
Catechin (26)



Flavanone (27)



Isoflavone (28)

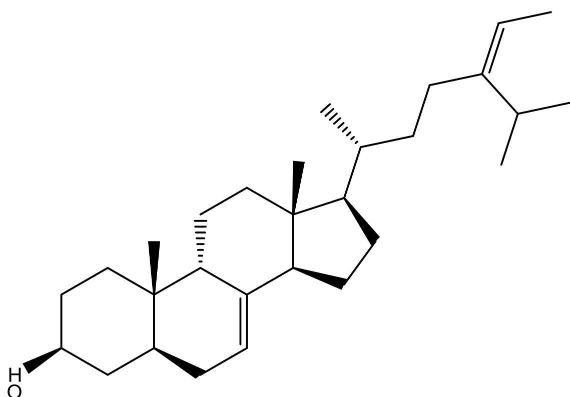


Flavan-3-ol (29)

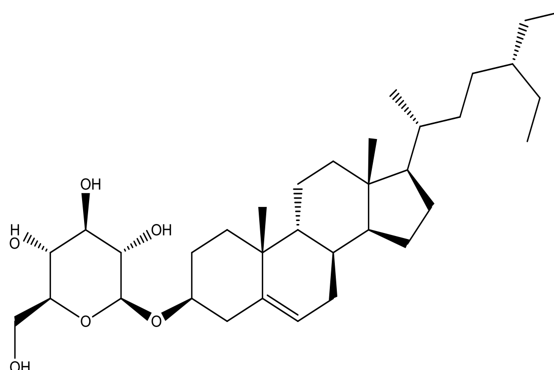
1.4.2.3. Sterols

Most of the sterols and its derivatives are found in the seed part of the cucurbitaceae fruits, mostly in *Benincasa cerifera*, *Cucumis sativus*, *Cucurbita maxima*, *Cucurbita pepo*, *Cucumis sativus*. Sterols mainly occur as 24-ethyl-Δ(7) and Δ(7,22)-sterols as major components, whereas a small amount of saturated and Δ(5)-and Δ(8)-sterols are present

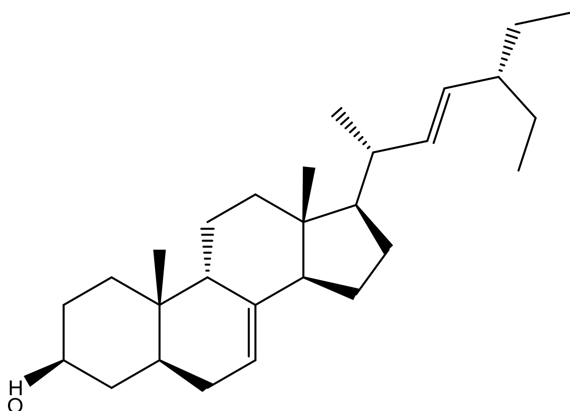
in the seeds. The major sterol compounds reported in the plants are avenasterol **(30)**, sitosterol glucoside **(31)**, spinasterol **(32)**, stigmasterol **(33)**, β-sitosterol, fucosterol, campesterol etc.



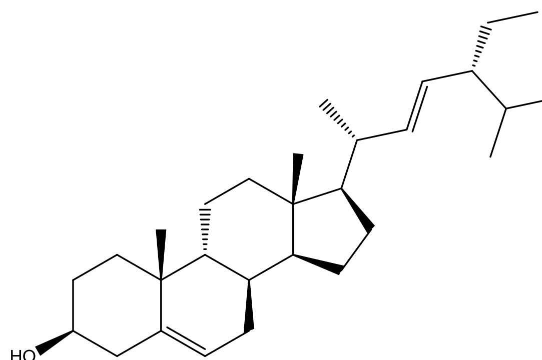
Avenasterol (30)



Sitosterol glucoside (31)



Spinasterol (32)

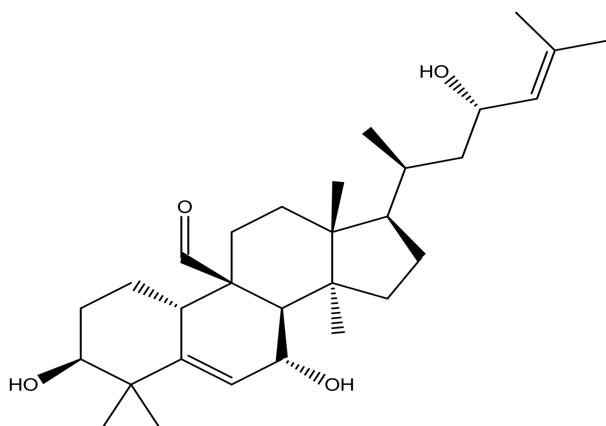


Stigmasterol (33)

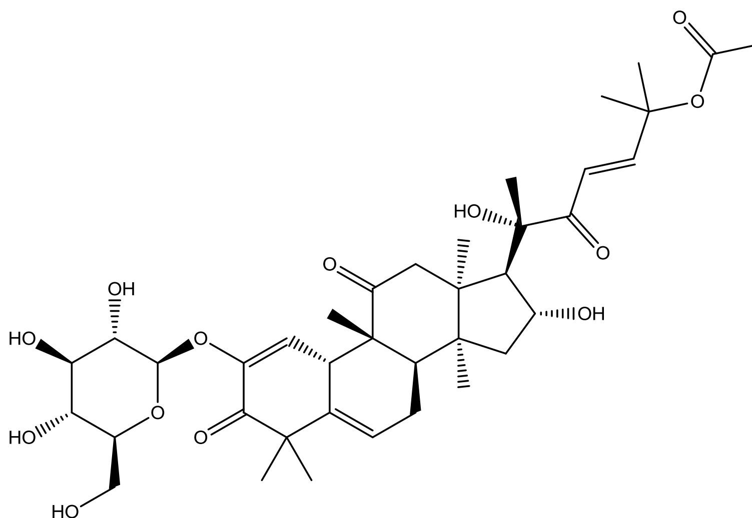
A steroidal glycoside, charantin was isolated from *M. charantia* which exist as an equal mixture of stigmasterol glucoside and β-sitosterol glucoside (Desai & Tatke, 2015). In addition, *Cucurbita pepo*, *Citrullus vulgaris* seeds contain a trace amount of cholesterol (Badr et al., 2011; Hannah and Krishnakumari, 2015).

1.4.2.4. Alkaloids

Among the cucurbitaceae family, the genus *Momordica* contains a significant amount of alkaloids. The alkaloid, momordicine **(34)** was isolated from fruits of *Momordica charantia* reported by Supraja and his co-workers (Supraja et al., 2015). A glycol alkaloid, vicine was reported from the seed extract of *Momordica charantia* (Haixia et al., 2004). One alkaloidal compound 1-tert-butyl-5,6,7-trimethoxy isoquinolene was isolated from the methanolic extract of *Coccinia grandis* (Choudhury et al., 2013). The cytotoxic effect of alkaloid rich fraction of fruits of *Citrullus colocynthis* was studied in MCF-7 and HEPG-2 cell line (Mukherjee and Patil, 2012). A bitter principle colocynthin **(35)** (a mixture of alkaloid and crystalline alcohol) was reported in *Citrullus colocynthis*.



Momordicin I (34)



Colocynthin (35)

Table 1.1: The Tribes and Genera of edible fruits of Cucurbitaceae family (Renner et al. 2013**)**

Tribe	Genus	Wild (W) /Cultivars (C)	Cultivars/ Binomial name	English name	Geographical distribution in India
Benincaseae	*Bennincasa*	C	*Benincasa hispida*	Wax Gourd	Tropical & Subtropical regions of India
			Benincasa fistulosa	Apple Gourd	Punjab, Rajasthan, Uttar Pradesh
Benincaseae	*Citrullus*	W	*Citrullus colocynthis*	Bitter Apple	Andhra Pradesh, Assam, Bihar, Jharkhand, Delhi, Goa, Gujarat, Karnataka, Kerala, Maharashtra, Odisha, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh
			Citrullus lanatus	Watermelon	Andaman & Nicobar Islands, Assam, Bihar, Jahrkhand, Delhi, Gujarat, Karnataka, Madhya Pradesh, Maharashtra, Punjab, Rajasthan, Tamil Nadu, Tripura, Uttar

					Pradesh, Uttarakhand, West Bengal
Benincaseae	*Coccinia*		*Coccinia grandis*	Ivy Gourd	Andhra Pradesh, Assam, Bihar, Kerala, M.P., Maharashtra, Manipur, Odisha, Rajasthan, Tamil Nadu, W.B., U.P., Gujrat,Goa
			Coccinia indica		
			Coccinia palmate		
Benincaseae	*Cucumis*		*Cucumis hystrix*		Arunachal Pradesh, Assam, Meghalaya, Mizoram
			Cucumis indicus		Kerala, Maharashtra
			Cucumis melo	Muskmelon	Andhra Pradesh, Assam, Karnataka, Madhya Pradesh, Maharashtra, Manipur, Rajasthan, Tamil Nadu, Uttar Pradesh.
			Cucumis sativus	Cucumber	Northern India (Ganges region)
			Cucumis javanicus		Assam
			Cucumis ritchiei	Ritchie melon	Karnataka, Kerala, Maharashtra, Punjab, Tamil Nadu

Benincaseae	*Lagenaria*	C	*Lagenaria siceraria*	Bottle gourd	Throughout India
			Lagenaria vulgaris		
Cucurbiteae	*Cucurbita*	C	*Cucurbita ficifolia*	Figleaf gourd	Andhra Pradesh, Arunachal Pradesh, Assam, Bihar, Goa, Gujarat, Karnataka, Kerala Maharashtra, Rajasthan, Tamil Nadu, Tripura, Uttar Pradesh, Uttarakhand, West Bengal
			Cucurbita maxima	Cultivated squash	
			Cucurbita moschata	Crookneck pumpkin	
			Cucurbita pepo	Field Pumpkin	
			Cucurbita argyrosperma	Cashew pumpkin	
Sicyoeae	*Luffa*	W & C	*Luffa acutangula*	Ridge gourd	Native and cultivated throughout India
			Luffa cylindrical	Sponge Gourd	
			Luffa echinata	Bitter sponge gourd	Assam, Bihar, Gujarat, Himachal Pradesh, Madhya Pradesh, Maharashtra, Tamil Nadu, Uttar Pradesh, Uttarakhand, West Bengal
			Luffa graveolens		Bihar, Maharashtra, Sikkim, Uttar Pradesh
Sicyoeae	*Trichosanthes*		*Trichosanthes cordata*		Andhra Pradesh, Arunachal

					Pradesh, Assam, Bihar, Chhattisgarh, Jharkhand, Madhya Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Rajasthan, Sikkim, Tamil Nadu, Tripura, Uttar Pradesh, Uttarakhand, West Bengal
			Trichosanthes cucumerina	Snake gourd	Native and cultivated throughout India
			Trichosanthes dioica	Pointed gourd	Arunachal Pradesh, Assam, Bihar, Delhi, Himachal Pradesh, Jammu & Kashmir, Meghalaya, Punjab, Rajasthan, Uttar Pradesh, West Bengal
			Trichosanthes lobata	--	Andhra Pradesh, Karnataka, Kerala, Puducherry, Tamil Nadu, Uttar Pradesh, West Bengal

			Trichosanthes ovigera	--	Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Meghalaya, Sikkim, Tripura, Uttar Pradesh, West Bengal
			Trichosanthes tricuspidata	--	West Bengal
			Trichosanthes truncata	--	Andhra Pradesh, Arunachal Pradesh, Assam, Meghalaya, Sikkim, West Bengal
Momordiceae	*Momordica*	W	*Momordica balsamina*	Balsam apple	Gujarat, Haryana, Rajasthan
			Momordica charantia	Bitter melon	Western and Eastern Ghats, all over Central and South India
			Momordica cochinchinensis	Gac	Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Bihar, Karnataka, Manipur, Nagaland, Tripura, Uttar Pradesh, West Bengal

			Momordica cymbalaria		Andhra Pradesh, Karnataka, Madhya Pradesh, Maharashtra, and Tamil Nadu
			Momordica dioica	Spine gourd	Deccan plateau and Central India
	Sechium		*Sechium edule*	Winter Squash. chayote	Northeast India

Table 1.2. Phytochemical and pharmacological aspects of some edible plants of cucurbitaceae family

Scientific name	Common name	Major phytoconstituents	Pharmacological activity
Benincasa hispida	Wax Gourd	Pentacyclic triterpene, bryonolic acid, lupeol, beta-sitosterol, cucurbitin, avenasterol, multiflorenol, isomultiflorenyl acetate, stigmasterol, stigmasterol 3-*O*-β-D-glucopyranoside, α-spinasterol 3-*O*-β-D-glucopyranoside, daucosterol, 2,5-dimethyl pyrazine (Han et al., 2013; Ghosh et. al., 2011).	Antiulcer, anti-angiogenic, antioxidant, bronchodilator antipyretic (Qadrie et al., 2009), antidiraheal, antiobesity, antimicrobial (Natarajan et al., 2003) antibacterial, antihistaminic, hypoglycemic (Chakraborty et al., 2018)
Citrullus colocynthis	Bitter Apple	Flavone-C-glycosides, ursolic acid, Cucurbitacin I 2-*O*-beta-D-glucopyranoside, cucurbitacin E 2-*O*-β-D-glucopyranoside, colocynthoside A, B, hexanocucurbitacin I 2-*O*-β-	Antidiabetic, antilipidemic (Hussain et al., 2014a), antibacterial, antimicrobial (Marzouk et al., 2009) analgesic, anti-inflammatory (Pashmforosh et al., 2018), anticancer,

		D glucopyranoside, khekadaengoside E, flavonoid glycosides (isosaponarin), isovitexin (Rajasree et al., 2016; Hussain et. al. 2014; Miao et al., 2012)	cytotoxic (Rezai et al., 2017)
Citrullus lanatus	Watermelon	Vitamin A, C, lycopene, cucurbitacin E, flavonoids, vitamin, thiamine, riboflavin, polyphenols, terpene, steroid, flavonoid, vicilin (Rajasree et al., 2016; Gupta et al., 2018)	Anti-urolithiatic, diuretic (Siddiqui et al., 2018), antimicrobial (Adunola et al., 2015), hepatoprotective, cardioprotective, analgesic, anti-inflammatory (Gupta et al., 2018)
Coccinia grandis	Ivy Gourd	Polyprenol 1, saponin, flavonoids, glycosides, taraxerone, taraxerol, 24R-24-ethylcholest-5-en-3β-ol glucoside, Cephalandrins A and B, β-sitosterol, stigma-7-en-3-one, cucurbitacin B, coccinoside (saponin), flavonoid glycoside, Lupeol, β-amyrin, β-sitosterol (Pekamwar et al., 2013)	Antidyslipidemic (Singh et. al., 2007), mast cell stabilizing, antianaphylactic, antihistaminic (Taur et. al., 2011), analgesic (Hossain et. al., 2014b), antibacterial & cell proliferative (Sakharkar et al., 2017); antiproteolytic, leishmanicidal (Das et al., 2015), antidiabetic, insulinotrophic (Meenatchi et al., 2017)
Coccinia indica	Ivy gourd	Steroids, terpenoids, saponins, flavonoids alkaloids, tannins, glycosides, phenol and mucilage compounds (Pushpa Rani et. al., 2016)	Antihyperglycemic, hypolipidemic (Balaraman et al., 2010), antifungal (Shaheen et al., 2018)

Cucumis melo	Muskmelon	Phenolic glycosides, fatty acids, amino acid, gallic acid, ellagic acid, catechin, quercetin, vanillin, eugenol, vanillic acid, luteolin-7-glucoside, naringenin glycosides , apigenin-7-glycoside, oleuropein, m-coumaric acid, phenylacetic acid, linoleic acid, tocopherols, oleic acid (Mallek-Ayadi et al., 2017, Mallek-Ayadi et al., 2018; Rajasree et al., 2016)	Promote skin hydration, antioxidant, anti-inflammatory (Dhiman et al., 2012), antiproliferative (Rolim et al., 2018)
Cucumis sativus	Cucumber	Glycoside, steroid, saponin, tannin, flavonoid, flavone glycosides (isovitexin, saponarin), acylated flavone-C-glycosides, 9-ß-methyl-19-norlanosta-5-ene type glycosides, cucurbitacins, cucumegastigmanes I and II, cucumerin A and B, vitexin, orientin, isoscoparin 2″-*O*-(6‴-(E)-*p*-coumaroyl) glucoside, apigenin 7-*O*-(6″-*O*-*p*-coumaroylglucoside) (Mukherjee et al., 2013; Rajasree et al., 2016).	Diuretic, antihelmintic, hypolipidemic (Sudheesh et al., 1999), anti-wrinkle, antiaging, (Nema et al., 2011), antimicrobial, antidiabetic (Dixit et al., 2010), hepatoprotective, anti-parasitic (Mukherjee et al., 2013), antiurolithiatic (Pethakar et al., 2017), antimicrobial (Sotiroudis et al., 2010)
Cucurbita maxima	Cultivated squash	Spinasterol, 24-ethyl-5α-cholesta-7, 22, 25-trien-3β-ol, flavonoids, polyphenolics, Cucurbitaxanthin, gibberellin and α-tocopherol, β-carotene, carotenoids, γ-amino butyric acid, 11E-octadecatrienoic acid, polysaccharides,	Antidiabetic, antihyperlipidemic (Sharma et al., 2013), CNS stimulant (Doke et al., 2011), diuretic (Saravanan et al., 2012), immunosuppressive, antitumor, antihypertensive, anti-inflammatory,

		13-hydroxy-9Z octadecatrienoic acid, phenolic glycosides, protocatechuic, caffeic, syringic, vanillic, *p*-coumaric, ferulic, oleic, linoleic, palmitic acids (Muchirah et al., 2018; Rezig et al., 2012)	antibacterial, antihypercholestramia (Rajasree et al., 2016)
Cucurbita pepo	Field pumpkin	Linoleic acid, oleic acid, Δ7-sterols (avenasterol, spinasterol), Δ5-sterol (sitosterol, stigmasterol), triterpenoids, sesquiterpenoids, squalene, tocopherols, hexanorcucurbitane glycosides, lutein, α-carotene, β-carotene, violaxanthin, auroxanthin epimers, flavoxanthin, luteoxanthin, chrysanthemaxanthin, α-cryptoxanthin, β-cryptoxanthin, cucurbitacin I, J, L,K,M (Gutierrez et al., 2016; Rajasree et al., 2016)	Diuretic, antiandrogenic, immunological, anti-inflammatory, hepatoprotective, anti-ulcer, antileprotic (Dhiman et al., 2012; Rajasree et. al., 2016), antibacterial, antioxidant, antitumor, antidiabetic, hypolipidemic (Adnan et al., 2017)
Lagenaria siceraria	Bottle gourd	Cucurbitacins B, D, G and H, saponins, flavone-C-glycoside, polyphenol, campesterol, fucosterol, sitosterol, Lagenin (Prajapati et al., 2010), D: C-Friedooleanane-type triterpenoids (Chen et al., 2008).	Antimicrobial (Dash & Ghosh, 2018), lipase inhibitory activity (Ahmed & Malik, 2017), xanthine oxidase and alpha-amylase inhibitory activities (Ahmed & Malik, 2017), antitumor, cardioprotective, diuretic immunoprotective, antiproliferative, hepatoprotective, CNS depressant, , antihyperglycemic,

			antihyperlipidemic, anticancer, immunomodulatory (Rajasree et. al. 2016; Prajapati et al., 2010).
Luffa acutangula	Ridge gourd	Luffangulin, luffaculin, saponin glycosides, gallic acid, *p*-coumaric acid, ferulic acid, protocatechuic acid, acutoside C, acutoside D, unsaturated aliphatic alcohols , carboxylic acids, fatty acids and their esters (Nagarajaiah et al., 2015; Suryanti et al., 2017)	Hepatoprotective (Jadhav et. al., 2010), antidiabetic, antimicrobial, cytotoxic, antibacterial, immunomodulatory (Manikandaselvi et al., 2016)
Luffa cylindrica	Sponge gourd	Lucyin A, lucyoside, maslinic acid, ginsenosides , luffin P1, luffin S, luffacylin, apigenin-7-*O*-D-glucuronidemethyl ester; luteolin-7- *O*--D-glucuronide, methyl ester, *p*-coumaric acid, chlorogenic acid, caffeic acid (Du et al., 2006; Lucy et al., 2012)	Antiischemic , Antihyperlipidemic, immunomodulatory (Khajuria et al., 2007), antimicrobial (Indumathy et al., 2011), antiviral, antidiabetic (Partap et al., 2012)
Luffa echinata	Bitter sponge gourd	Echinatin, saponin, cucurbitacin B and E, echinatol A and B, oleanolic acid (Dogar et al., 2018)	Antihepatotoxic , analgesic, anti-inflammatory (Dogar et al., 2018; Modi & Kumar, 2014), hepatoprotective (Ahmed et al., 2001)
Momordica charantia	Bitter gourd	Momordicin I, momordicin IV, aglycone of Momordicoside, kuguacin, charantoside, vicine, goyaglycoside, quercetin-, kaempferol- and	Antidiabetic, antihyperlipidemic, anticarcinogenic, hepatoprotective, antiviral potential, wound healing activity, anti-

		isorhamnetin-*O*-glycosides (Jia et al., 2017; Grover et al., 2004)	inflammatory, analgesic (Jia et al., 2017; Grover et al., 2004)
Momordica cochinchinensis	Gac	Hydroxybenzoic acids, hydroxycinnamic, gallic acid, *p*-hydroxybenzoic acid, apigenin, oleic, palmitic, linoleic acids, carotenoids (Ishida et al., 2004), lycopene, momordin, peptides MCoTI-II, (Jittawan et al., 2011; Chuyen et al., 2015; Müller-Maatsch et al., 2017)	Antioxidant, antimicrobial, antiproliferative (Yu et al., 2017), anticancer, provitamin A activity (Chuyen et al., 2015), trypsin inhibitor (Felizmenio-Quimio et al., 2001)
Momordica dioica	Spine gourd	Pleuchiol, momodicaursenol triterpenes of ursolic acid (Ali et al., 1998), hederagenin, oleanolic acid, α-spiranosterol, stearic acid, gypsogenin (Sadyojatha and Vaidya, 1996), steroidal tritrpenoids (Luo et al., 1998)	Hepatoprotective, antihepatotoxic, antidiabetic, antibacterial (Pingle et al., 2018), immunostimulant (Venkateshwarlu et al., 2017)
Trichosanthes cucumerina	Snake gourd	Palmitic, stearic, arachidic, behenic, lignoceric acid, lutein, zeaxanthine, cucurbitacins, coumaric acid, *p*-coumaric acid, caffeic acid, chlorogenic acid (Adebooye et al., 2008)	Antidiabetic (Devi, 2017), antibacterial (Reddy et al., 2013), cytotoxic, anticancer, (Kongtun et al., 2009), gastroprotective (Arawwala et al., 2010)
Trichosanthes dioica	Pointed gourd	Trichosanthin, lectin, euphol, α-amyrin, β-amyrin, butyrospermol, lupeol, taraxerol, betulin, and karounidiol, cucurbitacin B, cucurbitacin E, sterols, steroidal saponin, tannin, flavonoids (Khandaker et	Antihyperglycemic (Rai et al., 2008), antihyperlipidemic (Sharmila et al., 2007), antitumor (Bhattacharya et al., 2011), antiinflammatory (Bhattacharya & Haldar,

		al., 2018)	2013), immunomodulatory (Bhadoriyal & Mandoriya, 2012), wound healing (Shivhare et al., 2010)
Trichosanthes tricuspidata	Bitter snake-gourd	Cucurbitane glycosides (cucurbitacin K 2-*O*-beta glucopyranoside), methyl palmitate, palmitic acid, suberic acid, bryonolic acid, cucurbitacin B, isocucurbitacin B, 3-epi-isocucurbitacin B, 23,24-dihydrocucurbitacin D, isocucurbitacin D, hexanorcucurbitane octanorcucurbitane glycosides (Duvey et al., 2012; Kanchanapoom et al., 2002)	Anthelmintic (Duvey, 2013), antioxidant, antibacterial (Xavier & Dhanasekaran, 2018), antihyperglycemic (Kulandaivel et al., 2013)
Sechium edule	Chayote/ Mirliton squash	Cucurbitacins B and D, cucurbitacin I, vitexin, luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucoside, phloridzin, naringenin, phloretin, apigenin, carboxylic acids and esters, chlorogenic , vanillic, *p*-hydroxybenzoic acid, sterols, amino acids, 3-octadecenoic acid, trilinolenin, α-linolenic acid (Ragasa et al., 2014; Aguiñiga-Sánchez et al., 2017	Antioxidant, α-glucosidase inhibitory, antidiabetic (Sulaiman et.al., 2013), cardioprotective (Neeraja et al., 2015), antiatherosclerotic (Ragasa et al., 2014), hepatoprotective (Firdous et.al., 2012a), antibacterial (Ordonez et al., 2009), antiepileptic, CNS depressant (Firdous et.al., 2012b), antiproliferative (Aguiniga-Sanchez et al., 2017)

1.5. Pharmacological and therapeutic potential

The plants belonging to cucurbitaceae family provide an excellent source of bioactive functional components with various therapeutic importances. The ethnopharmacolgical uses of the cucurbiatceae plants are also well known in Indian traditional medicinal system. The plant extracts and compounds obtained from the cucurbitaceae family are very useful for their extensive biological activity includes antidiabetic, anti-inflammatory, cytotoxic, hepatoprotective, antimicrobial effects etc. Various types of pharmacological and therapeutic potential of cucurbitaceae plants have been discussed in this section.

The effect of several fruit extracts of cucurbitaceae species on central nervous system (CNS) was studied in *in vivo* model. The anxiolytic activity of alcoholic extract of *Benincasa hispida* on various behavioral models was reported by Nimbal and his colleagues (Nimbal et al., 2011). Report has been found that *Lagenaria siceraria* exhibited anti-stress, adaptogenic and CNS depressant property (Pawar et. al., 2009). In another study, *Sechium edule* and *Cucumis sativus* were reported to possess antiepileptic and CNS depressant activity in a dose-dependent manner (Firdous et al., 2012b; Nasrin et al., 2013).

The analgesic and antiinflammatory property of some cucurbitaceous plants *viz. Citrullus colocynthis* (Hussain et al., 2014), *Cucumis melo* (Dhiman et al., 2012), *Momordica charantia* (Ahamad et al., 2017), *Trichosanthes dioica* (Kumar et. al., 2012, Kulkarni & Raghavan, 2013) have been reported by several researchers. It has been found that *Citrullus lanatus*, *Cucurbita maxima* and *Cucurbita pepo* possesses anti-inflammatory potential (Rajasree et al., 2016). In another study, the aqueous and methanolic extract of *Lagenaria siceraria* fruits reported to possess analgesic and anti-inflammatory properties (Shah et al., 2012; Ghule et. al., 2006 (a). The anti-inflammatory activity of *Benincasa hispida* was observed in rat model (Rachchh et. al., 2011) in a dose-dependent manner.

The hypolipidemic activity of several cucurbitaceae plants has been reported till date. The lipid-lowering activity of these plants thus alleviates clinical complications, such as insulin resistance, diabetes, hypertension and dyslipidemia related to lipid metabolism. *Momordica charantia* have a prominent effect on lipid metabolism and thus used to address hyperlipidemia (Nerurkar et al., 2010). The hypolipidemic activity of *Coccinia*

indica was reported in the streptozotocin-induced diabetic rat model (Balaraman et al., 2010). The ethnopharmacological uses of *Citrullus colocynthis* as lipid-lowering agents were also documented in some literatures (Hussain et al., 2014). The alcoholic and aqueous extract of *Lagenaria siceraria* showed potential activity in lowering total cholesterol, triglyceride and low-density lipoproteins and as well as in increasing HDL level (Ghule et. al., 2006b). Some other plants of this family *viz. Cucurbita maxima* (Rajasree et al., 2016), *Luffa cylindrica* (Partap et al., 2012) and *Momordica charantia* (Hossain et al., 2012) also having an antihyperlipidemic effect.

The antihyperglycemic effect of cucurbitaceae species is well reported in the management of diabetes. The globulins isolated from the seeds of *Citrullus lanatus* and *Cucurbita moschata* was found effective as antihyperglycemic compounds (Teugwa et al., 2013). The polysaccharides obtained from some cucurbitaceae species were reported to possess antidiabetic activity (Dabaghian et al., 2012). Some peptides, sterol glucosides, saponins isolated from *M. charantia* exhibits beneficial effects in diabetes management (Raman and Lau, 1996). In another report, *Coccinia indica* exerts hypoglycemic effects by altering the activity of the gluconeogenic enzyme. Some phytocompounds *viz*. ß-sitosterol, and pectin were found as major bioactive components against hyperglycemia (Hossain et al., 1992). Moreover, the fruits of *Sechium edule* and *Benincasa hispida* showed potential antidiabetic effect (Sulaiman et al., 2013; Saeed et al., 2007). The antidiabetic activity of the cucurbitaceae plants attributed to the phenolic compounds present as their active constituents.

The antioxidant potential of cucurbitaceae family plants are observed owing to a large number of phenolic and flavonoid compounds present in it. The higher content of phenolic and flavonoid compounds in cucurbitaceae plants were estimated through Folin-Ciocalteu method, mostly expressed as gallic acid equivalent (for phenols) and rutin equivalent (for flavonoids). The antioxidant potential of fresh and dried fruits of *Lagenaria siceraria* was evaluated (Kubde et al., 2010). A report has been found that *Momordica cochinchinensis* is a reach source of antioxidant constituents (Chuyen et. al., 2015). The polyphenol and flavonoid components exert their antioxidant activity observed from free radical scavenging as well as ferric reducing antioxidant power (FRAP) assay. In 2014, Sabo and his co-workers reported antioxidant activity of the

seeds of eighteen varieties of edible Cucurbitaceae plants (Sabo et al., 2014). It has to be noted that most of the nutritional benefits of these plants are attributable to their phenolic and flavonoid content. Moreover, the antioxidant property of these plants can be largely correlated with their several enzyme inhibition (*viz.* α-glucosidase and lipase) capacities.

The anticancer activity of some dietary plants of cucurbitaceae family has been extensively reported in the literature (Sharma et al., 2015). A large number of cucurbitacin and cucurbitane-type triterpene glycosides isolated from cucurbitaceae plants were found effective as cytotoxic and anti-tumoral agents. Some other triterpenes, α-momorcharin, trichosanthin, cyclic bisdesmosides, gypenosides, ribosome-inactivating protein, trichosanthin etc. obtained from this species possess cytotoxic and antitumoural activity. The antitumor activity of trichosanthin isolated from *Trichosanthes kirilowii* were reported in the literature. Luffins a and b, Luffaculin isolated from *Luffa cylindrica* exerts inhibitory effects on different tumor cell lines by reducing the proliferation of cancer cells (Ng et al., 1992; Méndez-Cuesta et al., 2018). The cytotoxic activity of cucurbitaceous plants *viz. Cucumis sativus*, *Benincasa hispida*, *Coccinia indica, Cucurbita maxima*, and *Luffa acutangula* were studied on HeLa Cell Line (Varalakshmi and Rao, 2012). The seed extract of *Momordica cochinchinensis* showed anti-proliferative effect on human lung cancer (Yu et al., 2017). The aqueous extract of *Momordica charantia* was reported to prevent skin carcinoma (Ganguly & Das, 2000) as well as it possesses antimutagenic activity (Kusamran et al., 1998). The root extract and fruit juice of *Trichosanthes cucumerina* having potential cytotoxic activity (Kongtun et al., 2009). The bitter apple fruit (*Citrullus colocynthis*) also possesses anticancer activity (Hussain et al., 2014).

Some proteins isolated from cucurbitaceae plants were found effective antiviral, antitumor, and antimicrobial agents. The antimicrobial activity of *Momordica charantia* extract was evaluated against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* (Costa et al., 2011). In addition, the anti-HIV activity of α- and ß- momorcharin has also been reported by Au and his co-workers (Au et al., 2000). Another cucurbitaceous plant, *Citrullus colocynthis* (L.) Schrad was reported to exhibit potential antimycobacterial activity against some drug-sensitive and drug-resistant *Mycobacterium tuberculosis* strain (Mehta et al., 2013). Moreover, *Momordica cochinchinensis* (Chuyen et al., 2015),

Trichosanthes cucumerina (Reddy et al., 2013), *Trichosanthes dioica* (Bhattacharya and Halder, 2010) and *Sechium edule* (Ordonez et al., 2009) were screened for their antimicrobial activity. The compounds isolated from leaves and stems of *Cucumis sativus* exert antifungal activity (Das et al., 2012). Several antifungal proteins *viz.* cucurmoschin, hispin, luffacylin, vicillin isolated from the plants have been reported as potential biofungicide agents (Yadav et al., 2013). The synergistic antifungal activity was observed of metronidazole while combining with *M. charantia* extract, reported by Santos and his research group (Santos et al., 2012). The antifungal activity of the leaf and stem parts of *Solena amplexicaulis* (creeping cucumber) was noticed against nine fungal species (Moorthy et al., 2013).

The cardioprotective activity of the fruit of *Trichosanthes cucumerina* was observed in case of doxorubicin-induced cardiotoxicity in the rat model (Shah et al., 2012). In another study, *Lagenaria siceraria* reported to reduce the doxorubicin-induced cardiotoxicity, hence proved as effective cardioprotective agent (Fard et. al., 2008). The cardiotonic activity of *Lagenaria siceraria* fruit juice was also reported in an study (Dhiman et al., 2012). Some other plants *viz. Citrullus lanatus* and *Cucurbita pepo* have also been found to be therapeutically active against cardiovascular disease (Rajasree et al., 2016)

Several plants of cucurbitaceae family posseses immunomodulatory effects reported till date. For instances, *Lagenaria siceraria* showed immunoprotective, immunomodulatory as well as immunostimulative effect (Rajasree et al., 2016; Deshpande et al., 2008). The saponin constituents isolated from *Lagenaria siceraria* fruits are also studied for its immunomodulatory activity (Gangwal et al., 2008a). In a recent report, it was found that the crude extract of *Luffa acutangula* exhibited immunomodulatory activity in a dose-dependent manner (Shendge and Belemkar, 2018). Some phenolic compounds, *viz.* gallic acid, *p*-hydroxybenzoic acid, etc. present in *L. acutangula* var amara showed potential immunomodulatory activity (Kalaskar and Surana, 2014). The other Luffa species, *Luffa cylindrica* also possesses immunomodulatory activity (Partap et al., 2012). The exploration of immunomodulatory activity of *Cucurbita pepo* and *Momordica dioica* was reported in the literature earlier (Rajasree et. al., 2016; Jafarian et al., 2012).

Several cucurbitaceous plants possess significant hepatoprotective activity. The extract and fractions of *Sechium edule* reported inhibiting carbon tetrachloride-induced hepatic injury in rats (Firdous et al., 2012a). Both of the Momordica species, *Momordica charantia*, and *Momordica dioica* act as hepatoprotective as well as antihepatotoxic agents (Ahamad et al., 2017; Talukdar & Hossain, 2014). It was also observed that *Trichosanthes dioica* prevents gastric ulcers and liver necrosis, studied in rat model (Kulkarni & Raghavan, 2013). In addition, *Luffa acutangula* exhibited hepatoprotective activity against CCl_4 and rifampicin-induced liver toxicity (Jadhav et al., 2010). Other cucurbitaceous plant, *Cucurbita pepo*, and *Lagenaria siceraria* extract also possess hepatoprotective activity (Rajasree et al., 2016).

1.6. Nutritional and economical importance of Cucurbitaceae family

Different parts of cucurbitaceae plants are used in human diet for their several nutritional benefits. Some major species of Cucurbitaceae family e.g. *Cucurbita* (pumpkins, squashes, gourds, marrows, courgettes), *Cucumis* (melons, cucumbers), *Benincasa* (Wax gourd, Tinda), *Citrullus* (watermelon), *Lagenaria* (calabash, bottle gourd) and *Luffa* (sponge gourd, sponge bitter gourd, angular gourd) are widely used for edible purposes (Ajuru & Nmom, 2017). Some cucurbitaceous plants are used for making items of utility such as drinking vessels, cooking pots, utensils, bath sponge, in industrial filters and in sound insulation. Most of the members of this family contain cucurbitacins, which are bitter and having high medicinal value. *Benincasa hispida* (wax gourd) was found in a tropical atmosphere with moderate rainfall. The white, chalky wax which covers around the fruit prevents the occurrence of microorganisms. The flesh part of the fruit is used to make the soup stock. *Citrullus colocynthis* (L.) Schrad (bitter apple) help in obtaining a balanced diet and the seeds are also edible but the fruits are very much bitter (Badifu et al., 1991). The leaves, shoots, and immature fruits of *Coccinia cordifolia* or Ivy gourd are used in culinary purposes. The fruits, shoots, and leaves of *Lagenaria siceraria* and *Luffa acutangula* are widely used in cooking purpose and used in making icing for cakes. The fruit flesh of *Cucurbita ficifolia* is used with sugar to make candy or it can be fermented to make beer. The immature fruits of *Sechium edule* are used to prepare salads and both in cooked form, having a good source of vitamin C. *Cucumis sativus* contains a good amount of water, with small fiber content. In addition, it provides a good source of

vitamins A, K, and C, as well as a large amount of potassium. The fruit of *Cucumis melo* consists of juicy flesh, sweet in taste, used mainly in preparing dessert. The flowers, stems, and fruits of *Cucurbita pepo* are consumed as vegetable. Pumpkins provide a number of beneficial nutrients and minerals. They contain a high level of vitamin A, thiamin, niacin, vitamin B6, iron, magnesium, and phosphorus. They also contain vitamin C, vitamin E, potassium, copper and manganese. The flesh is a good source of dietary fiber (Ajuru & Nmom, 2017). Though the seeds of pumpkin possess more fat, they make up for it with high levels of protein, magnesium, and zinc. The medium-sized calabashes or *Lagenaria siceraria* fruits are used for the production of ladles, boxes, water jugs, planters, flutes, sitars, and other musical instruments. The dry rinds are used as containers for palm wine, water, and floats by fishermen for fishnets and rafts, gun powder and seeds (Jimoh et al., 2013). Another potential use of cucumber is being observed in cosmetic industry because of the fruit is used in rubbing over the skin for softness and whiteness; it offers cooling, healing, and soothing effect to the irritated and used to prepare soap. The mature fruits of *Luffa aegyptiaca* were known as loofahs are used for sponges and filters, and for stuffing pillows, saddles, and slippers (Vouldoukis et al., 2004).

Apart from that, there are several ethnoveterinary uses of cucurbitaceae plants reported in the literature. The tuber of *Cucumis ficifolius* is used in the treatment of blackleg, colic and emaciation in cow, bovine, etc (Tamiru et. al., 2013). The leaves of *Cucurbita pepo* is used in the treatment of trypanosomosis in animals. The leaves and seeds of *Lagenaria siceraria* are used to treat rabies and trypanosomosis; *Momordica foetida* (wild cucumber) is useful in the management of fracture, rabies, trypanosomosis, myiasis, lice and some ectoparasite infestation (Sori et al., 2004). It also has sedative effects on animals. The root decoction of *Citrullus colocynthis* is usually given to the animals to cure constipation. Sometimes, the plant extract is also mixed with honey, mustard oil and applied internally for easy opening of the uterus during delivery (Galav et al., 2013).

1.7. Toxicity

Although the plants of cucurbitaceae family are well recognized for their nutritional and therapeutic benefits, sometimes their uses should be restricted due to adverse reactions which may come from different uptakes, processing methods, physical differences and

other conditions (Jia et al., 2017). To overcome the problem, the toxicity profile of the plants need to be checked which may be developed during the uses of the herbal therapy. There are several reports have been found in the toxic effects of plants of cucurbitaceae family. The toxicity of juice and alcoholic extract of *M. charantia* was observed in normal and alloxan induced diabetic rat. In post-mortem examination, congestion of internal organ and change in blood color was found (Batran et al., 2006). The antifertility activity of the proteins isolated from *Momordica charantia* was reported by altering spermatogenesis and by inducing histological changes in both testis and accessory reproductive organs of albino mice. Some reports have been found that high-dose ingestion of *M. charantia* fruit caused abdominal pain and diarrhea in diabetic patients. Due to the cytotoxic behavior, *M. charantia* causes significant inhibition of DNA and protein synthesis and thus act as an anti-metabolite (Jia et al., 2017). In another study, the *Citrullus colocynthis* extract exhibited liver toxicity by inhibiting the ferric stimulated liver peroxide (LPO) in a dose-dependent manner (Barth et al., 2002). The toxic effects of *Lagenaria siceraria* are well reported due to consumption of the high amount of Cucurbitacin, causes dehydration and gastrointestinal injury and renal toxicity (Puri et al., 2011). In another study, the leaf extract of *Coccinia grandis* showed severe toxicity alloxan-induced diabetic Wister rats in higher doses (Attanayake et al., 2013). In many cases the therapeutic dose of cucurbiatcin tends to impart toxicity in animals. It has to be noted that, some structural modification of the functional groups may help in reducing toxic effects of cucurbiatcin and their related compounds and found to be effective lead compounds in drug discovery research.

Chapter - 2

2. Scope, objective and plan of work

2.1. Scope and rationale
2.2. Objective
2.3. Plan of work

2.1. Scope and rationale

According to World Health Organization (WHO), a large number of people are suffering from several non-communicable diseases (also known as lifestyle-related disease) which contributes around 70% of all deaths globally (WHO, 2017). The NCDs are chronic in nature and depends on several factors including genetics, physiology, environment, occupational habits, diets etc. The major types of NCDs are cardiovascular disease, hypertension, stroke, diabetes, dyslipidemia, obesity etc. The prevalence of most of the NCDs are noticed in low- and middle-income countries due to the imbalance in regular diet and physical activity (Tabish et al., 2017). According to WHO report, 61% morbidity rate was observed in the Indian population, whereas 23 % of the Indian population was suffered from global burden of NCDs. The NCDs are commonly mediated through several metabolic pathways in which numerous enzymes are involved. It was hypothesized that the catalytic power of enzymes is mostly related to the metabolic process of human body. There are several enzymes involved in major biochemical pathways of disease pathogenesis *viz.* α–glucosidase, angiotensin-converting enzyme (ACE), pancreatic lipase, HMG - CoA reductase, carbonic anhydrase, and aldose reductase etc. The inhibition of these enzymes are found to be a major approach to combat these disease by addressing transport of proteins (amino acids), carbohydrates (sugars and starches), or lipids (fatty acids) (Meisinger et al., 2006).

In the Hippocratic medicine, it was believed that the dietetic intervention plays a major role in human well-being. The therapeutic importance of natural products, alternatively nutraceuticals have been established due to their wide spectrum of pharmacological potentials, higher safety margin as well as lesser costs than synthetic drugs. There are several reports indicating that high intake of fruits and vegetables reduce the risk of NCDS owing to the presence of functional secondary and primary metabolites (Nasri et al., 2014). It has been observed that the widespread occurrence of natural phenolic compounds in the human diet (mainly fruits, vegetables, and beverages) play a vital role in reducing the risk of metabolic syndrome and the related complications involved in NCDs (Lin et al., 2016). The plant polyphenols serve as effective dietary antioxidants might offer some protection against oxidative damage. In addition, there are some specific bioactive compounds present in the plants which in term responsible for preserving their bioactivities. In this context, standardization of the plant materials by

employing several analytical techniques such as high-performance liquid chromatography (HPLC), High performance thin layer chromatograpgy (HPTLC), LC-MS/MS is essential for profiling of to the bioactive metabolites. Thus the standradisation will help in chemical fingerprinting as well as dereplication of plant extract to establish quality, safety and efficacy parameters

Medicinal plants provide a wealth of phytoconstituents having diverse biological activities, which are found to be highly effective in the management of metabolic diseases. Based on the literature survey it was found that plants of cucurbitaceae family have been highly recognized for effective control of lifestyle diseases such as diabetes, obesity and related disorders, owing to the presence of a significant amount of phenolic glycosides, flavonoids, terpenoids, and minerals, water-soluble polysaccharides, dietary fibers, etc. (Chanda et al., 2018). It has been noticed that most of the metabolic disorders are originated due to the irregularity of some enzymatic action. For example, carbonic anhydrase is linked with hypertension, edema, obesity, cardiac hypertrophy etc. whereas pancreatic lipase is linked with hyperlipidemia, diabetes, obesity and related disorders. The pancreatic lipase inhibitor hampers the digestion of triglyceride and thus inhibits fat absorption and averts obesity. Another enzyme, α-glucosidase also plays a major role in our body. Inhibiting α-glucosidases enzyme results in delaying carbohydrate digestion, prolongs the overall carbohydrate digestion time, and subsequently reduces the rate of glucose absorption. Thus this enzyme are found to be helpful in treating post-prandial hyperglycemia and related complications like diabetic nephropathy, neuropathy, and retinopathy. Based on the observation, the therapeutic evaluation of the cucurbitaceae plants was performed through several in-vitro enzyme inhibition models. Thus the scientific validation of the wide therapeutic uses of these plants was established to assess quality, efficacy, and safety. With this background, the aim of the study was therapeutic evaluation and standardization of some selected plants of cucurbitaceae family. The schematic diagram of the workflow has been presented in Figure 2.1.

2.2. Objective of the work

- ✓ Standardization and quality evaluation of the plants of cucurbitaceae family *viz. Lagenaria siceraria, Luffa acutangula, Momordica charantia* and *Coccinia grandis.*
- ✓ Evaluation of therapeutic efficacy of the selected plants of cucurbitaceae family through *in vitro* carbonic anhydrase, lipoprotein lipase, α-glucosidase.enzyme inhibition assays
- ✓ Exploration of underlying enzyme inhibitory mechanism of the extract, fractions and its principal constituents
- ✓ Bioactivity guided identification and dereplication of the active principles linked with the enzyme inhibitory activity
- Establishment of the potential benefits of plants of cucurbitaceae family as functional food components

2.3. Plan of work

The specific plan of work has been described as follows:

- ✓ Collection and authentication of the four plants (*Lagenaria siceraria, Luffa acutangula, Momordica charantia* and *Cocinia grandis*) of cucurbitaceae family.
- ✓ Shade drying, pulverization of the plant materials.
- ✓ Extraction and successive fractionation with different solvents based on polarity.
- ✓ Phytochemical evaluation of the extracts and frcations of the plants of cucurbitaceae family.
- ✓ Reverse phase high performances liquid chromatography (RP-HPLC) standardization of *L. siceraria*, *B. hispida, M. charantia, C. grandis, C. pepo* and *L. acutangula* with marker compound.
- ✓ High performance thin layer chromatography (HPTLC) standardisation standardization of *L. siceraria*, *B. hispida, M. charantia, C. grandis, C. pepo* and *L. acutangula* with marker compound
- ✓ Evaluation of carbonic anhydrase inhibition potential of the four cucurbitaceous plants by spectrophotometric assay.
- ✓ Evaluation of pancreatic lipase inhibition potential of the four cucurbitaceous plants by spectrophotometric assay.
- ✓ Evaluation of alpha glucosidase inhibition potential of the four cucurbitaceous plants by spectrophotometric assay.

- ✓ Study of enzyme inhibition mode and type (e.g. reversible or non-reversible/ competitive, non-competitive, and mixed competitive) by both non-linear (Michellis Menten equation) and and linear (Lineweaver Burk plot) mode.
- ✓ Molecular docking study to explore the enzyme-ligand interaction mechanism.
- ✓ Correlation analysis between total phenol and flavonoid content and bioactivity.
- ✓ Bioactivity guided LC-QTOF-MS based dereplication of secondary metabolites.

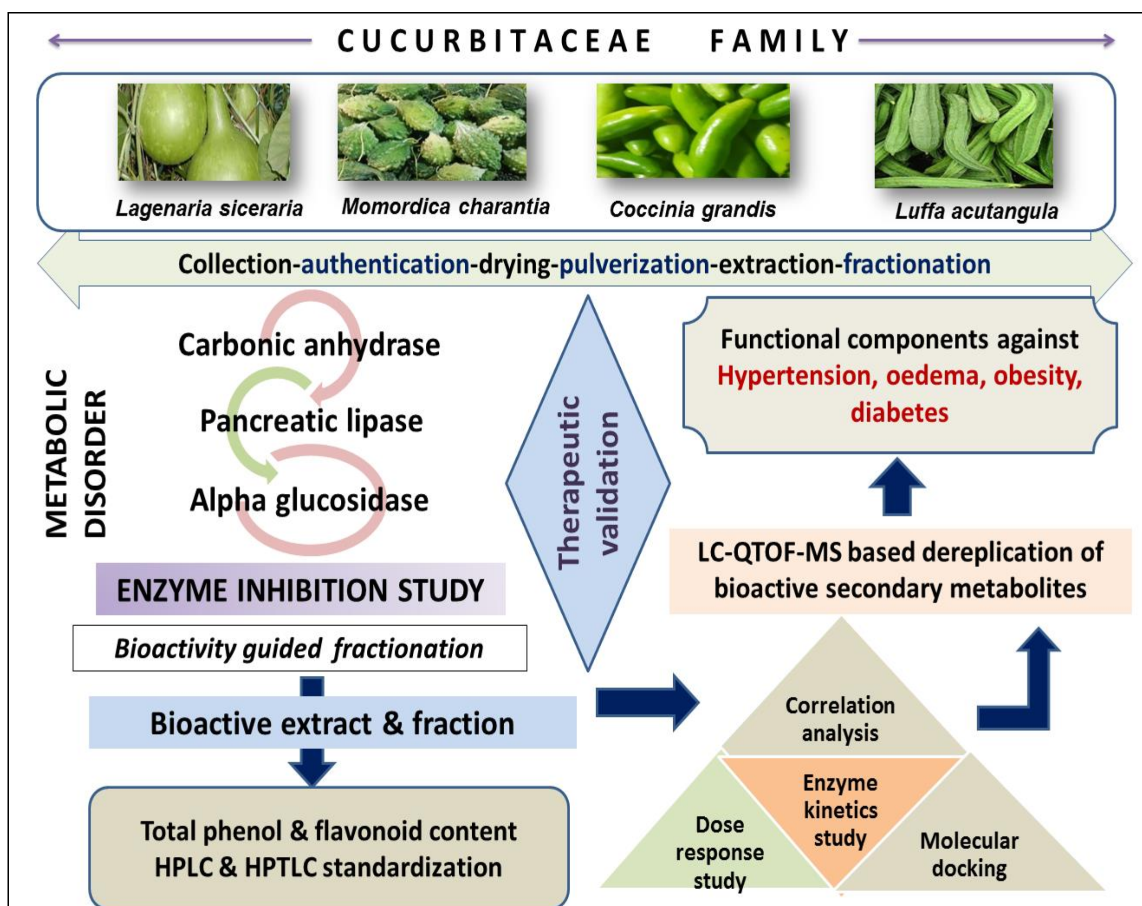


Figure 2.1. Schematic diagram of workplan of the study

Chapter 3

3. Plant profile, collection, extraction and phytochemical screening

3.1. Rationale for selection of the plants
3.2. *Lagenaria siceraria*
3.3. *Luffa acutangula*
3.4. *Momordica charantia*
3.5. *Coccinia grandis*
3.6. Extraction and phytochemical screening

3.1. Rationale for selection of plants

A large number of population from Indian subcontinent including India, Pakistan, and Bangladesh relies on the richest sources of their traditional medicinal practices. India comprises of a rich biodiversity of medicinal plants which is being fostered by the indegenous knowledge based on the ethnopharmacolgical practices of community. Overall, the alternative medicinal systems of India utilizes more than 7500 plant species in the treatment of various ailments. There are several reports available on the indigenous knowledge of most commonly used medicinal plants of cucurbitaceae family used in traditional system of medicine. It was noticed that, the whole plants, or some parts of plants of cucurbitaceae family (fruits, leaves, roots, stems) have been reported to possess potential therapeutic efficacy (Nishteswar, 2014).

In 2012-2013, a group of reserachers from both India and Bangladesh participated in a survey on ethnomedicinal practices of cucurbitaceae family plants in the treatment of several ailments. The survey was done based on the information shared by the tribal medicinal practitioners from various regions of both the countries. The study mainly highlights on the ethnomedicinal importance of the cultivated cucurbitaceae family plants, largely consumed as vegetables. Among them, *Citrullus lanatus*, *Coccinia grandis*, *Cucumis melo*, *Momordica charantia*, *Momordica cochinchinensis*, *Trichosanthes kirilowii*, *Lagenaria vulgaris* are mostly indicated in the treatment of diabetes and edema wheras *Benincasa hispida, Citrullus lanatus, Coccinia grandis* and *Lagenaria vulgaris* are reported to prescribe in the treatment of heart disorders. Another ethnopharmacological uses of cucurbitaceae plants *viz. Luffa cylindrica*, *Momordica charantia*, *Momordica cochinchinensis*, *Trichosanthes dioica*, *Trichosanthes kirilowii* were observed in the trertament of cancer and tumor (Rahmatullah et al., 2012; Shrivastava et al., 2013).

Based on the above literature reports and ethnopharmacological relevances, the present study aim to validate the ethnopharmacological claim of the selected plants *viz. Lagenaria siceraia*, *Luffa acutangula*, *Momordica charantia* and *Coccinia grandis* based on their phytochemical and therapeutic evaluation. In this chapter the morphological, phytochemical and pharmacological properties of the selected plants have been discussed in details.

3. 2. *Lagenaria siceraria*

3.2.1. Botanical taxonomy

Scientific classification

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Violales
Family	:	Cucurbitaceae
Genus	:	*Lagenaria* Ser.
Species	:	*Lagenaria siceraria* (Molina) Standl.

Vernacular names

English	:	Bottle Gourd
Sanskrit	:	Alabu
Hindi	:	Lauki
Bengali	:	Ladu
Tamil	:	Surakkai
Telugu	:	Sorakkaya
Marathi	:	Dudhi

Figure 3.1.a. *L. siceraria* leaves and flowers

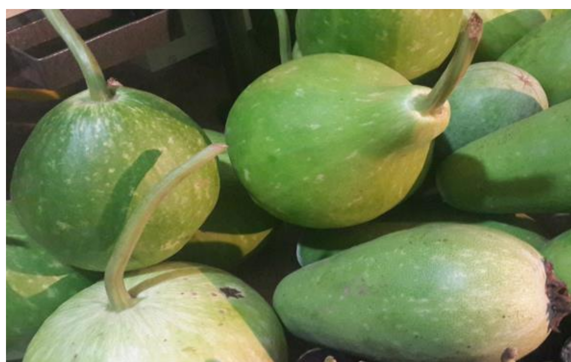


Figure 3.1.b. *L. siceraria* fruits

Figure 3.1.c. Voucher specimen of *L. siceraria*

3.2.2. Plant description

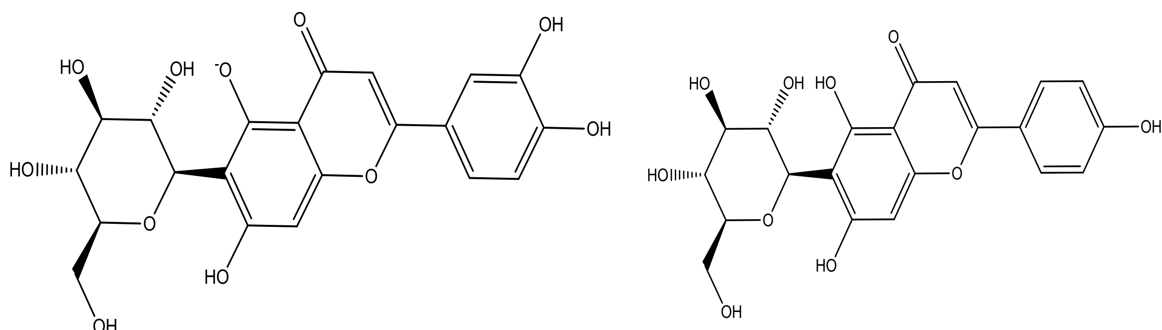
The bottle gourd plant is a vigorous, annual, running or climbing vine with large leaves and a lush appearance. It grows fast and may begin to flower only 2 months after seeding. The vine is branched and climbs by means of tendrils along the stem. The foliage is covered with soft hairs and has a foul musky odor when crushed. The leaves of the bottle gourd plant are up to 15 inches wide, circular in overall shape, with smooth margins, a few broad lobes, or with undulate margins. Leaves have a velvety texture because of the fine hairs, especially on the undersurface. The flowers are borne singly on the axils of the leaves, the males on long peduncles and the females on short peduncles. The flowers are white and attractive, up to 4 inches in diameter, with spreading petals. The ovary is inferior and in the shape of the fruit. Otherwise, the male and female flowers are similar in appearance. The anthers are borne on short filaments grouped at the center of the flower. The stigmas are short, thickened, and branched. The brownish seeds are numerous in a whitish green pulp. Each seed is somewhat rectangular in shape with grooved notches near the attached end (Flowers of India). The pictorial description of the fruits and leaves of *L. siceraria* has been presented as Figure 3.1 (a-b). The voucher specimen of *L. siceraria* has been presented in Figre 3.1.c.

3.2.3. Morphology of fruits

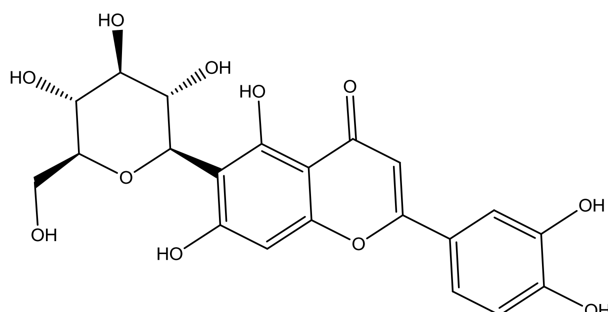
The young fruits are hairy and usually light green but become glabrous and beige or brown at maturity. The moist, white flesh of the immature fruit dries out as the fruit matures. The fruits are large, up to 1.8m. Long, fruit bottle shaped with a hard shell- like epicarp when ripe, numerous seeds, long, white, smooth, 1.6- 2.0 cm long, horizontally compressed with marginal groove. Almost 25 cultivars of *L. siceraria* (Molina) Standl.are present of different shapes and sizes based on the morphological variation in fruit shape and fruit *viz.* Big Apple Gourd, Blister Gourd, Indian Serpent Gourd, Bottle Gourd, Giant Bottle Gourd, Chinese Bottle Gourd, India Long Hybrid, Big Green Sausage Gourd, Harvest Bowl Gourd, Penguin Gourd, Long Handle Dipper Gourd, Green Bell Hybrid, Speckled Swan, Cave Man's Club, Dinosaur Gourd, Powder Horn, Long Siphon, Basket Gourd, Large Kentucky Bushel Gourd, Sugar Bowl, Bushel Gourd, Martin House Gourd, Bule, Opo Long Bottle Gourd, Asia Short Hybrid Bottle Gourd (Tiwari, 2016).

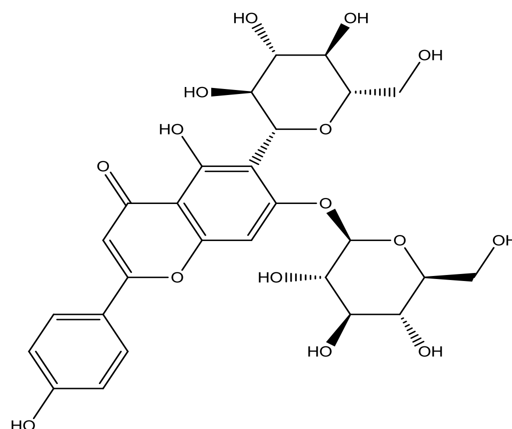
3.2.4. Phytochemical profile

Lagenaria siceraia contains a large number of primary and secondary metabolites. The primary metabolties contains carbohydrate, protein (0.2%), fat (0.1%) (ether extract), fibers (0.6%), mineral matter (0.5%), calcium and phosphorous (<0.01%) along with iron (0.7 mg/100 g), sodium (11.0 mg/100 g), and iodine (4.5 µg/kg). The presence of several amino acids have been observed in the fruit. The vitamins present in the fruits are thiamine, riboflavin, niacin, ascorbic acid etc. The fruit also contains a significant amount of choline. The fruit skin contains crude protein, cellulose, and lignin (Prajapati et al., 2010). A large number of flavonoids are reported *viz.* 4-C-glycosylflavone: 7-0-glucosyl-6-C-glucoside apigenin, 6-C-glucoside apigenin, 6-C-glucoside luteolin **(1)**, and 7,4'-O-diglucosyl-6-C-glucoside, isovitexin **(2)**, isoorientin **(3)**, saponarin **(4)**, saponarin 4'-O-glucoside, and saponarin 4'-O-glucoside in the plant. A protein, lagenin was isolated from seeds of the plant as novel ribosome-inactivating protein with ribonucleolytic activity (Wang et al., 2000).

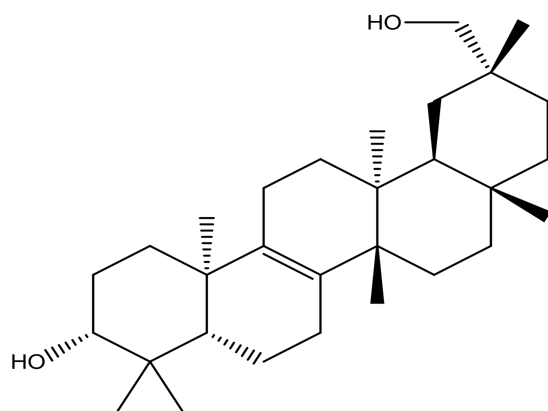


6-C-glucoside luteolin (1) **Isovitexin (2)**

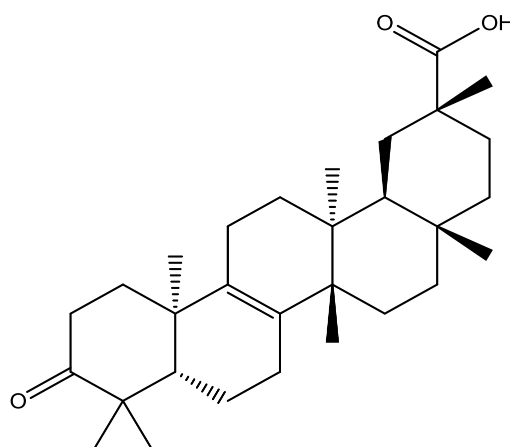




Isoorientin (3) **Saponarin (4)**



Bryonolol (5)



Bryononic acid (6)

Several triterpenoids were isolated from the methanol extract of the stems of plant as d:*C*-friedooleanane-type triterpenes, 3 b-*O*-(*E*)-feruloyl-D:*C*-friedooleana-7, 9 (11)-dien-29-ol , 3b-*O*-(*E*)-coumaroyl-D:*C*-friedooleana-7,9(11)-dien-29-ol , 3b-*O*-(*E*) coumaroyl-d:*C*-friedooleana-7,9 (11)-dien-29-oic acid, and methyl 2 b,3 b-dihydroxy-D:C-friedoolean-8-en-29-oate , together with five known triterpenes with the same skeleton, 3-epikarounidiol, 3-oxo-d:C-friedoolena-7, 9 (11)-dien-29-oic acid, bryonolol **(5)**, bryononic acid **(6)**, and 20-epibryonolic acid etc. (Chen et al., 2008). Several aliphatic aldehydes *viz.* octanal, nonanal, and decanal were reported along with 1,4-benzenediol, 2-pentadecyn-1-ol, 9,12-octadecadienal and fatty acids (such as palmitic acid and stearic acid) were reported as volatile constituents (Chatterjee et al., 2009).

3.2.5. Pharmacological activity

L. siceraria (Molina) Standley is an annual herbaceous climbing plant with a long history of traditional medicinal uses in many countries, especially in tropical and subtropical regions. The pharmacological activities are described in Table 3.1.

Table 3.1. Pharmacological activities of *L. siceraria*

Parts used	Pharmacological activity	References
Leaves, seeds, and fruit	Antimicrobial activity	Dash & Ghosh, 2018
Fruits	Lipase inhibitory activity	Maqsood et al., 2017
	Xanthine oxidase and alpha-amylase inhibitory activities	Ahmed et al., 2017

Fruits	Antistress and adaptogenic property	Lakshmi et al., 2009
Seed	Anthelmintic activity	Thube et al., 2009
Fruits	Immunomodulatory activity	Gangwal, 2008b
Fruits	Antihyperlipidemic activity	Ghule et al., 2006b
Fruits	Analgesic and anti-inflammatory activity	Ghule et al., 2006a
Fruits	Diuretic activity	Ghule et al., 2007
Fruits	Antioxidant activity	Desahpande et al., 2007
Fruits	Antihepatotoxic activity	Elisha et al., 1987

3.3. *Luffa acutangula*

3.3.1. Botanical taxonomy

Scientific classification			Vernacular names		
Kingdom	:	Plantae	English	:	Ridge gourd
Division	:	Magnoliophyta	Sanskrit	:	Kosataki
Class	:	Magnoliopsida	Hindi	:	Jhimani
Order	:	Cucurbitales	Bengali	:	Jhinga
Family	:	Cucurbitaceae	Tamil	:	Itukari
Genus	:	Luffa	Telugu	:	Adavibeera
Species	:	*Luffa acutangula (L.) Roxb*	Marathi	:	Divali

Figure 3.2.a. ***L. acutangula*** **leaf**

Figure 3.2.b. ***L. acutangula*** **fruit**

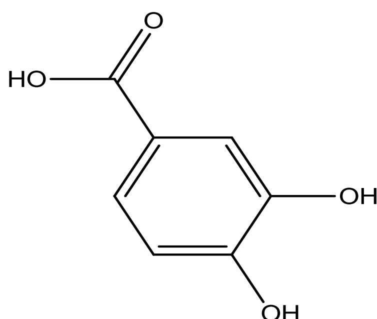
Figure 3.2.c. Voucher specimen of *L. acutangula*

3.3.2. Plant description

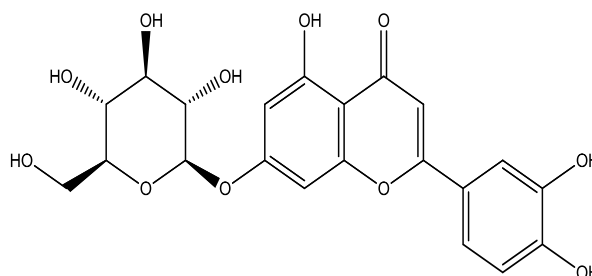
Ridge gourd is a tropical running vine with rounded leaves and yellow flowers. The roots of plants are yellowish, brown in colour, almost cylindrical in shape, having 8- 12cm length and 0.7cm thickness. They are rough in touch because of longitudinal wrinkles and also showed new adventitious roots. The stems are with 5 angled glabrous stem and 3-fid tendril. The leaves are orbicular in outline with the length 15-20 cm long, palmately 5-7 angled or sublobate, scabrid. Veins and vein lets are prominent. The fruits are obovate, pale yellowish brown in color with 4-10 cm long, 2-4cm broad and outer surface being covered with 8-10 prominent longitudinal ribs. It is tapering towards the base and longitudinally ribbed. The fruits are divided into 3 chambers. The inner part is fibrous in nature and easily detachable as a whole from the outer part. The fruits are bitter in taste. Transverse section of fruit through a rib shows a single layer of papillose epidermis covered with thick striated cuticle, followed by 4-6 layers of parenchymatous cells. The seeds are black in colored with bitter taste and having ovoid- oblong shape. The length is generally 0.6-0.8cm with width of 0.5-0.6 cm (Pingale et al., 2018). The pictorial description of the fruits and leaves of *L. acutangula* has been presented as Figure 3.2 (a-b). The voucher specimen of *L. acutangula* has been presented in Figure 3.2.c.

3.3.3. Morphology of fruits

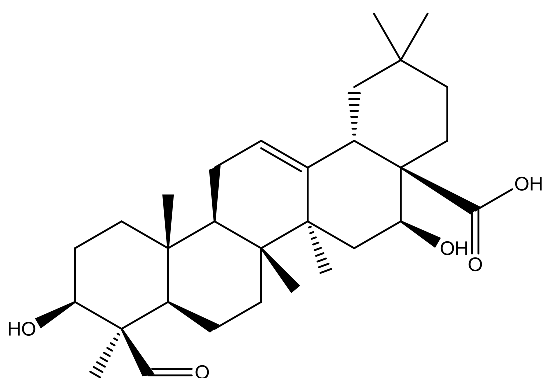
The matured fruits of *L. acutangula* are dry, fibrous, splitting from a lid-like structure, known as an operculum, located at the apex of the fruit. The fruits are identified with the presence of club-shaped, ten prominent ribs running along its length, a fibrous spongy skeleton network inside, and containing numerous flattened seeds that are pitted and black without a narrow wing-like margin. The fruits are green in colour when young, the fruit turns dry and brown when mature, together with the disappearance of its soft internal tissue. There are three botanical varieties have been reported *viz. L. acutangula* var. *acutangula*, (large-fruited cultivated types), *L. acutangula* var. *amara* (Roxb.) (wild or feral type with extremely bitter fruits) and *L. acutangula* var. forskalii (Harms) (Heiser and Schilling, 1988).



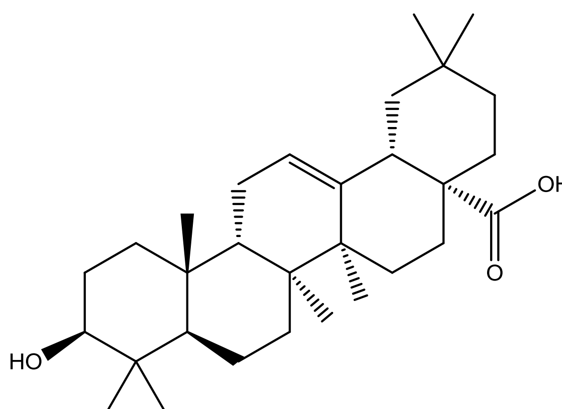
Protocatechuic acid`(7)



Luteolin-7-glucoside (8)



Sapogenin (9)



Oleanolic acid (10)

3.3.4. Phytochemical profile

The primary metabolites present in *L. acutangula consists of* protein, fat, amiono acids, carbohydrates, carotene, and saponins. The plant contains a significant amount of polyphenols (mostly phenolic acids *viz.* gallic acid, p-coumaric acid, ferulic acid, protocatechuic acid **(7)**, apigenin-7-glucoside, Luteolin-7-glucoside **(8)**, flavonoids (catechin, quercetin, anthocyanins), especially in its fruits (Swetha & Muthukumar 2016). Luffeine is present in the fruits as bitter principle. The presence of oligosaccharide compound, *viz.* lectin was reported from *L. acutangula*. The seeds are a rich source of fixed oil *viz.* glycerides of palmitic, stearic, and myristic acids. A ribosome inactivating peptide, Luffangulin was also found in the seeds of *L. acutangula* (Junkai et al., 2002). Another protein, Luffaculin 2 was reported from the seeds of *L. acutangula* (Wang and Ng., 2002). The presence of sapogenin (9), oleanolic acid (10) and a bitter principle, Cucurbitacin B, E were also identified from the seeds of *L. acutangula* (Anitha & Mrithula, 2014).

3.3.5. Pharmacological activity

Several pharmacological activities of *L. acutangula* has been reported in literature. According to Ayurvedic pharmacopeae, ridge gourd possess significant health benefits, useful as diuretic and in splenic enlargement and also in the treatment in of vata, kapha, anaemia, leucoderma, tumors. The pharmacological activities of *L. acutangula* has been described in Table 3.2.

Table 3.2. Pharmacological activities of *L. acutangula*

Parts used	Pharmacological activity	References
Fruits	Antihyperlipidemic activity	Shendge & Belemkar, 2018
Fruits	Antidiabetic Activity	Shendge & Belemkar, 2018; Pimple et al., 2011
Fruits	Hepatoprotective activity	Mishra et al., 2017
Fruits	Antioxidant property	Suryanti et al., 2015
Seed	Antiulcer activity	Pimple et al., 2012
Fruits	Antibacterial and Antifungal Activity	Bulbul et al., 2011
Leaves, seeds, and fruit	Anti-inflammatory activity	Gill et al., 2011
Fruits	Antitumor Activity	Dashora & Chauhan, 2005

3.4. *Momordica charantia*

3.4.1. Botanical taxonomy

Scientific classification			Vernacular names		
Kingdom	:	Plantae	English	:	Bitter Gourd
Division	:	Magnoliophyta	Sanskrit	:	kaarvellakah
Class	:	Magnoliopsida	Hindi	:	Karela
Order	:	Violales	Bengali	:	karala
Family	:	Cucurbitaceae	Tamil	:	Iraca-valli
Genus	:	Momordica L.	Telugu	:	Kakara
Species		*Momordica charantia L*	Marathi		Ambalem

Figure 3.3.a. *M. charantia* leaf and flower

Figure 3.3.b. *M. charantia* fruit

Figure 3.3.c. Voucher specimen of *M. charantia*

3.4.2. Plant description

Bitter Gourd is an annual or perennial, monoecious, herbaceous a tendril-bearing vine, climber which is up to 5 m in length. It bears a simple, alternate leaves 4-12 cm across, with 3-7 deeply separated lobes, separate yellow male and female flowers, about 2-3 cm in diameter. Male flowers, more numerous, have a yellow center and conical base, while female flowers have a green center and small bump at the base. The stems are green, well-branched, slender and usually slightly five angled or ridged and carries unbranched tendrils in the leaf axils. Root: The central taproot comes to the apex where the stem spreads to climb. The fruit has a distinct warty looking exterior and an oblong shape. It is hollow in cross-section, with a relatively thin layer of flesh surrounding a central seed cavity filled with large flat seeds and pith. Seeds and pith appear white in unripe fruits. The fruit is most often eaten green. Although it can also be eaten when it has started to ripen and turn yellowish, it becomes more bitter as it ripens. When the fruit ripens and turns orange and mushy, it is too bitter to eat. It splits into segments which curl back dramatically to expose seeds covered in bright red pulp (Gupta et al., 2011). The pictorial description of the fruits and leaves of *M .charantia* has been presented as Figure 3.3 (a-b). The voucher specimen of *M. charantia L. siceraria* has been presented in Figre 3.3.c.

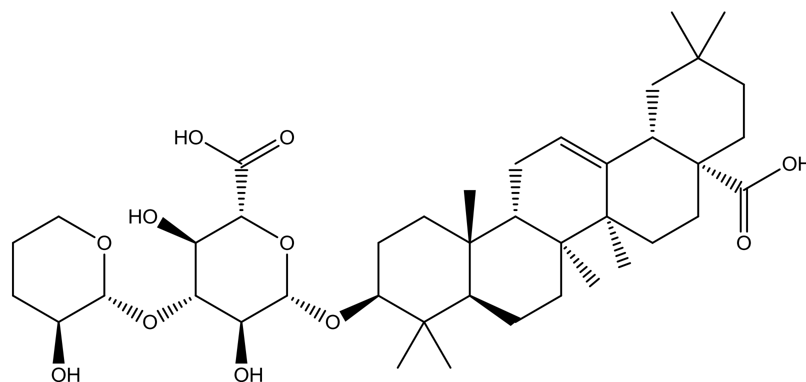
3.4.3. Morphology of fruits

The fruits of *M. charantia* are pendulous cylindrical, egg-shaped and 2-10 cm long (up to 20 cm in cultivated varieties), and covered with longitudinal ridges and warts. At maturity, they turn orange to yellow, and the tips split into three and turn back to reveal the yellow pulp and the bright red arils that enclose the seeds which adhere to the inside of the fruit. Each of the flattened woody seeds is 5-9 mm long, and has finely pitted surfaces. The seedlings show epigeal germination, and resemble cucumber seedlings. The thick, brittle hypocotyl is 2-3 cm long, the cotyledons thick, firm, convex on the lower surface and almost without veins, the epicotyl 1-2 cm long and the first leaves shortly stalked, rounded, bluntly lobed and finely toothed. There are two botanical varieties of *M. charantia* reported viz. *M. charantia* var. muricata (syn. var. *abbreviata*) and *M. charantia* var. *charantia*, the former are mostly wild whereas the latter is mostly cultivated.

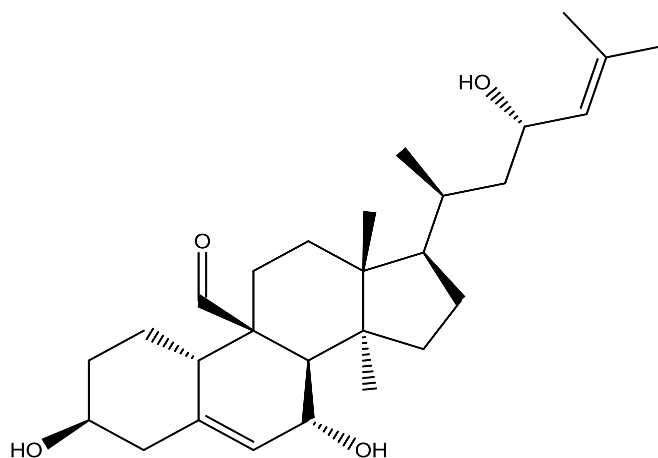
The wild variety (*M. charantia* var. *muricata*) is considered as the progenitor of cultivated *M. charantia* var. *charantia*.

3.4.4. Phytochemical profile

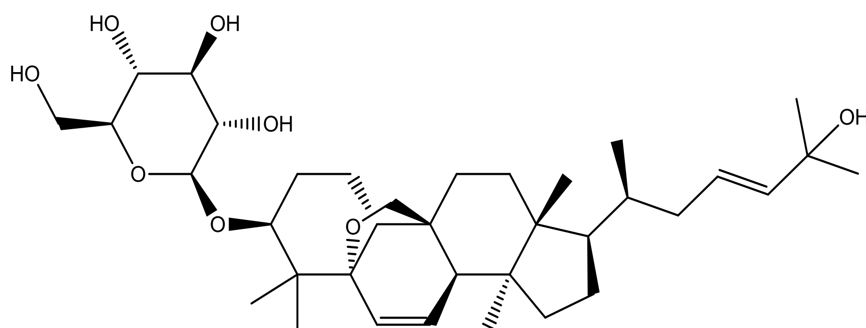
The chemical constituents present in *M. charantia* fruit are classified as carbohydrates, proteins, lipids and more as primary metabolites. In addition a large number of bioactive secondary metabolites *viz.* triterpenoids, saponins, polypeptides , flavonoids, alkaloids and sterols are found in *M. charantia* fruits. The details of chemical constituents present in *M.charantia* fruit have been summarized as follows:



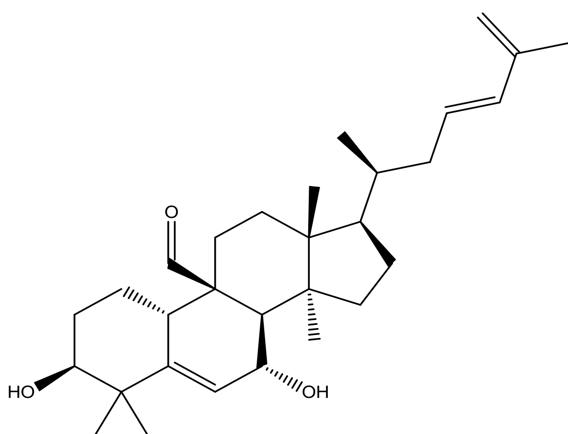
Momordin (11)



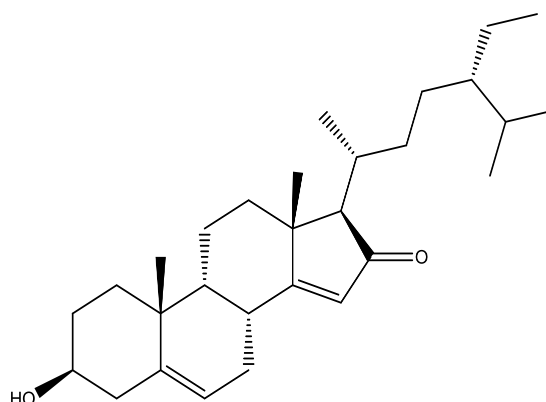
Momordicine I (12)



Momordicoside I (13)



Kuguacin J (14)



Momordenol (15)

Table 3.3. Major phytoconstituents found in *M. charantia*

Name of compounds	References
Ribosome-inactivating proteins (RIPs)	Chang et al., 2017
Momorcharins, momordenol (15), momordicilin, momordicins, momordicinin, momordin (11), momordolol, charantin, charine, cryptoxanthin,cucurbitins, cucurbitacins, cucurbitanes, cycloartenols, diosgenin, elaeostearic acids, erythrodiol, galacturonic acids, gentisic acid, goyaglycosides, goyasaponins, multiflorenol	Jia et al., 2017; Grover et al., 2004
D-galactose and D-methyl galacturonate	Panda et al., 2015
Kuguasaponins A–H	Zhang et al., 2014

Karavilagenin F, karavilosides, momordicines VI-VIII	Zhao et al., 2014
Peptides, MCh-1 and MCh-2	He et al., 2013
Kuguacin J (14)	Pitchakarn et al., 2012
Charantal	Panlilio et al., 2012
Taiwacin A, taiwacin B	Lin et al., 2011
Pentanorcucurbitacins, octanorcucurbitacin, trinorcucurbitacins	Chen et al., 2009
Momordicatin	Gupta et al., 2010
Dicarboxylic acids, octanedioic, nonanedioic and decanedioic acids, capric acid	Wu et al., 2009
Kuguacins A–E	Chen et al., 2008
Momordicine I (12) and Momordicine II	Ling et al., 2008
Kuguacins A–E	Chen et al., 2008
Momordicosides I (13) F1, F2, G, , K and L	Okabe et al., 1982

3.4.5. Pharmacology

Over the years scientists have reported several therapeutic benefits of *M. charantia* due to presence of a large number of primary and secondary metabolites including proteins, polysaccharides, flavonoids, triterpenes, saponins, ascorbic acid and steroids etc. Various biological activities of *M. charantia* have been reported, such as antiviral, antioxidant, antihyperglycemic, antibacterial, antitumor, immunomodulation, antidiabetic, anthelmintic, antimutagenic, hepatoprotective, anticancer and anti-inflammatory activities (Jia et al., 2017). The major pharmacological activities of *M. charantia* has been described in Table 3.4.

Table 3.4. Pharmacological activities of *M. charantia*

Parts used/Compounds	Pharmacological activity	References
α and ß-momorcharin, lectin and MAP 30	Antiviral activity	Jia et al., 2017
Dry powder	Antiinflammatory	Bao et al., 2013
Leaves	Antibacterial activity	Costa et al., 2011
Fruits	Immunomodulatory activity	Juvekar et al., 2009
Seed	Antimicrobial	Braca et al., 2008

Alpha momorcharin	Anti-HIV activity	Chen et al., 2008
Fruit pulp, seed, leaves and whole plant	Antidiabetic activity	Fernandes et al., 2007
Crude extract	Anticancer activity	Akihisa et al., 2007
Fruits	Hypocholesterolemic and anti-oxidant potential	Chaturvedi et al., 2005
Ribosome-inactivating proteins and MAP30	Antiherpes activity	Grover et al., 2004
Fruits	Antipoliovirus activity	
Aqueous extract	Abortifacient and antifertility	
Fruits	Anti-ulcer activity	
	Anthelmintic study	
	Antimalarial activity	

3.5. *Coccinia grandis*

3.5.1. Botanical taxonomy

Scientific classification			**Vernacular names**		
Kingdom	:	Plantae	English	:	Ivy Gourd
Division	:	Magnoliophyta	Sanskrit	:	Bimbika
Class	:	Magnoliopsida	Hindi	:	Kunduru
Order	:	Violales	Bengali	:	Telakucha
Family	:	Cucurbitaceae	Tamil	:	Kovai
Genus	:	Coccinia Wight & Arn	Telugu	:	Donda kaya
Species	:	*Coccinia grandis (L.) Voigt*	Marathi	:	Tondli

Figure 3.4.a. *C. grandis* leaf and flower

Figure 3.4.b. *C. grandis* fruit

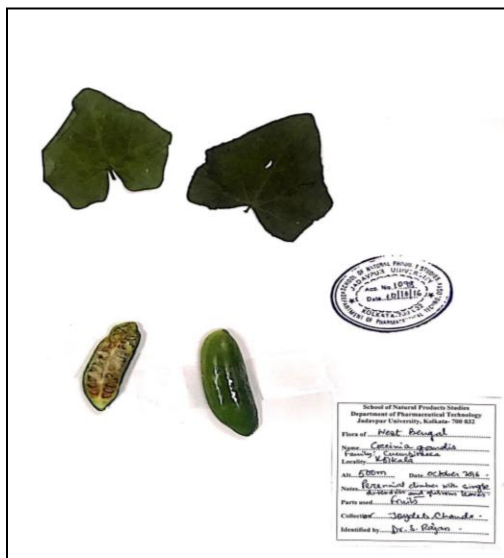


Figure 3.4.c. Voucher specimen of *C. grandis*

3.5.2. Plant description

Ivy gourd is a tropical plant of the pumpkin family, an aggressive climbing vine that can spread quickly over trees, shrubs, fences and other supports. It is an outdoor plant but prefers a sunny sheltered position and a sandy soil. The stem is a herbaceous climber or perennial slender climber with occasional adventitious roots forming where the stem runs along the ground. The tendrils are long, elastic with coil-like springy character that can wrap around the host to the entire length. The leaves are classified as palmately simple with five lobes while the shape varies from the heart to pentagon form. The size of the leaves is approximately 5-10 cm in width and length. The flower is large and white about 4 cm in diameter and contains five long tubular petals. The ivy gourd fruit belongs to the berry type: oval and hairless with thick and sticky skin. The raw fruit is green in color and turns bright red when it is ripe. The mature fruit is usually from 25 to 60 mm long by 15-35 mm in diameter and contains several pale, flattened seeds (Flowers of India). The pictorial description of the fruits and leaves of *M .charantia* has been presented as Figure 3.4 (a-b). The voucher specimen of C. grandis has been presented in Figure 3.4.c.

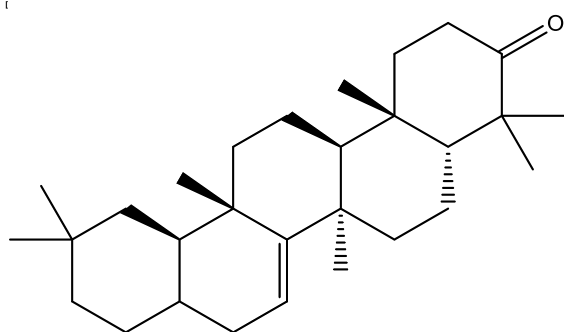
3.5.3. Morphology of fruit

The fruit of *C. grandis* is berry like with red flesh and a red skin that rarely exhibits a white longitudinal mottling. The fruit size is approximately 4-5 x 2-2.5 cm, ovoid-oblong

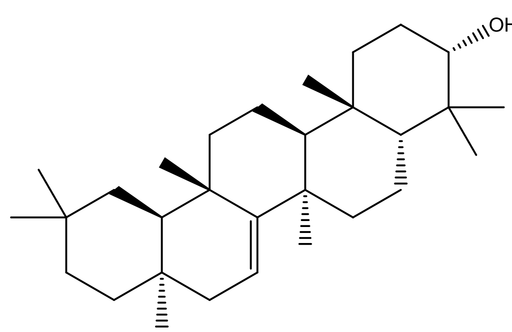
elliptical in shape. The fruits are green and have white stripes on them and turn red when mature. The widely used coccinia species is *C. grandis* (L.) *Voigt* also known as *C. grandis* var. wightiana.

3.5.4. Phytochemical profile

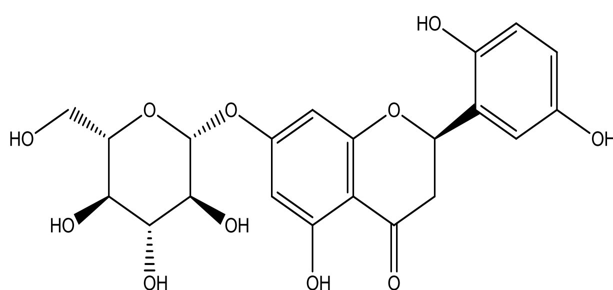
GC-MS analysis of methanolic extract of *C. grandis* revealed the presence of several phytoconstituents, *viz.* 2(3H)-furanone, 2-methoxy-4-vinylphenol, benzofuranone, 9,12-Octadecadienoic acid, tocopherol, campoesterol, stigmatosterol, ethisteron, n-pentadecanoic acid, hexadecanoic acid, linoleic acid, oleic acid, and α-tocopherol (Kondhare et al., 2017). The fruits are reported to possess taraxerone **(16)**, taraxerol **(17)**, 24R-24-ethylcholest-5-en-3β-ol glucoside, cephalandrins A and B, β-sitosterol, stigma-7-en-3-one, cucurbitacin B, coccinoside (saponin) **(18)**, flavonoid glycoside, lupeol **(19)**, β-amyrin, β-sitosterol etc (Bambal et al., 2011; Pekamwar et al., 2013).



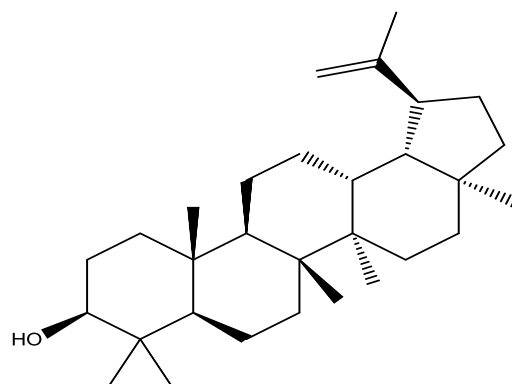
Taraxerone (16)



Taraxerol (17)



Coccinoside (18)



Lupeol (19)

3.5.5. Pharmacological activity

C. grandis posseses a wide range of nutritional and therapeutic importance. Several clinical reports on fruit and leaves of *C. grandis* haver been documented for its antidiabetic, antibacterial, anti-inflammatory, analgesic and antipyretic activity (Kumar et al., 2018). The details of pharmacological activites of *C. grandis* have been reported in Table 3.5.

Table 3.5. Pharmacological activities of *C. grandis*

Parts used	Pharmacological activity	References
Leaves	Anti-inflammatory	Nachimuthu et al., 2018
Leaf	Antibacterial, cell proliferative	Sakharkar et al., 2017
Fruits	Antidiabetic and insulinotrophic	Meenatchi et al., 2017
Fruits	Antiproteolytic and leishmanicidal activity	Das et al., 2015
Fruits	Hypoglycemic activity	Pekamwar et al., 2013
Seed	Hepatoprotective activity	Pekamwar et al., 2013
Fruits	Antimalarial activity	Ravikumar et al., 2012
Fruits	Wound healing activity	Bambal et al., 2011
Leaves, seeds, and fruit	Antihelminthic activity	Yogesh et al., 2011
Fruits	Alpha-amylase inhibitory activity	Sudha et al., 2011
Fruits	Antibacterial activity	Bhattacharya et al., 2010
Fruits	Antidyslipidemic activity	Singh et al., 2007

3.6. Extraction and phytochemical screening of plants

3.6.1. Collection and authentication

Th fresh fruits of the selected curubitacaea plants were collected from the local market of Jadavpur, Kolkata and authenticated by the field botanist. The voucher specimen was pepared with each plant sample for future refrence in Figure 3.1.c (*L. siceraria*), 3.2.c (*L. acutangula*), 3.3.c (*M. charantia*), 3.4.c (*C. grandis*). The details of collection and authentication of plants has been described in Table 3.6.

Table 3.6. Collection and authentication of plants

Name of the plant	Collection area	Voucher specimen number	Collection season
L. siceraria	Kolkata, West Bengal	SNPSJU/2014/1095	September, 2014
L. acutangula	Kolkata, West Bengal	SNPSJU/2015/1096	April 2015
M. charantia	Kolkata, West Bengal	SNPSJU/2016/1097	October 2016
C. grandis	Kolkata, West Bengal	SNPSJU/ 2016/1098	October 2016

3.6.2. Extraction of the plant material

In order to get optimum separation of the phytomolecules, successive extraction with different solvents (based on the polarity) was found effective in order to isolate and characterize the active principles. Among various types of extraction process, cold maceration is the simplest mode of extraction where the powdered plant material is taken in a stoppered container and soaked with the solvent for a specified period of time until the soluble portions are dissolved in the solvent. It is a widely used technique, adopted in medicinal plants research. The purpose of maceration technique is to extract the therapeutically desirable portion and eliminate the inert material by treatment with a selective solvent known as the menstruum. Maceration involves three principal steps. Firstly, plant materials are converted to powder form by grinding. As the surface area increases, they allow good contact between solvent and material for proper mixing with the solvent. After grinding, a chosen solvent is added in a closed vessel. Then, the liquid is strained off but the solid residue of this extraction process is pressed to recover a large number of occluded solutions. During the process of maceration occasional shaking facilitate the extraction yield by increasing diffusion and remove the concentrated solution from the sample surface. The efficacy of the maceration process is determined by two main factors, solubility, and effective diffusion. Another critical factor of cold maceration process is solvent polarity. For example, in case of extraction of non-polar compounds like fats, oils, and lipids, non-polar solvents are used whereas the extraction of highly polar compounds like glycosides, phenolics, sugars, amino acids, proteins and polysaccharides are mostly done with polar solvents such as ethanol and water.

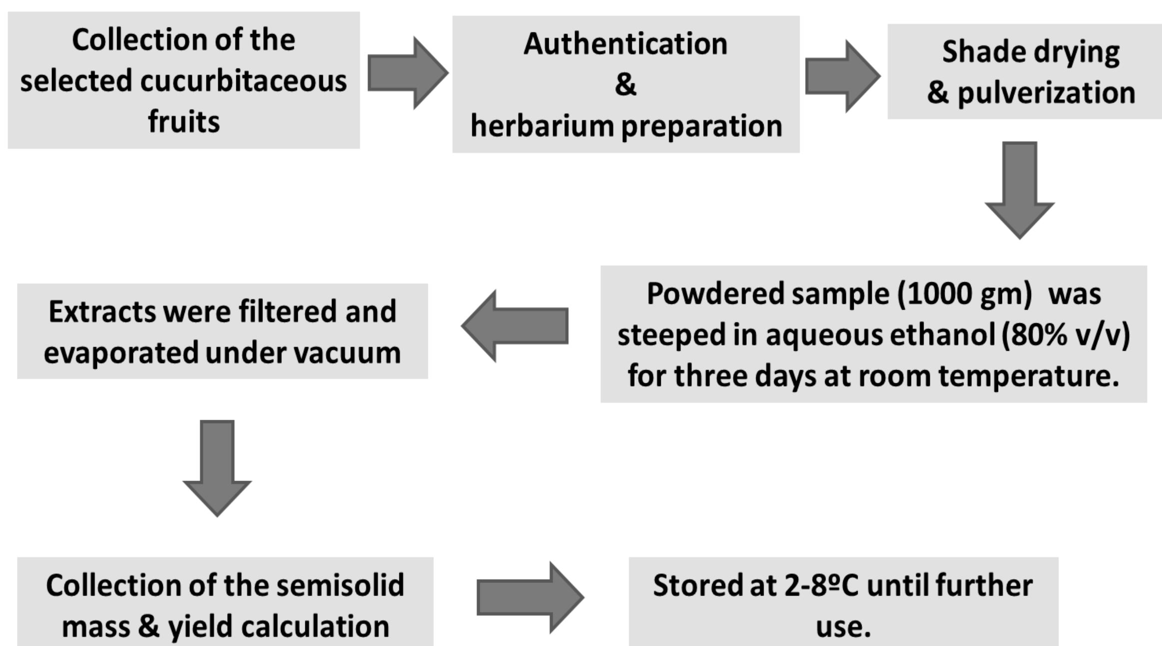


Figure 3.5. Schematic diagram of extraction process

In this study the plant materials were shade dried and cut into cut into small pieces. Subsequently, pulverization was performed by using a grinder. 1000 gm of each powdered sample was steeped with aqueous ethanol for three days at room temperature. The process was repeated for three times. The collected solvent extracts were filtered and evaporated under vacuum at 45-50ºC through the rotary evaporator (Eyela N-1100S-W, USA). The extract was stored at 2-8ºC in a refrigerator until further use. The schematic diagram of extraction process has been represented in Figure 3.5. The yields for each extracts were calculated based on the following equation yield (g/100 g) = (W1 × 100)/W2 where W1 is the weight of the extract residue obtained after solvent removal and W2 is the weight of plant material taken. The yield was expressed as % (w/w) in Table 3.7.

Table 3.7. Percentage yields of the four plants

Name of the plant	Initial quantity taken	Final weight of extract	Extrcation solvent	% Yield (w/w)
L. siceraria	1000 g	164.0 g	Aqueous ethanol	16.4%
L. acutangula	1000 g	233.6 g	Aqueous ethanol	23.36 %
M. charantia	1000 g	127.0 g	Aqueous ethanol	12.70 %
C. grandis	1000 g	289.0 g	Aqueous ethanol	28.90 %

3.6.3. Phytochemical screening

The plant extracts were subjected to a qualitative test (Mukherjee, 2002) for identification of various phytoconstituents including carbohydrates, amino acids, vitamin-C, saponins, flavonoids etc. The test procedures are described in Table 3.8. The results of the phytochemical tests confirmed the presence of different secondary metabolites as described in Table 3.9. The results indicated that all the four plants contain a significant amount of phenolic and flavonoid compounds whereas a very low/absence of alkaloidal compounds was observed in all cases. The phytochemical study revealed that the plants contain terpenoids, carotenoids, carbohydrates, and tannins.

Table 3.8. Qualitative phytochemical test

Detection of alkaloids (Trease and Evans, 2009; Mukherjee, 2002) The crude aqueous ethanol extract were dissolved individually in dilute hydrochloric acid and filtered. The filtrates were used to test for the presence of alkaloids.	
Dragendroff's test	0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendroff's reagent were added in 2 ml filtrates in a test tube. Development of orange brown colored precipitate suggested the presence of alkaloid.
Hager's test	2 ml filtrates were treated with Hager's reagent (saturated solution of picric acid solution). Formation of yellow colour precipitate indicates the presence of alkaloids.
Mayer's test	0.2 ml of dilute hydrochloric acid and 0.1 ml of Mayer's reagent (saturated solution of Potassium Mercuric Iodide=1.36 gm of mercuric iodide in 60 mL of water mixed with a solution which contains 5gm of potassium iodide in 20 ml of water) were added to 1.2 ml of extract. Formation of yellowish buff colored extract precipitate indicates presence of Alkaloids.
Wagner's test	2 ml of filtrates was treated with dilute HCl and 0.1 ml of Wagner's reagent (saturated solution iodine in potassium iodide). Formation of reddish brown precipitate indicated the positive response for alkaloid.

Detection of glycosides (Harborne, 1998) The crude aqueous ethanol extract was hydrolysed with dil. Hydrochloric acid, and then subjected to test for glycosides.	
Borntrager's test	Dilute H_2SO_4 was added to the extract and boiled. The filtrate the extracted with ether or chloroform. Ammonia was added to the organic layer. Pink red color in organic layer indicated positive response.
Modified Borntrager's Test	The aqueous ethanol extract were treated with Ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.
Legal test	Sodium nitroprusside solution was added to the extract and dissolve in pyridine and made it alkaline. Pink red color indicated positive response.
Baljet test	Sodium picrate solution was added to the extract. Yellow to orange color indicated positive response

Detection of phytosterols (Mukherjee, 2002)	
Libermann-Burchard Test	10 mg of aqueous ethanol extract was dissolved in 1 ml of chloroform. 1 ml of acetic anhydride was added in that, following 2 ml of concentrated sulphuric acid, a reddish violet color developed, indicating the presence of steroids.
Salkowski Test	Concentrated sulphuric acid was added to 10 mg of extract, dissolved in chloroform. A reddish-blue color exhibited by chloroform layer and green fluorescence by the acid layer suggested the presence of steroids.
Detection of triterpenoids (Harborne, 1998)	
Noller's test	Extract solution was added to, 2 ml of 0.01% anhydrous stannous chloride in thionyl chloride solution. Purple colour formed changed to deep red colour after few minutes indicates the presence of triterpenoids.

Detection of flavonoids (Mukherjee, 2002; Trease and Evans, 2009)	
Zinc hydrochloric acid reduction Test	To the alcoholic solution of aqueous ethanol extract, a pinch of Zinc dust and Conc. HCl was added. Appearance of magenta colour after few minutes indicates presence of flavonoids.
Gelatin Test	To the aqueous ethanol extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of flavonoids.
Lead acetate Test	The aqueous ethanol extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Detection of phenolic compounds and tannins (Mukherjee, 2002)	
Ferric chloride test	5 ml of aqueous ethanol extracts solution was allowed to react with 1 ml of 5% ferric chloride solution. Greenish black coloration indicated the presence of phenolic compounds.
Potassium dichromate test	5 ml of the aqueous ethanol extracts were treated with 1 ml of 10% aqueous potassium dichromate solution. Yellowish brown precipitate suggested the presence of tannin.
Alkaline Reagent Test	The aqueous ethanol extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of tannins.

Detection of reducing sugars (Mukherjee, 2002)	
Fehling's test	5 ml of the extract solution and 5 ml of Fehling's solution were mixed and boiled for 5 minutes. Brick red colored precipitate confirmed the presence of reducing sugars. Fehling's solution A: 34.64 g copper sulphate was dissolved in a mixture of 0.5 ml of H_2SO_4 and sufficient water to produce 500 ml.Fehling's solution B: 176 gm of sodium potassium tartarate and 77gm of NaOH are dissolved in sufficient water to produce 500 ml. Equal volumes of solution A & B are mixed at the time of use.

Benedict's test	5 ml of the filtrate and 5 ml of Benedict's solution (1.73 gm of cupric sulphate, 1.73 gm of sodium citrate and 10 gm anhydrous sodium carbonate are dissolved in water and the volume was made up to 100 ml with water) was mixed in a test tube and boiled for few min. Development of brick red precipitate confirmed the presence of reducing sugars.
Molisch's test	2 ml of conc. sulphuric acid was added carefully along the sides of the test tube containing 2 ml of filtrate. Then it was treated with Molisch's reagent (2.5 gm of pure α-naphthol was dissolved in 25 mL of ethanol). Formation of a reddish violet ring at the junction of two layers indicated the presence of Carbohydrates.

Detection of saponins (Harborne, 1998)	
Foam test	2 gm aqueous ethanol extracts were diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. Development of stable foam suggested the presence of saponins.
Potassium dichromate test	The aqueous ethanol extracts was treated with 1% lead acetate solution. White precipitate indicated the presence of saponin.

Detection of protein and amino Acid (Harborne, 1998)	
Ninhydrin test	Aqueous ethanol extracts were treated with 0.25% ninhydrin (Tri-ketohydrindene hydrate) at the pH range of 4-8. Development of purple color indicated the positive response for amino acids.
Biuret test	The aqueous ethanol extracts were treated with 1 ml of 40% NaOH mixed with 2 drops of 1% copper sulphate. A violet color indicated the presence of proteins.
Xanthoproteic Test	The aqueous ethanol extracts were treated with few drops of concentrated Nitric acid solution. Formation of yellow colour indicates the presence of proteins.

In this chapter, the botanical and morphological characteristics of *L. siceraria, L. acutangula, M. charantia* and *C. grandis* were described. The collection, authentication and extraction of the plants were performed and a significant amount of yield was observed.

Table 3.9. Results of qualitative phytochemical tests

Metabolites	*L. siceraria*	*L. acutangula*	*M. charantia*	*C. grandis*
Alkaloid	-	-	-	-
Phenolics	+	+	+	+
Flavonoids	+	+	+	+
Saponins	+	+	+	-
Anthroquinone glycosides	-	-	-	-
Cardiac glycosides	-	-	-	-
Steroid	-	-	-	-
Triterpenoid	+	+	+	+
Carbohydrates	+	+	+	+
Tannins	+	+	+	+
Monoterpenoids	-	-	-	-
Carotenoids	+	+	+	+

The plant extract was subjected to qualitive phytochemical screening which confirmed the presence of phenolics, flavonoids, glycosides and tannins etc, Further the standardization of the plant extract was done by high performance thin layer chromatography and high performance liquid chromatography. The *in vitro* enzyme inhibition study and kinetics mechanism was carried out with the extract and the partioned fractions for therapeutic validation of the plants against diabetes, obesity, hyperlipidemia, cardiovascular and related disorders.

Chapter 4

4. RP-HPLC standardization of *L. siceraria*, *B. hispida, M. charantia*, *C. grandis*, *C. pepo* and *L. acutangula* with marker compound

4.1. HPLC in medicinal plant analysis
4.2. Importance of cucurbitacin E as marker compound
4.3. Instrumentation and reagents
4.4. Extraction of plant material
4.5. RP-HPLC method development
4.6. RP-HPLC method validation
4.7. Results
4.8. Discussion
4.9. Publication

4.1. HPLC in medicinal plant analysis

High performance liquid chromatography (HPLC) is a versatile chromatographic separation technique for qualitative and quantitative evaluation of targeted marker compound in plant extracts or fractions. The major goal of HPLC is to separation, identification and quantification of phytoconstituents present in plants, in one words complete fingerprinting of a mixture of herbal components. In natural product research, HPLC technique is widely used in authentication, standardization and quality evaluation based on the marker compounds which in terms ensure its quality, safety, and efficacy of medicinal plants/herbal products. Nowadays, HPLC has been hyphenated with various detection techniques, *viz.* UV-Vis, PDA, MS, and NMR to obtain higher sensitivity and detection capabilities of the phyto-compounds for quality control of herbal medicines. The hyphenated chromatographic techniques along with chemometric analysis often offer an excellent approach for evaluation of quality as well efficacy of medicinal plants.
An HPLC system consists of a pump to move the eluent and sample through the system, an injection device to allow sample introduction, a column(s) to provide solute separation, a detector to visualize the separated components and finally a data collection device to assist in interpretation and storage of results. The HPLC analysis may be divided into two phases; normal-phase and reversed phase based on the type of stationary and mobile phase. The selection of any chromatographic mode depends on chemical nature and the compatibility of the analytes with both stationary and mobile phase. The reversed-phase HPLC (RP-HPLC) is the most commonly used mode of HPLC. Apart from that, the HPLC based separation is based on two elution methods; In isocratic method the mobile phase composition and the flow rate is unchangeable throughout the run whereas in gradient elution the mobile phase composition changes over time. The application of isocratic method is in identification of known/selective compounds in a mixture whereas the gradient elution is useful in separating a complex mixture (Sarker & Nahar, 2015).

4.2. Importance of cucurbitacin E as marker compound

The importance of cucurbitaceae species has been highly recognized for effective control of lifestyle diseases such as diabetes, obesity and related disorders (Patel & Rauf, 2017). The fruits of cucurbitaceae family are a good source of glucose, fructose, essential amino acids, vitamins, water-soluble polysaccharides, dietary fibers, phenolic

glycosides, flavonoids, terpenoids, and minerals etc. Apart from the diverse chemical constituents, this family is very well characterized by their presence of cucurbitacin. Cucurbitacin consists of a tetracycline cucurbitane nucleus skeleton with a variety of oxygenation functionalities at different positions with diverse chemical categories. The cucurbitacins are present as non-glycosylated or glycosylated triterpenoids and divided into twelve categories, incorporating cucurbitacins A-T (Chen et al., 2005). Various biochemical studies suggested that cucurbitacins have a potential cytotoxic property which is responsible for making it a prominent lead for anti-cancer drug development (Alghasham, 2013). The hydrophobic property of the cucurbitacin nucleus is a major regulating factor for their cytotoxic effects and it increases linearly with their hydrophobicity (Bartalis & Halaweish, 2005). In particular, cucurbitacin E (Figure 4.1) and their glycosides are the most widely distributed chemical constituents in food plants of cucurbitaceae family (Dhiman et al., 2012). Cucurbitacin E has been reported to possess anti-inflammatory (Abdelwahab et al., 2011), anti-angiogenic, immunomodulatory, cytotoxic (Attard & Cuschieri, 2004), cytostatic and hepatoprotective (Shyam et al., 2010) properties in both *in-vitro* and *in-vivo* model. It has been observed that the combination of cucurbitacin E with other synthetic anti-cancer drugs results in synergistic action in terms of cytotoxicity with a greater efficacy in tumor growth inhibition (Sadzuka et al., 2008). Despite the potential therapeutic activity of Cucurbitacin E and cucurbitacin E glycoside, their chronic exposure is undesirable due to their extremely bitter and disagreeable taste as well as their toxicological effects found in experimental animals (Rupachandra & Sarada, 2013). It has been presumed that back mutated fruits produce more toxicity and bitterness whereas the suppressor gene is responsible for the absence of cucurbitacins (Barham, 1953).

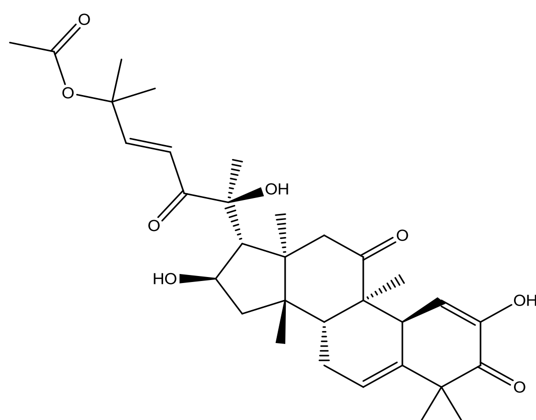


Figure 4.1. Cucurbitacin E

Although a large number of gourd family plants are grown and consumed, six species namely *Lagenaria siceraria*, *Benincasa hispida*, *Momordica charantia*, *Coccinia grandis*, *Cucurbita pepo* and *Luffa acutangula* have potential nutraceutical benefits. The therapeutic benefits of these plants are also well documented in Ayurveda. The present study was aimed to develop a validated RP-HPLC method for standardization of the selected fruits of the cucurbitaceae family by using cucurbitacin E as a marker compound. The validation of RP-HPLC method was further carried out based on the ICH guidelines. This validated method can be applied for quantitative estimation of cucurbitacin E in the cucurbitaceae food plants and their related preparations. The workflow of RP-HPLC study has been described in Figure 4.2.

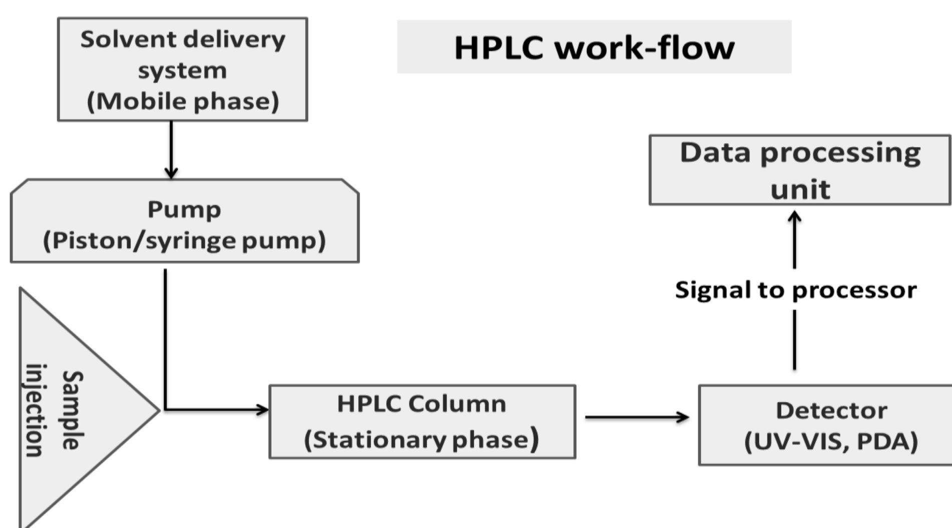


Figure 4.2. Workflow of HPLC study

4.3. Instrumentation and Reagents

The RP-HPLC system (Waters, Milford, MA, USA) consisted of a 600 controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) detector equipped with an in-line degasser AF 2489 and a rheodyne 7725i injector having 20 µl loop volume. Membrane filters (0.45 µm pore size) (Millipore) were used for filtration of the mobile phase. Quantitative estimation was performed with Empower 2 software programs using the external standard calibration method. Methanol (HPLC grade), glacial acetic acid (HPLC grade), petroleum ether and ethyl acetate (analytical grade) were procured from Merck (Mumbai, India). All the other solvents (AR grade) procured from Merck. Cucurbitacin E

(purity ≥ 95 % HPLC) was purchased from Chromadex Inc. USA. All aqueous solutions were prepared using purified water (resistivity of 18.2 MΩ.cm at 25°C) from a Mili-Q filtration system.

4.4. Extraction of plant material

The mature fruits of *L. siceraria*, *B. hispida*, *M. charantia*, *C. grandis*, *C. pepo* and *L. acutangula* were collected from the local market of Jadavpur, West Bengal, India. They were authenticated and the voucher specimen of all of them has been retained in the School of Natural Product Studies (Details in Chapter 3, Section 3.6). The juice was squeezed from the fruits and then filtered through Whatman no. 1 filter paper. The aqueous extract was lyophilized and stored at -20 °C for further use. The % yield of the extracts was calculated. The schematic diagram of extraction has been presented as Figure 4.3.

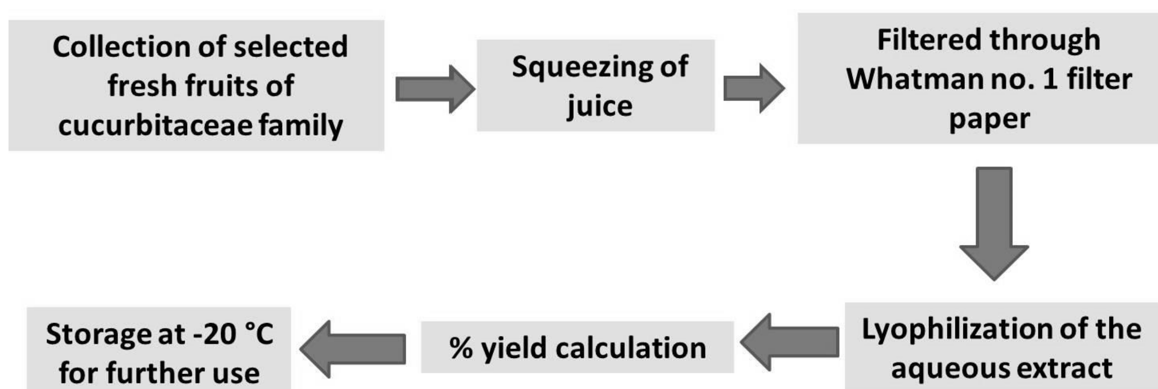


Figure 4.3. Schematic diagram of aqueous extraction of cucurbitaceous fruits

4.5. RP-HPLC method development

The chromatographic method was developed based on the previous method with some modification (Krepsky et al., 2009). The RP-HPLC method was refined by changing the mobile phase composition in a gradient manner and finally the isocratic method was optimized with the mobile phase of acetonitrile (solvent A) and water (solvent B) in the ratio of 70: 30 (v/v). The pH of the solvent B was adjusted at 3.8 by using 1% (v/v) glacial acetic acid. The mobile phase was filtered through 0.45 μm pore size (Millipore) membrane filter followed by sonication to degas the solvent. The separation was carried

out on a Waters Spherisorb 5mm ODS2 column (C18, 250" × 4.6", 5µm particle size). The temperature of the column was kept at 25°C and the injection volume was 20 µl. The total run time was set at 10 minutes. The flow rate was set at 1.0 ml/min and the λ_{max} was set at 230 nm for maximum absorption of the compound. A baseline was recorded with the optimized chromatographic method for about 15 min prior to standard and sample injection. Each chromatographic analysis was followed by a blank run to wash out any carryover from the previous analysis. A standard stock solution of Cucurbitacin E was prepared by dissolving approximately 1 mg of cucurbitacin E in 1 ml methanol. Further dilution was carried out to prepare calibration samples in the concentration range of 1-100 µg/ml. The sample solutions were prepared by taking 10 mg of extract in 1 ml methanol. The solution was filtered through 0.45 µl syringe filter prior to injection. A standard operating procedure (SOP) of the HPTLC method has been developed and maintained in our laboratory.

4.6. RP-HPLC method validation

The RP-HPLC method validation was carried out by determining linearity, specificity, accuracy and precision, limit of quantification and limit of detection on the basis of International Conference on Harmonization guidelines (ICH 1996).

4.6.1. Linearity

The linearity of lapachol and apigenin were found in the range of 10 - 100 µg/ml and 1 - 80 µg/ml respectively, in the optimized chromatographic conditions. The calibration curve was made by plotting the main peak area (Y-axis) against the concentration (X-axis) and linearity was determined by the linear regression analysis.

4.6.2. Specificity

The specificity of the method was determined by comparing the retention time of both standard and test samples. This mainly ensures the identity and the purity of the analyte and to minimize the error due to the contamination of the sample.

4.6.3. Limit of detection (LOD) and limit of quantification (LOQ)

The Limit of Detection (LOD) and Limit of quantification (LOQ) was calculated based on the ICH guideline by determining the SD of the response (σ) and the slope of the linear

equation (S). The LOD and LOQ were calculated by the following equation LOD = 3.3 σ/S, LOQ = 10 σ/S.

4.6.4. Accuracy and precision

Intra-day and inter-day assay accuracy and precision for each analyte were determined at LQC (low quality control), MQC (medium quality control) and HQC (high quality control). Intra-day and inter-day assay, both data were assessed by comparing data from within one run (n = 6). Accuracy of the method was determined by standard addition technique and expressed in terms of % RSD for mean recovery of the theoretical concentration. The samples were spiked with three different amounts of standard compounds in triplicate and analyzed under the previously established optimal condition. The precision of the analytical method was assessed by performing intra-day and inter-day variation, assessed by injecting six replicates at three different concentrations of the reference compounds. Values were represented as % RSD of intra-day and inter-day runs.

4.6.5. System suitability parameters

System suitability testing was performed by using six replicates of test concentrations. The variations in number of theoretical plates, capacity factor and tailing factor were calculated as average of six replicates.

4.6.6. Robustness

Robustness study was performed by changing different mobile phase composition, flow rate and detection of wave length to determine their influence on the retention time. Statistical analysis was performed using the Graph Pad Prism Version 5.0. The results are represented as the mean ± % RSD.

4.7. Results

4.7.1. Extraction yield

The extracts were weighed and the percentage yields were calculated. The percentage yield (%) the aqueous extracts was found maximum for *M. charantia* whereas *C. pepo* was found to be lowest. The extraction yield of the lyophilized aqueous extract was presented in Table 4.3.

4.7.2. Method validation

In RP-HPLC, the linearity range of the response was found to be 1-100 µg/ml. The correlation coefficient was found from the calibration curve as > 0.99, which confirms that the data is closer to the line of best fit. The regression equation was found to be Y=19111X-54747 (Figure 4.4). The specificity of the proposed method confirmed no interference among the peak of standard and test samples. The limits of detection (LOD) and limit of quantification (LOQ) were estimated to be 3.45 and 8.82 µg/ml respectively, which reflect the high sensitivity of the method. The % recovery value (95.35–97.23%) indicated the good accuracy of the method (Table 4.1).

Table 4.1. Accuracy study of RP-HPLC method

Excess CuE added (ng)	Expected CuE in extract (ng)	Average CuE found (ng)	Average Recovery (%)	RSD (%)
0	66.3	63.21	95.35	1.25
10	77.3	74.20	95.99	0.98
40	107.3	103.8	96.82	1.41
80	147.3	143.2	97.23	1.05

The % RSD of intra-day and inter-day precision was reported to be < 2% for in cases of both peak area (response) and retention time, which confirms high repeatability of the method (Table 4.2). The robustness of the experimental method was found to be in the range < 2 %. The number of theoretical plates, capacity factor and tailing factor were found to be 4092 (desirable > 2000), 6.72 (desirable 2–10), 1.35 (desirable < 1.5), respectively, from the mean of six determinations of test concentration.

4.7.3. Estimation of cucurbitacin E by RP-HPLC

The content of cucurbitacin E in the lyophilized extract was determined using the calibration curve by plotting the mean peak area (y-axis) against the concentrations (x-axis). The study confirmed that *C. pepo* contains the highest amount of cucurbitacin E (0.0663% w/w) whereas lowest amount of was reported in *L. siceraria* as 0.0356 % (w/w). The content of cucurbitacin E in the other species varied

Table 4.2. Intra-day and inter-day precision study of RP-HPLC method

Intra-day (n=6)				Inter-day (n=6)			
RT (min)		Response (AU)		RT (min)		Response (AU)	
Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD
4.70	0.87	4753208	1.20	4.68	1.50	4593228	1.28
4.65	1.47	7612069	1.30	4.55	1.17	7292664	1.81
4.69	1.46	16198361	1.25	4.70	1.10	18105372	1.50

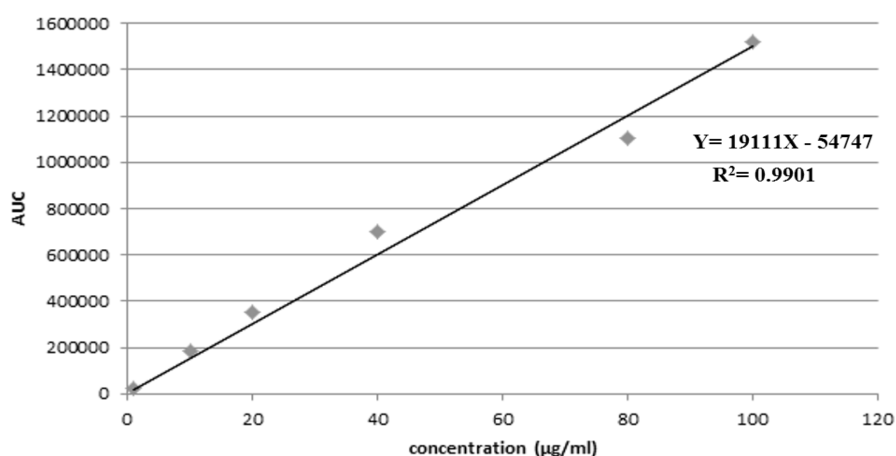
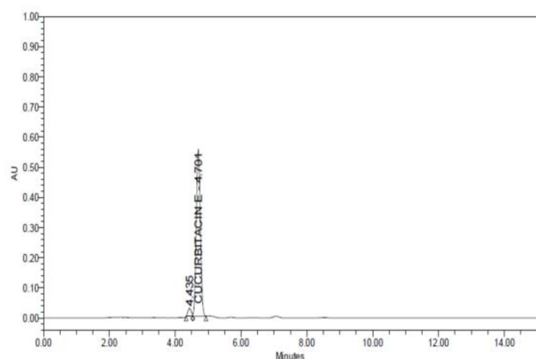


Figure 4.4. Calibration curve of Cucurbitacin E

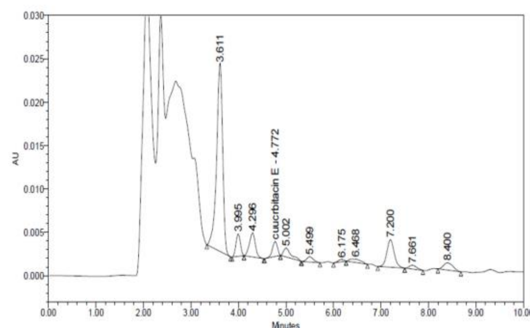
Table 4.3. Content estimation of Cucurbitacin E by RP-HPLC

Plant name	Common name	Extraction yield	Cucurbitacin E content (%w/w per dry plant)
L. siceraria,	Bottle gourd	5.21	0.0356
B. hispida	Wax gourd	4.08	0.0446
M. charantia	Bitter gourd	7.25	0.0523
C. grandis	Ivy gourd	5.88	0.0511
C. pepo	Pumpkin	3.83	0.0663
L. acutangula	Ridge gourd	4.20	0.0556

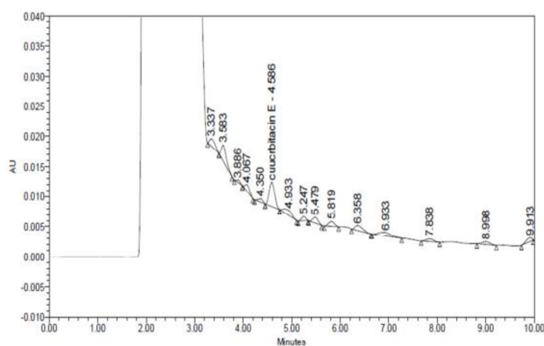
within this range. The content of cucurbitacin E was presented in Table 4.3. The RP-HPLC chromatograms of standard cucurbitacin E and the six species have been shown in Figure. 4.5 (A-G).



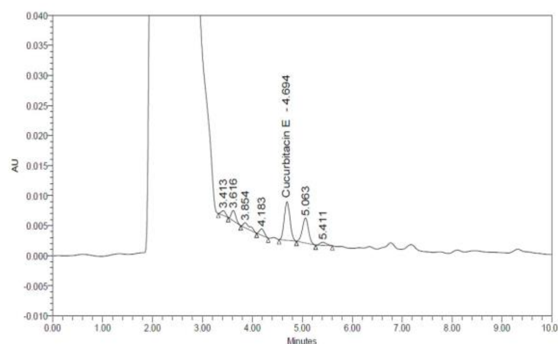
A. Chromatogram of Cucurbitacin E standard



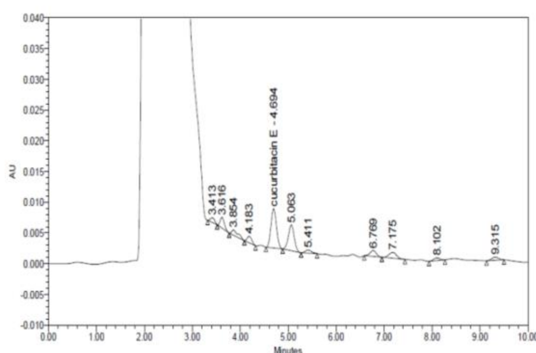
B. *L. siceraria*



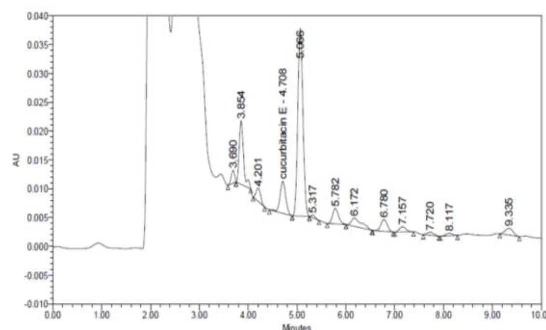
C. *B. hispida*



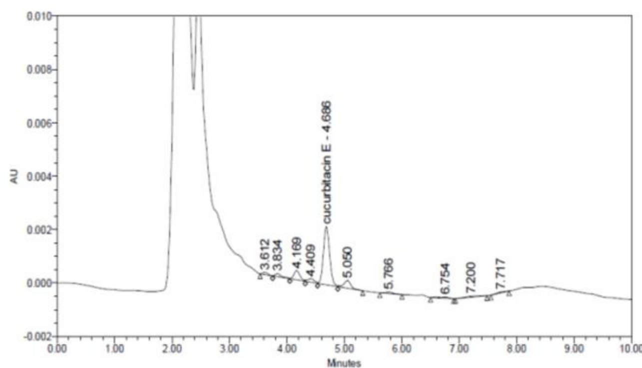
D. *M. charantia*



E. *C. grandis*



F. *C. pepo*



G. *L. acutangula*

Figure 4.5. (A-G) RP-HPLC/UV chromatogram of Cucurbitacin E standard and lyophilized extracts of cucurbitaceae plants. [HPLC condition: Mobile phase of acetonitrile (solvent A): water (solvent B):: 70: 30 (v/v) at λ_{max} of 230 nm]

4.8. Discussion

The aqueous extracts of *L. siceraria*, *M. charantia* are widely used by practitioners of Ayurveda in Indian system of medicine as well as dietary supplements. In India, the fresh juice of *L. siceraria* and *M. charantia* are consumed for their anti-obesity and anti-diabetic properties (Efird et al., 2014; Katare et al., 2014). Although cucurbitacin class of compounds (specifically Cucurbitacin D & E) possesses immense pharmacological potential *viz.* antitumor, hepatoprotective, anti-inflammatory etc. (Miro, 2015), their unpredictable occurrence may lead to be to colitis with bloody diarrhea, severe abdominal cramps, vomiting and hypotension (Bajcsik et al., 2017). In October 2010, Indian Council of Medical Research (ICMR), Ministry of Health & Family Welfare, Government of India conducted a pilot study on the adverse effects of *L. siceraria* after consumption of its juice. The patients were reported to have suffered from diarrhoea, vomiting, elevated levels of liver enzymes and excessive ulceration in distal esophagus (Indian Council of Medical Research Task Force, 2012). There are several other cases of toxicity of the plants of cucurbitaceae family which have been reported in India as well as in other countries like Australia, Alabama and California (Puri et al., 2011). The probable cause of the toxicity lies in is the presence of the active principle, cucurbitacin. It was further observed that the toxicity of cucurbitacin was closely related to their chemical structure, specifically due to the presence of a double bond at C-23 and acetyl group at C-25 in their structure (Kaushik et al., 2015). Reports have been found that,

cucurbitacin and their glycoside exerts potential cytotoxicity in several cell lines. In specific, cytotoxic behaviour of cucurbitacin E was reported at lower IC_{50} value, when studied in human hepatocellular carcinoma HepG2 cell line (Bartalis & Halaweish, 2005). The *in-vivo* toxicity study reported the LD_{50} values of cucurbitacin E at a dose of 2-12.5 mg/kg body weight in mice after oral administration of cucurbitacin derivatives. The toxic effects of cucurbitacin are rendered with increasing the blood pressure and subsequently accumulate fluid in thoracic and abdominal cavities by enhancing capillary permeability in human volunteers. It has been reported that maximum, tolerable limit of cucurbitacin should be restricted for human consumption, although the content of cucurbitacin may vary due to mutations, lack of irrigation and environmental factors. As a large population of India consumes fruit juices of the cucurbitaceae family regularly, the standardization of these fruits with cucurbitacin E as phytomarker is very necessary. This may help in preventing toxicity associated with the Cucurbitaceae food plants at a large. Thus, the RP-HPLC study confirmed the highest cucurbitacin E content in *C. pepo* whereas the lowest amount of was reported in *L. siceraria* fruit.

The developed RP-HPLC method was robust, accurate, precise and reproducible for quantification of cucurbitacin E with a narrow linear range. This validated method can be beneficial for the nutraceutical industry in establishing effective quality control of these fruits for safe human consumption.

4.9. Publication

- Determination of cucurbitacin E in some selected herbs of Ayurvedic importance through RP-HPLC. 2019. *Journal of Ayurveda and Intergrative Medicine* (Elsevier Science, USA). doi.org/10.1016/j.jaim.2019.01.002

Chapter 5

5. HPTLC standardization of *L. siceraria*, *B. hispida, M. charantia*, *C. grandis*, *C. pepo* and *L. acutangula* with marker compound

5.1. HPTLC in medicinal plant analysis
5.2. Instrumentation and reagents
5.3. Extraction of plant material
5.4. HPTLC method development
5.5. HPTLC method validation
5.6. Results & Discussion

5.1. HPTLC in medicinal plant analysis

HPTLC is a versatile, non-destructive, chromatographic separation technique based on the theory of thin layer chromatography. It is considered to be one of the most reliable methods for separation, identification, determination in order to ensure content uniformity, purity profile of the complex herbal medicines. The HPTLC fingerprint analysis helps in identification of marker compounds as well as adulterants present in herbal medicine. HPTLC has also been used to investigate the simultaneous assay of several components in a multicomponent herbal formulation, mostly used for evaluating the compounds with low or moderate polarities. The major advantages of HPTLC method are its reproducibility, simplicity, shorter development time, lower solvent consumption, and improved resolution with compared to conventional TLC method. HPTLC can be regarded as the advanced form of TLC. It uses HPTLC plates featuring small particles with a narrow size distribution. As a result, homogenous layers with a smooth surface can be obtained. HPTLC uses smaller plates (10 × 10 or 10 × 20 cm) with significantly decreased development distance (usually 6 cm) and analysis time (7–20 min). HPTLC plates are used to develop in twin-trough chambers, or horizontal

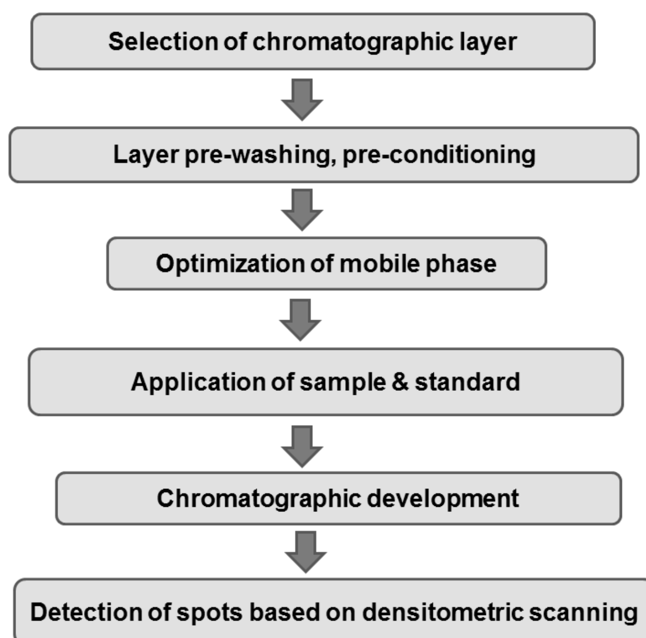


Figure 5.1. Workflow of HPTLC analysis

development chambers, fitted with filter paper to offer the better reproducibility. The detection of separated compounds on the sorbent layers is carried out in UV range (200-400 nm), specifically at 254 and 366 nm. The quantitative estimation of the targeted compounds is done by measuring the zones of samples based on the densitometric scanning. The workflow of a typical HPTLC analysis has been described in Figure 5.1.

HPTLC has a wide application in herbal industry in proper identification of plant materials used as raw materials, content estimation of plant materials after harvesting, storage, and drying stage and also monitoring the fingerprint during production (extraction, blending, packaging) process. Nowadays, HPTLC is hyphenated with mass spectroscopy (MS), Fourier transform infra-red (FTIR), and Raman spectroscopy etc in order to obtain better higher sensitivity for identification and characterization purpose (Srivastava, 2011). In this chapter, the HPTLC based standardization of *L. siceraria*, *C. pepo*, *B. hispida*, *L. acutangula*, *C. grandis* and *M. charantia* was performed with the marker compound, cucurbitacin E. The method was further validated based on following parameters *viz.* selectivity, sensitivity, precision, accuracy, ruggedness, detection limit as per ICH guidelines.

5.2. Instrumentation and Reagents

The CAMAG HPTLC system consisting of WINCATS software, LINOMAT V automatic sample applicator, scanning densitometer CAMAG scanner 3 and photo-documentation apparatus (CAMAG Reprostar 3) were used. The stationary phase was used as aluminium based Silica gel 60 F_{254} plate (Merck, Mumbai) (20 cm x 20 cm) with a particle size of 5-10 µm. A 100 µl syringe (Hamilton, Switzerland) was used for sample application on HPTLC plates. The quantitative estimation was performed with Wincats integration software 4.02 (Switzerland) using the external standard calibration method. Petroleum ether and ethyl acetate (analytical grade) were procured from Merck (Mumbai, India). Cucurbitacin E was purchased from Chromadex Inc.

5.3. Extraction of plant materials

The mature fruits of *L. siceraria*, *C. pepo*, *B. hispida*, *L. acutangula*, *C. grandis* and *M. charantia* were collected from local market of West Bengal. The juice was squeezed from the fruits, and then filtered through Whatman no. 1 filter paper. The details of extrcation, storage and yield value has been presented in Chapter 4.

5.4. HPTLC method development

In case of HPTLC, the mobile phase was optimized with petroleum ether and ethyl acetate in a ratio of 60:40 v/v. The external standard calibration curve for Cucurbitacin E was prepared with calibration solutions in a concentration range of 200 to 1000 ng/ml. Then standard solution was drawn into Camag Linomat syringe and applied through Linomat applicator at the concentrations of standard required. The extract was applied with the same method. The plate was then dried and developed in a glass chamber with the optimized mobile phase. After development, the plate was dried and scanned in Camag TLC scanner 3 at a wavelength of 254 nm. The standard stock solution of Cucurbitacin E was prepared by dissolving approximately 1 mg of cucrbitacin E in 1 ml methanol. Further dilution was carried out to prepare calibration samples in the concentration range of 1-100 µg/ml. The sample solutions were prepared by taking 10 mg of extract in 1 ml methanol. The solution was filtered through 0.45 µl syringe filter prior to injection. A standard operating procedure (SOP) of the HPTLC method has been developed and maintained in our laboratory.

5.5. Method validation

The HPTLC method validation was carried out by determining linearity, specificity, accuracy and precision, limit of quantification and limit of detection on the basis of International Conference on Harmonization guidelines. The validation parameters have been described in Chapter 4.

5.6. Results and Discussion

5.6.1. Extraction yield

The extracts were weighed and the percentage yields were calculated. The percentage yield (%) the aqueous extracts were calculated and the results were represented in Chapter 4. The % yield was found the maximum for *M. charantia* whereas *C. pepo* was found to be lowest.

5.6.2. Method validation

For HPTLC method validation, a calibration curve was prepared with the equation of Y = -328.027+3.881*X (R^2 value 0.99833) (Figure 5.2.a). The % RSD was calculated as 4.09 %. The R_f value of standard cucurbitacin E was found at 0.52. The specificity of the

method was confirmed by matching the R_f values of cucurbitacin E present in standard and sample solutions. The Limit of quantification (LOQ) and Limit of detection (LOD) were found at 10.53 and 3.47 ng per band, respectively, in the linearity range of 200 to 1000 ng per band. The recovery was found in the range of (99.42-99.85 %) indicates higher accuracy of the method (Table 5.1). The intra-day and inter-day % RSD values were found to be < 2%, which proves the higher precision of the method (Table 5.2 & Table 5.3).

Table 5.1. Recovery study of HPTLC method (n=6)

Excess Cucurbitacin E added to extract (ng)	Expected Cucurbitacin E (ng)	Cucurbitacin E found (ng)	Average Cucurbitacin E found (ng)	Average Recovery (ng)	RSD (%)
0	325	323.14 324.11 325.16 323.12 321.12 322.15	323.13	99.42	0.44
200	525	524.12 523.21 522.15 521.15 524.12 522.17	522.82	99.58	0.23
400	725	725.11 722.12 723.15 724.11 721.98 722.33	723.13	99.74	0.17
600	925	924.12 923.15 924.98 921.21 923.53 924.95	923.65	99.85	0.15

Table 5.2. Intra-day precision of HPTLC method (n=6)

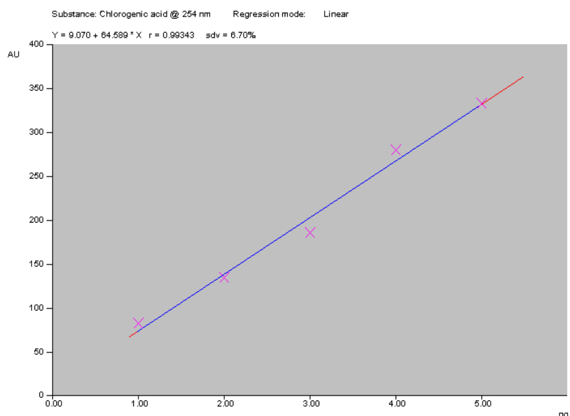
Amount (ng/spot)	Area	Mean	% RSD
200	1793.52 1783.21 1791.78 1799.21 1787.45 1765.98	1786.86	0.65
400	3953.12 3942.91 3941.67 3922.78 3925.12 3941.78	3937.89	0.30

Table 5.3. Intra-day precision of HPTLC method (n=6)

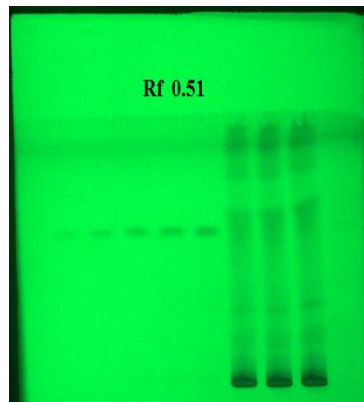
Amount (ng/spot)	Area	Mean	% RSD
200	1778.52 1758.21 1801.78 1711.21 1726.45 1732.98	1751.53	1.96
400	3932.12 3959.73 3921.67 3989.78 3948.16 3911.78	3943.87	2.303

5.6.3. Estimation of cucurbitacin E in lyophilized extract

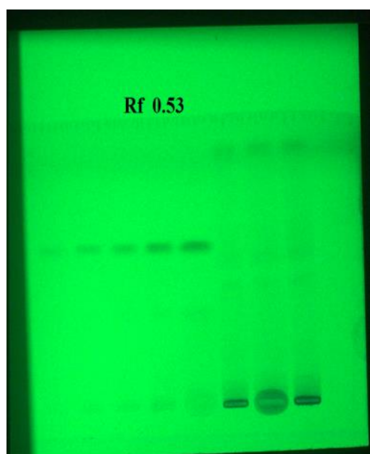
The content of cucurbitacin E in the lyophilized extract was determined using the calibration curve by plotting the mean peak area (y-axis) against the concentrations (x-axis). The % of cucurbitacin E in aqueous extract of *L. siceraria*, *B. hispida*, *M. charantia*, *C. grandis*, *C. pepo*, *L. acutangula* was found in the range of 0.024 to 0.057% w/w. The individual content of the plants has been described in Table 5.4. The picture of the HPTLC plate has been presented in Figure 5.2 (b-f). The HPTLC densitogram of standard cucurbitacin E and the six species have been shown in Figure 5.3 (a-g).



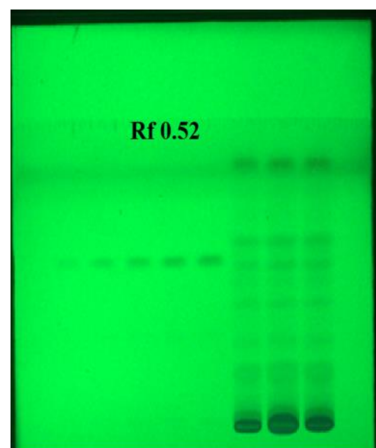
a. Calibration curve



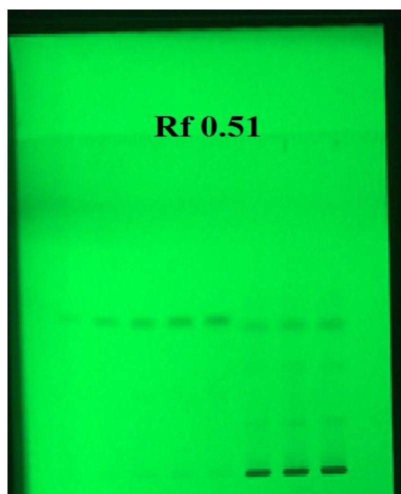
b. ***L. siceraria***



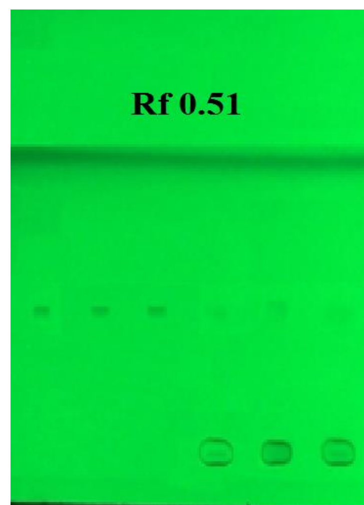
c. *B. hispida*



d. *M. charantia*

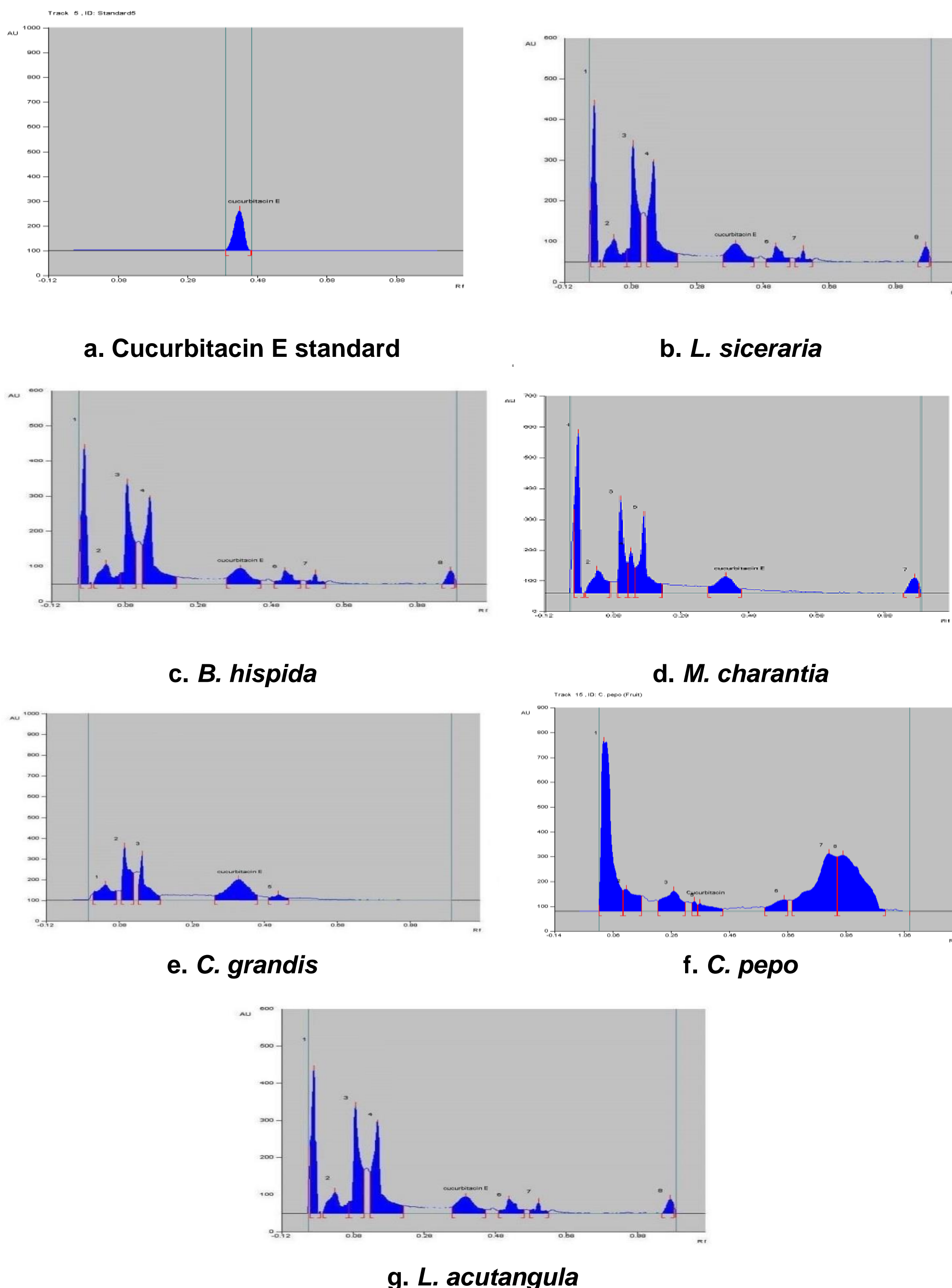


e. *C. grandis*



f. *C. pepo*

Figure 5.2.a. Calibration curve (b-f). Developed HPTLC plate at 254 nm



a. Cucurbitacin E standard **b. *L. siceraria***

c. *B. hispida* **d. *M. charantia***

e. *C. grandis* **f. *C. pepo***

g. *L. acutangula*

Figure 5.3. (a-g) HPTLC densitogram of Cucurbitacin E standard and lyophilized extracts [Condition: Mobile phase of Petroleum ether: ethyl acetate:: 60:40 v/v]

Thus the validated method was found very specific, accurate and reproducible for authentication and quality control of cucurbitaceous plants based on the content of biologically active marker compound, cucurbitacin E.

Table 5.4. Content estimation of Cucurbitacin E by HPTLC method

Plant name	Cucurbitacin E content by HPTLC (%w/w per dry plant)
L. siceraria	0.024
C. pepo	0.063
B. hispida	0.039
L. acutangula	0. 057
C. grandis	0.048
M. charantia	0.053

Nowadays, several analytical approaches are being developed for assessment of food safety that remains a high priority for industry stakeholders, regulatory agencies and consumers. In this context, the developed HPTLC method was very accurate, precise and reproducible for quantification of cucurbitacin E with a narrow linear range. For the optimal benefit of medicinally active cucurbitaceae plants, it is also recommended to ensure the cucurbitacin content in marketed formulation. Finally this study may be able to a way for quality control of the widely consumed fruits of cucurbitaceae family.

Chapter 6

6. Screening of carbonic anhydrase inhibitory activity of *Lagenaria siceraria*, *Luffa acutangula*, *Momordica charantia* and *Coccinia grandis*

6.1. Role of carbonic anhydrase inhibition

6.2. Study of *in vitro* carbonic anhydrase inhibitory activity of four plants

6.3. Carbonic anhydrase inhibition kinetics study of extract and fractions of *Luffa acutangula*

6.4. Carbonic anhydrase inhibitory activity of extract and bioactive constituents of *Lagenaria siceraria*

6.5. Publication

6.1. Role of carbonic anhydrase inhibition

Carbonic anhydrase or EC 4.2.1.1 (CA) isoenzymes are zinc-containing metalloenzymes found in all animals and photosynthesizing organisms. The enzyme was first discovered in red blood cells. The major role of the enzyme is interconversion of carbon dioxide (CO_2) and water $H_2O + CO_2 \leftrightarrow H_2CO_3$ to the bicarbonate ion and protons (or vice-versa). It is important in many physiological functions that involves in carboxylation or decarboxylation reactions. CA family have been classified into α, ß, and γ CA based on their structural and genetic varieties. Among them, α-CA is found mainly in higher vertebrates including humans. Carbonic anhydrase is present in a number of extrarenal tissues, including the eye, gastric mucosa, pancreas, central nervous system (CNS), and erythrocytes (Lindskog 1997). There are fifteen different types of α-CA isozymes (CA I – CA XV), which have been reported based on their amino acid sequences. They also differ in intracellular distribution as well tissue localization. Specifically, α-CA II is one of the most active and cytosolic bound enzymes present in proximal tubular epithelial cells, where they are endowed with the zinc metalloenzyme. CA type II (CAII) are composed of single peptide chains of approximately 260 amino acid residues in length and it coordinated to three imidazole ligands from His 94, His 96 and His 119 and a H_2O molecule which is believed to participate in the catalytic reaction. Carbonic anhydrase catalyses the interaction of CO_2 with H_2O in which the coordinated H_2O molecule deprotonated by the action of enzyme bound Zn. The OH ion acts as a nucleophile to CO_2 and form a bicarbonate complex. Subsequently the H_2O molecule replaces the zinc-bound HCO_3 to form enzyme-Zn-OH_2 complex (Figure 6.1) (Phan et al., 2015). CA also exerts sodium bi-carbonate ($NaHCO_3$) reabsorption and acid secretion by utilizing Na^+-H^+ antiporter in the luminal membrane to transport H^+ ion into the tubular lumen in exchange for Na^+ (Supuran, 2010).

Carbonic anhydrase regulates several physiological and pathological processes, including transportation of CO_2 and bicarbonate ions between metabolizing tissues and lungs, thus maintains the pH of blood and homeostasis. It also plays a significant role in bone resorption, renal hemodynamics, and electrolyte secretion in various tissues and organs and some other biosynthetic reactions *viz.* gluconeogenesis, lipogenesis and ureagenesis. It effects several neuronal cell in brain, mainly through GABA-releasing synapses in the hippocampal network. The therapeutic activities of α-CA isozymes are

widely involved in the treatment of obesity, hypertension, cardiac hypertrophy, oedema, glaucoma, epilepsy and osteoporosis (Supuran, 2010).

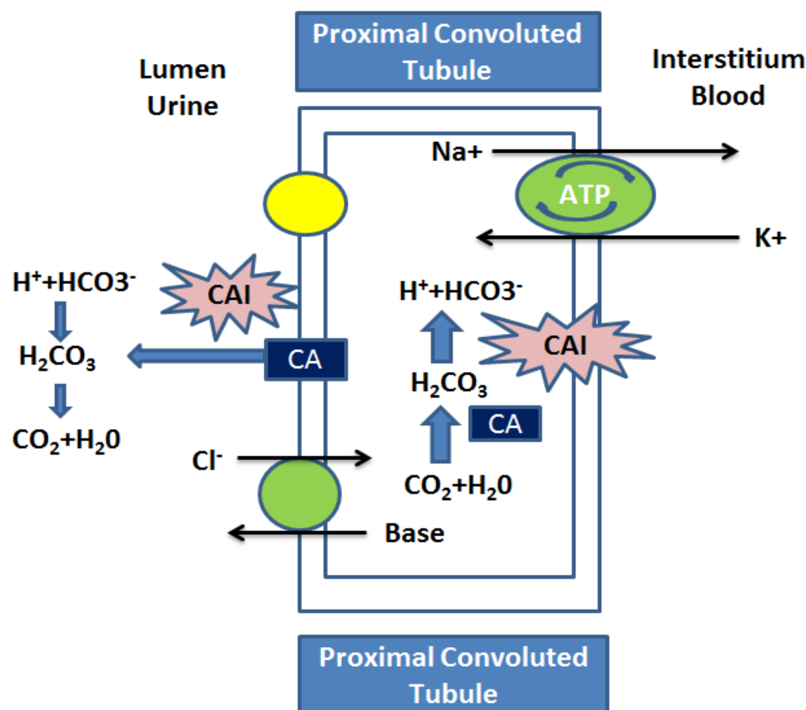


Figure 6.1. Mechanism of inhibition of carbonic anhydrase
[CAI: Carbonic anhydrase inhibitor]

Inhibition of the enzyme often lead to vasodilation of vascular smooth muscle, endothelium and thus may account for their significant physiological benefits. There are major three types of inhibition mechanism have been reported in the literature, Firstly, the inhibitor coordinated to the active site of the enzyme by replacing the zinc-bound water/hydroxide ion, secondly, the inhibitor can anchor with the Zn(II)-bound solvent molecule (water or hydroxide ion) and finally, by occluding the entrance to the cavity of CA active site (Innocenti et al., 2010). Sulfonamides like acetazolamide, dorzolamide, brinzolamide are the most widely used classical carbonic anhydrase inhibitors (mainly CA II mediated). However, they are non-specific to isoenzymes and associated with several adverse reactions like depression, malaise, gastrointestinal irritation, metabolic acidosis, renal calculi, and transient myopia (Lomelino et al., 2016). Hence, there is a need to develop potent and safe CA II inhibitors from natural resources. In this context, several natural phenolic compounds have been reported to possess potential CA II inhibition activity till date.

6.2. Study of *in vitro* carbonic anhydrase inhibitory activity of four plants

Carbonic anhydrase inhibitors offer potential therapeutic application against glaucoma, metabolic acidosis, edema, epilepsy, anti-obesity, and inflammation etc (Supuran 2010). The carbonic anhydrase inhibitory activity of several natural compounds isolated from various medicinal plants, mushrooms have been reported by Sahin et al., 2012. It has been observed that especially polyphenols (phenolic acids, coumarins) and flavones can act as lead compounds against carbonic anhydrase. Moreover, ethnopharmacological uses of some Cucurbitaceae plants against hypertension, edema, obesity insisted on the researchers to explore the pathophysiological mechanism behind the desired therapeutic effects.

In this chapter, the carbonic anhydrase inhibitory potential of *Lagenaria siceraria, Luffa acutangula, Momordica charantia* and *Coccinia grandis* have been studied. The plant with higher inhibition potential was further subjected for analyzing their dose-response, inhibition type, and mode and interaction mechanism. The identification of the bioactive constituents in the active fraction was performed through LC-QTOF-MS, which has been discussed in Chapter 9.

6.2.1. Reagents and chemicals

Carbonic anhydrase isozyme II from bovine erythrocytes (bCA II) (EC-232-576-6) (3848 W/A units mg/solid), 4-nitrophenyl acetate (4-NPA) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Acetazolamide IP (Batch No. AZM-V-P/131107) was procured as a gift sample from Mangalam Drugs and Organics Ltd., Mumbai, India. Tris buffer GR, H_2SO4 (98%), buffer capsules of pH 4.0 ± 0.05 and pH 7.0 ± 0.05 were procured from Merk, Mumbai for calibration of pH meter. All aqueous solutions were prepared using purified water from a Mili-Q filtration system. Tris buffer GR, sulphuric acid (purity 98%), acetone (HPLC grade), dimethyl sulfoxide, petroleum ether, dichloromethane, ethyl acetate, ethanol (synthesis grade) were bought from Merck (Mumbai, India). Acetonitrile, anhydrous acetonitrile (99.8%) and trifluoroacetic acid (HPLC grade ≥ 99.0%) were obtained from Sigma- Aldrich. All aqueous solutions were prepared using purified water (resistivity of 18.2 MΩ cm at 25°C) from a Mili-Q filtration system.

6.2.2. *In vitro* carbonic anhydrase inhibition assay

The carbonic anhydrase inhibitory activity of the plant extracts was assayed based on method described by Bijari et al. with 4-nitrophenyl acetate as substrate (Bijari et al., 2015). The method was based on the change in absorbance due to the liberation of 4-nitrophenol as the hydrolysis product of 4-NPA. In brief, 30 μl of bCA II (115 U/ml in 50 mM Tris-SO_4 buffer at pH 7.4) and 20 μl of test sample (in different concentrations) was added in the reaction mixture. The incubation period was set at 25° C for 15 min. The reaction was initiated by adding 10 mM 4-NPA (in anhydrous acetonitrile) in the inhibitor-enzyme mixture. The absorbance of the samples in each well was determined at 400 nm using a UV-visible spectroscopy (SpectraMax® M2e, Molecular Devices LLC, Sunnyvale, CA, USA). The assay procedure was carried out in triplicate. Acetazolamide was used as a positive control. The related carbonic anhydrase inhibitory activity was calculated based on the following equation:

Relative carbonic anhydrase activity (%) = (catalytic rate of esterase reaction with inhibitor)/ (catalytic rate of esterase reaction without inhibitor) × 100. IC_{50} values of inhibitors were determined by plotting the percentage of enzyme activity against the inhibitor concentration.

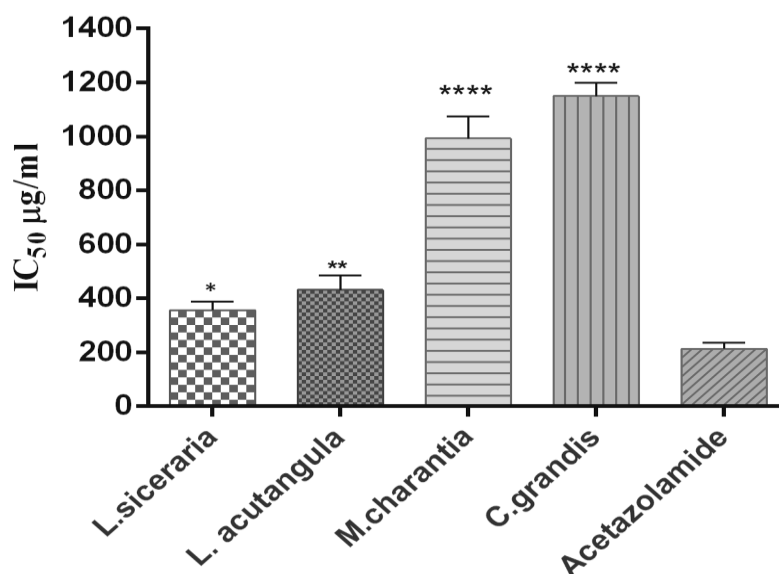


Figure 6.2. Half maximal inhibitory concentration (IC_{50} value) of *L. siceraria*, *L. acutangula*, *M. charantia*, *C. grandis* against carbonic anhydrase . [Acetazolamide —positive control; results are represented as mean ± SD (n = 3); One-way ANOVA with Dunnett's multiple comparisons, at P value 0.05].

6.2.3. Results and discussion

The aqueous ethanol extract of *L. siceraia* and three other plant extracts were tested for carbonic anhydrase inhibitory activity among which *L. siceraria* demonstrated highest inhibition capacity (IC_{50} value 356.03 ± 7.59 µg/ml). The IC_{50} values of other extract was found at 431.9±3.28 (*L. acutangula*), 993.2±5.34 (*M. charantia*) and 1150±7.9 µg/ml (*C. grandis*) respectively.The data was represented in Figure 6.2. Based on the above results *L. siceraria* and *L. acutangula* were furthered considred for dose response and enzyme kinetics study.

6.3. Carbonic anhydrase inhibition kinetics study of extract and fractions of *Luffa acutangula*

Luffa acutangula (L.) Roxb (Ridge gourd), belonging to Cucurbitaceae family, is a widely found vegetative climber throughout south-eastern Asia. The present study evaluated an extract and partitioned fractions of *L. acutangula* for carbonic anhydrase inhibition and total phenolic and flavonoid content; explored the mechanism of inhibition based on the reaction kinetics.

6.3.1. Methodology

6.3.1.1. Fractionation of *L. acutangula* aqueous ethanol extract

Fresh fruits of *L. acutangula* were collected from the local market of Jadavpur, Kolkata, India and extracted with aqueous ethanol based on the method described in Chapter 3. The aqueous ethanol extract was filtered and evaporated under vacuum by using rotary evaporator (EyelaN-1200, Japan) at 45–50°C, to yield the dry extract (23.36 % w/w). Afterwards, the dried extract (120 g) was suspended in water and partitioned successively with petroleum ether, dichloromethane and ethyl acetate (100 ml each). The fractionation was carried out in a repetitive manner followed by vacuum evaporation at 40ºC, to afford a yield of 2%, 4% and 8% (w/w) respectively. The fractions were stored at 4° C for 15 days prior to analysis.

6.3.1.2. Study of carbonic anhydrase inhibition kinetics parameters

Inhibition of carbonic anhydrase inhibition assay by the *L. acutangula* aqueous ethanol extract and subsequent partitioned fractions was determined using a previously reported method with some modifications described in section 6.2.1.4.The carbonic anhydrase

inhibitory activity (%) was calculated based on the following equation: Enzyme Activity = (ΔA_{400}nm/min Test - ΔA_{400} nm/min Blank) (Volume of assay, µl) / ($\mathcal{E}_{nitrophenolate}$= 18,400 L/mol/cm) (Volume of enzyme used, µl); Ɛ is the extinction coefficient of nitrophenolate. IC_{50} values of inhibitors were determined by plotting the percentage of relative enzyme activity (activity without inhibitor) against the inhibitor concentration. The enzymatic activity assay without inhibitor was defined as 100%. To explore the inhibitory mechanism (reversible or irreversible) of ethyl acetate fraction, plots of reaction velocity (ΔOD/min) vs. enzyme concentrations [E] at different sample concentrations were constructed (Biswas et al., 2017). The inhibition kinetics of ethyl acetate fraction was also performed using the in vitro carbonic anhydrase inhibition assay method with different concentrations of substrate (0.5–4 mM) and the inhibitors (0–450 µg/ml). The amount of reaction product formed was monitored at a 20-second interval for 10 mins. Afterward, the V_{max} and K_{app} were calculated according to the Michaelis-Menten equation and Lineweaver-Burk plot based on the following equation: For non-competitive, $1/V=K_m/V_{max}\times(1+[I]/K_i)\times1/[S]+1/V_{max}\times(1+[I]/\alpha K_i)$, where v is the reaction velocity; K_m is the Michaelis constant; Vmax is the maximal velocity; [I] is the concentration of inhibitor; K_i is the inhibitor constant; [S] is the concentration of substrate; α is the apparent coefficient. The Ki and αK_i values were determined by the secondary plot of the slope (K_m/V_{max}) and Y-intercept ($1/V_{max}$) vs. inhibitor concentration [I].

6.3.1.3. Estimation of total phenol and flavonoid content

Total phenolic and flavonoid content of the aqueous ethanol extract of *L. acutangula* fruits and the subsequent three partitioned fractions was determined using a previously described method with some modification (Biswas et al., 2016a). The sample was prepared by weighing approximately 1 mg of each fraction, dissolved in 1 ml of methanol followed by filtration with 0.45 µm syringe filter to get a concentration of 1mg/ml. The reaction mixture was prepared by mixing the sample (18 µl), 10% Folin-Ciocalteu's reagent (90 µl) and 7.5% $NaHCO_3$ (90 µl). The samples were thereafter incubated in a BOD incubator at 45°C for 45 min. Gallic acid was used as to generate a five-point calibration curve at the concentration range of 5-30 µg/ml. The absorbance was measured at 725 nm using a spectrophotometer (SpectraMax Plus, Molecular Devices LLC, USA). The total phenol content was calculated as mg gallic acid equivalent (GAE)/g

of sample. The total flavonoids content was also determined by spectrophotometric assay(Biswas et al., 2016a). Quercetin was used as standard. The five-point calibration curve of quercetin was prepared with different concentration range (10–75 µg/ml) with methanol. Initially, 3% NaNO2 (10 µl), 1M NaOH (50 µl) and 15% $AlCl_3$ (10 µl) were added in the reaction mixture and total volume of 170 µl was made by adding methanol. The absorbance of the mixture was determined at 415 nm. The content of total flavonoids was calculated as mg of quercetin equivalents (QE)/g of sample. The results were represented as mean ± standard deviation (SD) (n=3). In addition, to understand the interrelationship between carbonic anhydrase inhibitory activity (IC_{50} value) and the total phenol and flavonoid content of *L. acutangula* aqueous ethanol extract and subsequent fractions, Pearson's correlation coefficient was calculated.

6.3.1.4. Statistical analysis

The statistical analysis of all the experimental data were expressed as mean value ± standard deviation (SD) (n = 3). The significance of the difference between mean (control vs. test) was determined by Student's t-test at the P value < 0.05. The Pearson correlation test was employed to analyse the correlation coefficients between the content of polyphenols and bioactivities. The statistical analysis was performed by Graph pad prism 6.0 software.

6.3.2. Results and discussion

6.3.2.1. Effect of *L. acutangula* fractions on carbonic anhydrase activity

The carbonic anhydrase inhibitory activity was represented as half-maximal inhibitory concentration (IC_{50}). The IC_{50} values of the aqueous ethanol extract and the three subsequent partitioned fractions and acetazolamide (positive control) were estimated to be 286.0±2.41 (ethyl acetate fraction), 468.40±4.32 (aqueous ethanol extract), 609.60± 2.35 (dichloromethane fraction), 760.80±3.85 (petroleum ether fraction) and 203.6±2.08 (acetazolamide) µg/ml. The mean IC_{50} value of ethyl acetate fraction was found to be significantly similar (P value < 0.05) to the standard inhibitor acetazolamide. The decrease in relative enzyme activity with increasing concentration of ethyl acetate fraction confirmed the dose-dependent inhibition against carbonic anhydrase (Figure 6.3). The plots of the reaction velocity (v) vs. enzyme concentration at different inhibitor concentrations gave a group of straight lines. It was noted that all the straight lines

passed through the origin and the slope of the line decreased inversely with the increasing concentration of the inhibitors (Figure 6.4). This result suggested that ethyl acetate fraction inhibited carbonic anhydrase, reversibly (Biswas et al., 2017).

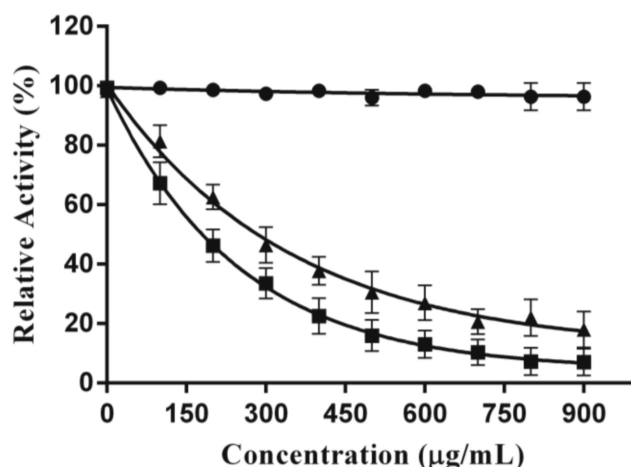


Figure 6.3. Dose dependent inhibition pattern of acetazolamide and ethyl acetate fraction on carbonic anhydrase inhibitory activity.[(●) without inhibitor; (▲) Ethyl acetate fraction; (■) acetazolamide; data are presented as mean ± SD (n = 3)].

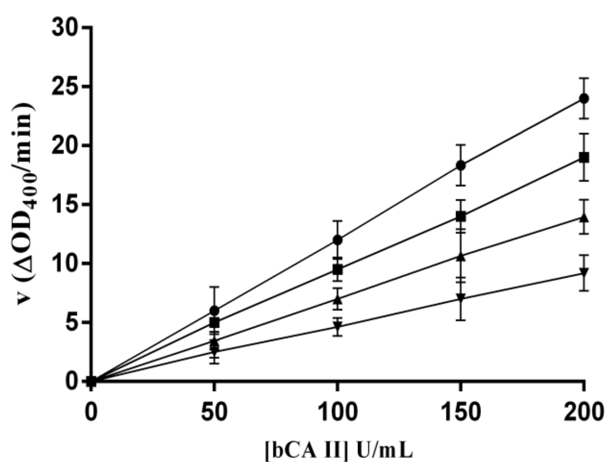


Figure 6.4. Reversible inhibition of ethyl acetate fraction against carbonic anhydrase activity [Plots of velocity vs. enzyme concentration]. [The v value indicates the change in absorbance at 400 nm/min at ethyl acetate fraction concentrations of 0 (■), 250 (▼), 500 (▲) and 750 (●) µg/ml. Data presented as mean ± SD (n = 3)].

6.3.2.2. Kinetics analysis of carbonic anhydrase inhibitory activity

The bCA II catalyzed 4-NPA esterase reaction rate was dependent on the concentration of ethyl acetate fraction as the initial reaction velocity was decreased with its increasing concentration. Based on the best-fit values of the Michaelis-Menten parameters, it was

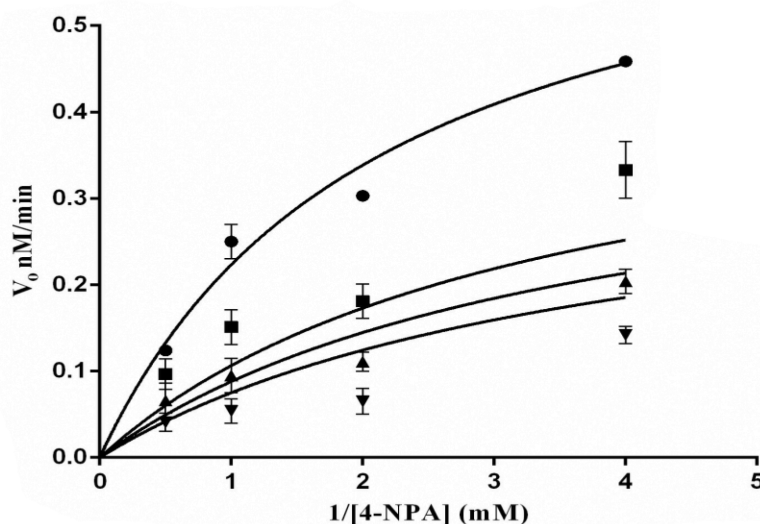


Figure 6.5.a. Kinetics study of ethyl acetate fraction against carbonic anhydrase activity [The data were fit to the Michaelis-Menten equation using nonlinear regression analysis];

observed that both the apparent values for V_{max} and the K_m changes in presence of inhibitors. The V_{max} and K_{app} values were calculated as 0.70 nM/min and 2.66 mM respectively (Table 6.1). In addition, Lineweaver–Burk plot was constructed by plotting 1/V (velocity) vs. 1/[S] [substrate] which gave a family of straight lines with different slopes Figure 6.5.a. The secondary re-plot of Y-intercept ($1/V_{max}$) vs. [ethyl acetate fraction] gave αK_i value as 2.14 ± 0.22 µg/mL whereas the K_i value was calculated as 182.50 ± 0.32 µg/ml from the replot of slope (K_m/V_{max}) vs. [ethyl acetate fraction] (Figure 6.5.b and 6.5.c). These two different K_i values indicated that the inhibitor can bind to both enzyme (K_i) as well as ES complex (αK_i), referring to mixed type of inhibition (Biswas et al., 2016b).

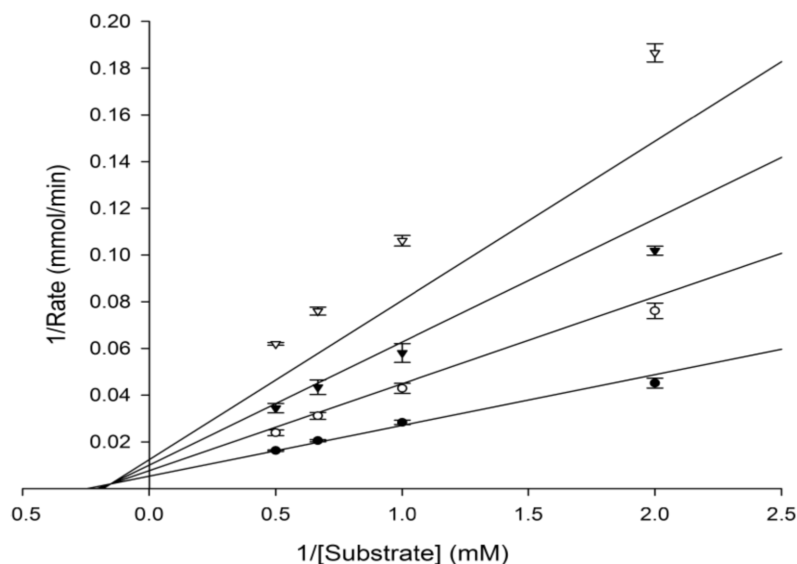


Figure 6.5.b. Lineweaver–Burk plot of the Michaelis–Menten data, represent mixed inhibition behaviour of ethyl acetate fraction on CA II. [The ethyl acetate fraction concentrations are (●) 0, (■), 250, (▲) 350 and (▼) 450 µg/ml. Data presented as mean ± SD (n = 3).

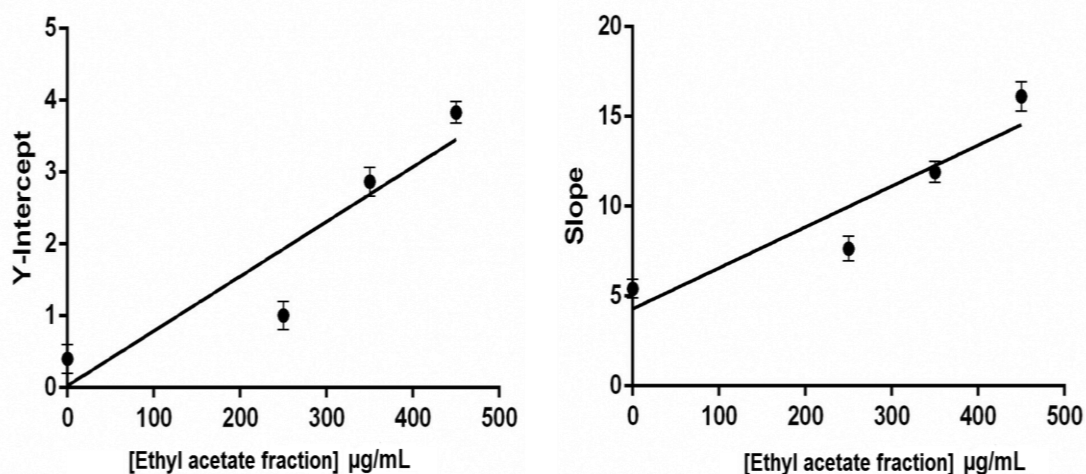


Figure 6.5.c. Plot of Slope (K_m/V_{max}) and Y intercept ($1/V_{max}$) vs. concentration of ethyl acetate fraction. Secondary replots were constructed from the data, collected from Lineweaver-Burk plot. [Concentrations of ethyl acetate fraction were 0, 250, 350, 450 µg/ml].

Table 6.1. Kinetics parameters of carbonic anhydrase inhibition of ethyl acetate fraction

Kinetics parameters	Value Mean ± SD (n=3)
$V_{max}{}^{a}$	0.70 nM/min
$K_{app}{}^{b}$	2.66 mM
$K_i{}^{c}$	182.50 ± 0.32 μg/ml
$\alpha K_i{}^{d}$	2.14 ± 0.22 μg/ml
IC_{50}	286.0 ± 2.40 μg/ml
Type of inhibition	Mixed

[$^{a}V_{max}$: Maximum reaction velocity; $^{b}K_m$:Apparent Michaelis-Menten constant; $_{c}K_i$- Inhibition constant; αK_i: Inhibition constant]

6.3.2.3. Correlation analysis between total phenol and flavonoid content and carbonic anhydrase inhibitory activity

The total phenol and flavonoid content were found to be maximum in ethyl acetate fraction at concentration 102.80 ± 3.03 mg GAE/g and 47.80 ± 8.41 mg QE/g of sample, respectively followed by the aqueous ethanol and dichloromethane fractions. The petroleum ether fraction had a very low total phenol and flavonoid content. A statistically significant correlation (r = 0.9581) was obtained between the total phenol content and carbonic anhydrase inhibition activity (IC_{50} value) of ethyl acetate fraction. However, no significant correlation was observed between total phenol and flavonoid content and carbonic anhydrase inhibitory actions of other fractions, which may be due to the difference in the contents and varieties of phenolic compounds (Table 6.2). Several phenolic compounds extracted from plants, mushrooms and honey inhibited carbonic anhydrase enzyme, which corresponds to their higher phenol and flavonoid content (Sahin et al., 2012). Some phenolic acids (viz. benzoic acid, chlorogenic acid, m-hydroxybenzoic acid, o-coumaric acid etc.) and flavonoids (viz. genistein) have also been reported to be active against carbonic anhydrase (Mocan et al., 2018). This study explored the abundant presence of phenolics as well as flavonoids in ethyl acetate fraction of *L. acutangula* fruit extract which may be effective to inhibit carbonic anhydrase.

Table 6.2. Correlation analysis between the total phenol and flavonoid content of *L. acutangula* fractions and their carbonic anhydrase inhibitory activity (IC_{50} value)

Correlations	Pearson correlation coefficient (*r* value)
Total phenol content and ethyl acetate fraction IC_{50}	0.9581**
Total flavonoid content and ethyl acetate fraction IC_{50}	0.5560^{ns}
Total phenol content and aqueous ethanol fraction IC_{50}	0.2393^{ns}
Total flavonoid content and aqueous ethanol fraction IC_{50}	0.5477^{ns}
Total phenol content and dichloromethane fraction IC_{50}	0.1017^{ns}
Total flavonoid content and dichloromethane fraction IC_{50}	0.6743^{ns}
Total phenol content and petroleum ether fraction IC_{50}	0.0296^{ns}
Total flavonoid content and petroleum ether fraction IC_{50}	0.0515^{ns}

[Correlation is significant at; ns- not significant (2-tailed); ** p-value < 0.01]

The ethyl acetate fraction of the aqueous ethanol extract of *L. acutangula* showed highest carbonic anhydrase inhibition activity. The enzyme kinetics analysis indicated a mixed mode of inhibition. The concentration of total phenolics and total flavonoids in the extract and each fraction thereof correlated with the level of carbonic anhydrase inhibition activity. Furthermore, the UPLC-QTOF-MS analysis of the ethyl acetate fraction identified a number of phenolic acids, hydroxycoumarins, flavones, flavanones, and flavonoids that might confirm that phenolic compounds of *L. acutangula* offer potential therapeutic benefits against carbonic anhydrase-related disorders.

6.4. Evaluation of carbonic anhydrase inhibitory activity of extract and bioactive constituents of *Lagenaria siceraria*

This study was designed to evaluate the carbonic anhydrase inhibitory activity of an extract, partitioned fractions and major phytoconstituents present in of *L. siceraria* fruits. The phytochemical profile of *L. siceraria* has been described in details in Chapter 3. This work was also targeted to explore the underlying carbonic anhydrase inhibitory mechanism of the bioactive compounds by enzyme kinetics and molecular docking study.

6.4.1. Methodology

6.4.1.1. Extraction and fractionation

The filtrate was dried under rotary evaporator, and the residue was collected. The % yield of aqueous ethanol extract (Fr.A) was found as 29.7% (w/w). Then the extract (dissolved in water) was sequentially fractionated by liquid-liquid partitioning with hexane and ethyl acetate. The obtained fractions were subjected to evaporation to get the semisolid mass. The yields of the aqueous (Fr.B), ethyl acetate (Fr.C) and n-hexane (Fr.D) fractions were calculated as 19.5, 12.8 and 8.0 % (w/w) respectively. These fractions were assayed for carbonic anhydrase inhibition. Among these fractions, highest inhibitory activity was observed in Fr.A, which subsequently targeted to flash chromatography (Biotage–Isolera one, Sweden) using a silica gel Cartridge (Biotage® SNAP cartridge KP-Sil 50 g). The workflow of extraction and fractionation of the plant material was described in Figure 6.6. The carbonic anhydrase inhibitory activity was carried out at the sample concentration of 400 µg/ml. A step gradient of ethyl acetate (mobile phase A) and methanol (mobile phase B) starting from 10 to 40% methanol in 45 min at a flow rate of 20 ml/min, was employed. The eluting fractions were monitored at the wavelengths of 280 and 320 nm. Total thirty sub-fractions (Fr.A.1-A.30) were separated and their carbonic anhydrase inhibitory activity was evaluated, in terms of half maximal inhibitory concentration (IC_{50}). Based on the IC_{50} values, six sub-fractions were selected with higher inhibition potential.

6.4.1.2. Estimation of total phenolic and flavonoid content

The total phenol and flavonoid content (TPC and TFC) was estimated according to the method reported earlier from our laboratory with some modification (Biswas et al., 2016a). The total phenol content (TPC) was calculated as mg gallic acid equivalent (GAE)/g of the sample. The total flavonoid content was calculated as mg of quercetin equivalents (QE)/g in the sample. The result was represented as the mean ± standard deviation (SD) (n=3).

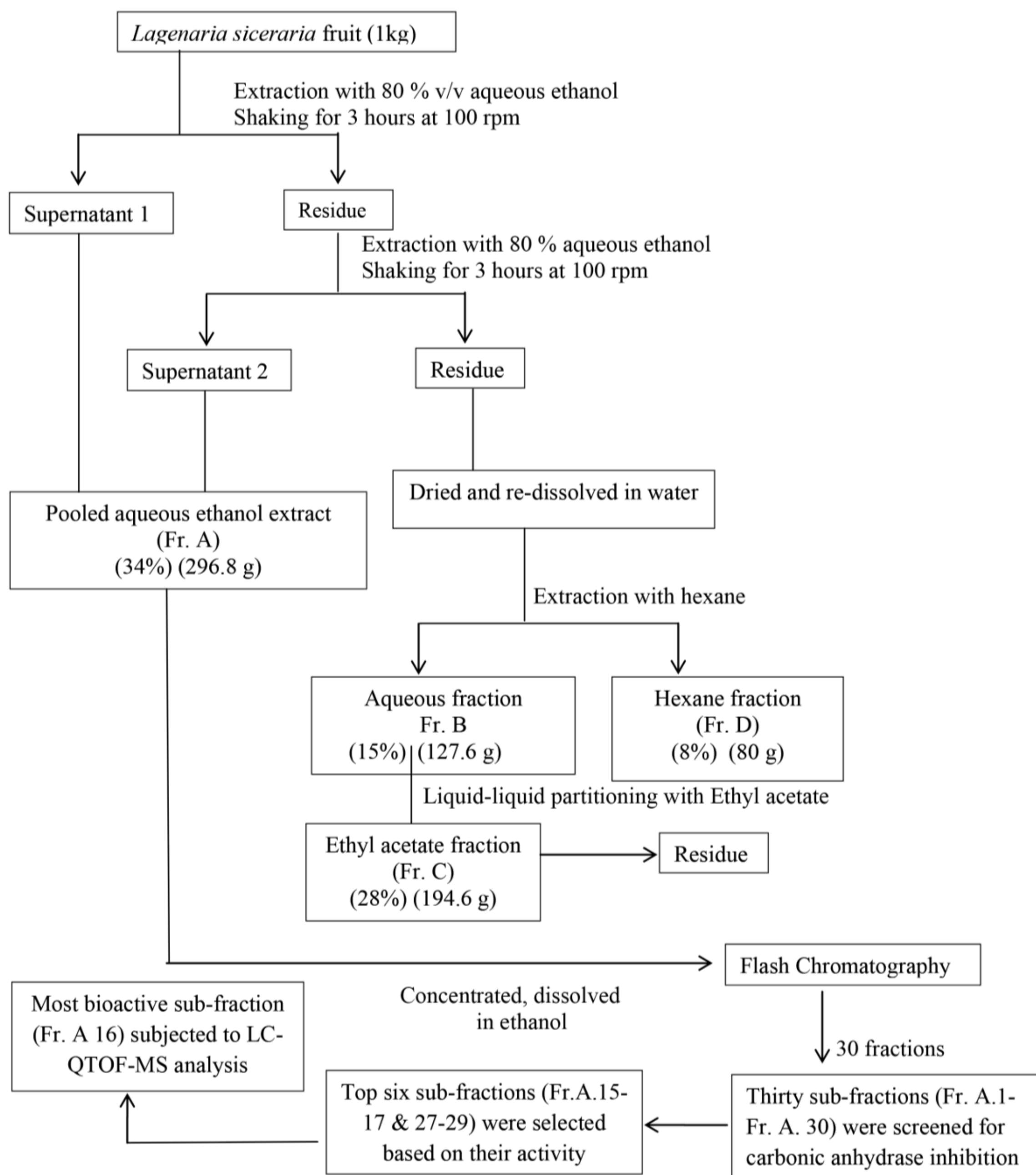


Figure 6.6. Schematic representation of extraction and fractionation of *L. siceraria* fruit. [Carbonic anhydrase inhibition activity represented as percentage (%); Yield of the fraction in (g)]

6.4.1.3. Carbonic anhydrase inhibition kinetics study

The carbonic anhydrase inhibitory activity of *L. siceraria* extract and fractions, coniferyl alcohol (CFA), ferulic acid (FA), p-coumaric acid (4-CA), hesperidin, and apigenin-7-glucoside was assayed based on the method described by Bijari et al. with 4-nitrophenyl acetate as substrate. The method was based on the change in absorbance due to the liberation of 4-nitrophenol as the hydrolysis product of 4-NPA. The details method of enzyme inhibition assay has been described in section 6.2.1.4. The absorbance of the samples in each well was determined at 400 nm using a UV-visible spectroscopy (SpectraMax® M2e, Molecular Devices LLC, Sunnyvale, CA, USA). The assay procedure was carried out in triplicate. Acetazolamide was used as a positive control. The related carbonic anhydrase inhibitory activity was calculated based on the following equation: Relative activity (%) = (catalytic rate of esterase reaction with inhibitor)/ (catalytic rate of esterase reaction without inhibitor) × 100. IC_{50} values of inhibitors were determined by plotting the percentage of enzyme activity against the inhibitor concentration.

The bioactive constituent found to be present in the active fraction, was subjected to enzyme inhibition kinetics study. To explore the inhibitory mechanism, reversibility of the enzyme-inhibitor reaction were studied. The plots of residual activity (expressed in ΔAbs/min) vs. bCA II concentrations (0-200 U/ml) [E] at different sample concentrations (0-300 mM) were constructed (Biswas et al., 2017). Also in order to determine the inhibition type, enzyme kinetics analysis was performed using the above spectroscopic method with different concentrations of substrate (0.5-4 mM) and the inhibitors (0-300 mM). The amount of reaction product formed was monitored at a 20-second interval for 10 min. The enzyme kinetics parameter was expressed linearly by using the Lineweaver–Burk equation in the double reciprocal form.

The V_{max}, apparent K_m and K_i values were calculated based on the equation as follows.
For competitive, $1/V= K_m/V_{max}(1+[I]/Ki)\ 1/S + 1/V_{max}$
For non-competitive, $1/V=K_m/V_{max}\times(1+[I]/K_i)\times1/[S]+1/V_{max}\times(1+[I]/\alpha K_i)$, where v is the reaction velocity; K_m is the Michaelis constant; V_{max} is the maximal velocity; [I] is the concentration of inhibitor; K_i is the inhibitor constant; [S] is the concentration of substrate; α is the apparent coefficient. The values were calculated by using the software SigmaPlot 12.5.

6.4.1.4. Identification and quantification of bioactive constituents

The LC-QTOF-MS analysis was carried out for putative identification of the major constituents present in the most potent bioactive fraction. The RP-HPLC analysis was further performed to quantify the bioactive compounds present in the active fraction.The chromatographic and mass spectrometric method has been described in Chapter 9.

6.4.1.5. Molecular docking study

The LC-QTOF-MS analysis of the bioactive fraction revelaed the presence of phenolic constituents *viz.* ferulic acid, p-coumaric acid, coniferyl alcohol (Data represented in Chapter 9). Molecular docking studies were carried out to investigate the binding modes of these phenolic compounds as well as the interactions in the active site. Docking simulations of molecules were performed using Schrodinger software suite 2013-3 (Schrödinger, LLC, New York, NY, 2013) which utilizes Glide module for predicting protein-ligand binding modes as well as binding affinity (Pagadala et al.,2017). Based on the earlier report, the crystal structure of bovine carbonic anhydrase-II (PDB ID: 1V9E) was considered for docking analysis (Bijari et al., 2015). The X-ray crystal structural coordinates (PDB ID: 1V9E) was retrieved for structural bioinformatics (RCSB) protein data bank (www.rcsb.org/). The search grid was generated by picking the co-crystal Zn atom and extended up to 20 Å. Here, Zn atom and nearby amino acid residues (Thr-200 and Gln-92) were found responsible for enzyme catalytic activity (Chiuri et al., 2009). The protein was prepared by the protein preparation wizard of Maestro, version 9.6 and finally minimized by using OPLS 2005 force field. The hydroxyl groups of search area were kept flexible during grid generation process. The 3D structures of coniferyl alcohol, ferulic acid, and p-coumaric acid were generated by using Chem3D Pro 11.0 (Cambridge Soft, 2008). The ligand preparation module (LigPrep) of Maestro 9.6 was used to prepare and optimize various tautomers, bond orders, ring conformations and stereochemistry of ligands. All the conformations generated were minimized using OPLS2005 force field before docking study. Molecules were docked by using Glide XP (extra precision) docking mode. The Glide docking score was used to determine the best-docked structure from the output. The best Glide scoring (i.e., lowest docking energy) was chosen to represent the most rationalized binding mode of ligands with bCA II.

6.4.2. Results and discussion

6.4.2.1. Carbonic anhydrase inhibitory activity guided fractionation

The aqueous ethanol extract (Fr. A) and the three fractions were tested for carbonic anhydrase inhibitory activity among which Fr. A demonstrated highest inhibition capacity. In addition, the carbonic anhydrase inhibition potential of the sub-fractions (of Fr. A) was calculated and subsequently six sub-fractions (Fr. A.15-Fr. A.17 and Fr. A. 27-Fr. A. 29) were selected on the basis of their bioactivity. Moreover, among the six major sub-fractions (from Fr. A), Fr.A.16 offered better inhibition profile, even higher than Fr. A. The statistical significance between the IC_{50} value of Fr.A.16 (328.87 ± 7.87 µg/ml) and acetazolamide (IC_{50} value 222.54 ± 4.25 µg/ml) was found at P value < 0.05 whereas the IC_{50} value of Fr. A was significantly higher at P value < 0.001 with compared to acetazolamide as positive control (IC_{50} value 222.54 ± 4.25 µg/ml). The result showed that Fr.A.16 was a potent fraction as the comparison to Fr. A. Other fractions were ranked in the following order based on their enzyme inhibition potential: Fr. A.15 > Fr. A.17 > Fr. A.28 > Fr. A. 29 > Fr. A.27. Further, Fr. A.16 was directed to LC-QTOF-MS analysis to identify the major constituents present in it.

6.4.2.2. Total phenolic and flavonoid content

All the six sub-fractions contained a significant amount of phenolic and flavonoid compounds. The TPC was found highest in Fr A.16 as 294.64±2.85 mg GAE/g of sample whereas TFC was found the maximum in Fr. A.17 (124.47±2.44 mg QE/g of sample) followed by Fr A.16 (104.11±1.32 mg QE/g of sample). The TPC and TFC were found lowest in both cases of Fr. A. 27 as 62.21±1.80 mg GAE/g and 90.21±3.16 mg QE/g of sample respectively. The TPC and TFC of all the six sub-fractions have been represented in Table 6.3. In addition the total phenol content of Fr. A. 16 was found highest with compared to other sub-fractions.

Table 6.3.Total phenol and flavonoid content of *L. siceraria* aqueous ethanol sub-fractions

L. siceraria **ethanol sub-fraction (Fr. A)**	**Total phenol content (mg GAE/g of sample) Mean ± SD (n=3)**	**Total flavonoid content (mg QE/g of sample) Mean ± SD (n=3)**
Fr. A. 15	232.06 ± 2.02	118.64 ± 1.79
Fr. A. 16	294.64 ± 2.85	104.11 ± 1.32
Fr. A. 17	198.42 ± 6.32	124.47 ± 2.44
Fr. A. 27	62.21 ± 1.80	90.21 ± 3.16
Fr. A. 28	69.13 ± 4.11	94.31 ± 5.13
Fr. A. 29	78.45 ± 3.26	100.13 ± 2.05

6.4.2.3. Kinetics analysis of bioactive constituents against carbonic anhydrase

The half maximal inhibitory concentration (IC_{50}) values of CFA, FA, 4-CA, hesperidin, and apigenin-7-glucoside were calculated. The results of the enzyme inhibition assay showed that that CFA, FA, and 4-CA inhibited carbonic anhydrase activity significantly (IC_{50} value range of 80-250 μM), whereas hesperidin and apigenin-7-glucoside (both are flavonoid glycoside) showed weak inhibition profile (IC_{50} value > 500 μM). The inhibitory property of CFA was found to be highest (IC_{50} value 80.38 ± 3.54 μM) whereas FA and 4-CA showed less inhibition with a higher IC_{50} value of 130.15 ± 5.38 and 256.52 ± 7.16 μM respectively. The summary of enzyme inhibition and kinetics data has been shown in Table 6.4. The carbonic anhydrase inhibitory activity of these compounds was found dose-dependent as well reversible in nature (Fig.6.7 and Fig.6.8).

In order to explore the inhibition mechanism, the kinetics of enzyme-ligand reaction was determined by analyzing Lineweaver-Burk plot. The Lineweaver-Burk plot suggested that vertical axis intercept ($1/V_{max}$) values were changed whereas K_m value remains constant after the addition of both CFA and FA in increasing concentration. This observation indicated that both CFA and FA inhibited carbonic anhydrase in a non-competitive manner (Figure 6.9). In contrast, the changes of K_m value with a constant V_{max} value was observed with increasing concentration of 4-CA, suggesting its competitive inhibition pattern. Interestingly, our observations also supported the previous finding of the inhibition mode of some phenolic acids against carbonic anhydrase (Sarikaya et al., 2010).

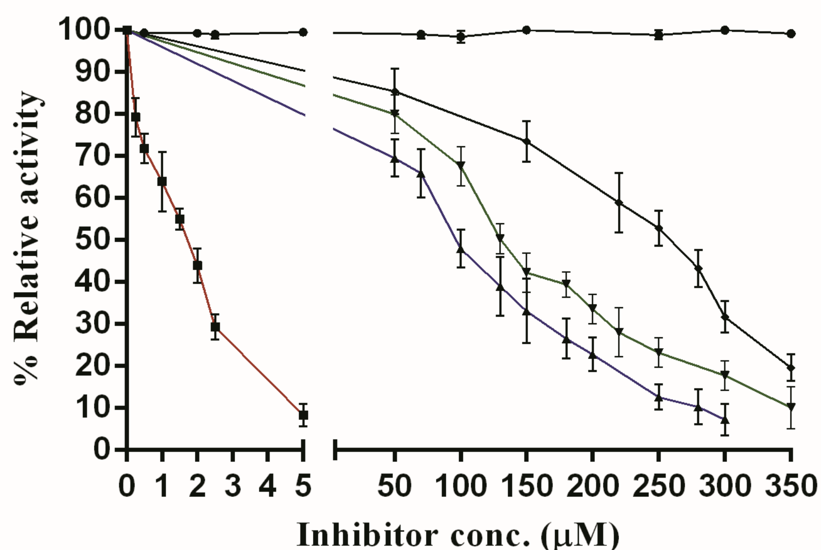


Figure 6.7. Dose response curve of carbonic anhydrase inhibitory activity
(●) without inhibitor; (▲) coniferyl alcohol; (▼) ferulic acid; (•) p-coumaric acid; (■) **acetazolamide** [Data are presented as Mean ± SD (n = 3). Relative carbonic anhydrase II inhibitory activity (without inhibitor) is considered as 100%].

Table 6.4. Kinetics and inhibition constants of phenolic compounds for carbonic anhydrase II

Compounds	IC_{50} (μM)	K_m[a] (mM)	V_{max}[b] (nmol/mg/min)	K_i[c] (μM)	Inhibition type
Coniferyl alchohol	80.38 ± 3.54	2.6	3.2	68	Non-competitive
Ferulic acid	130.15 ± 5.38	1.3	2.1	116	Non-competitive
p-coumaric acid	256.52 ± 7.16	0.4145	0.9406	132	Competitive
Acetazolamide	1.72 ± 1.29	--	--	--	--

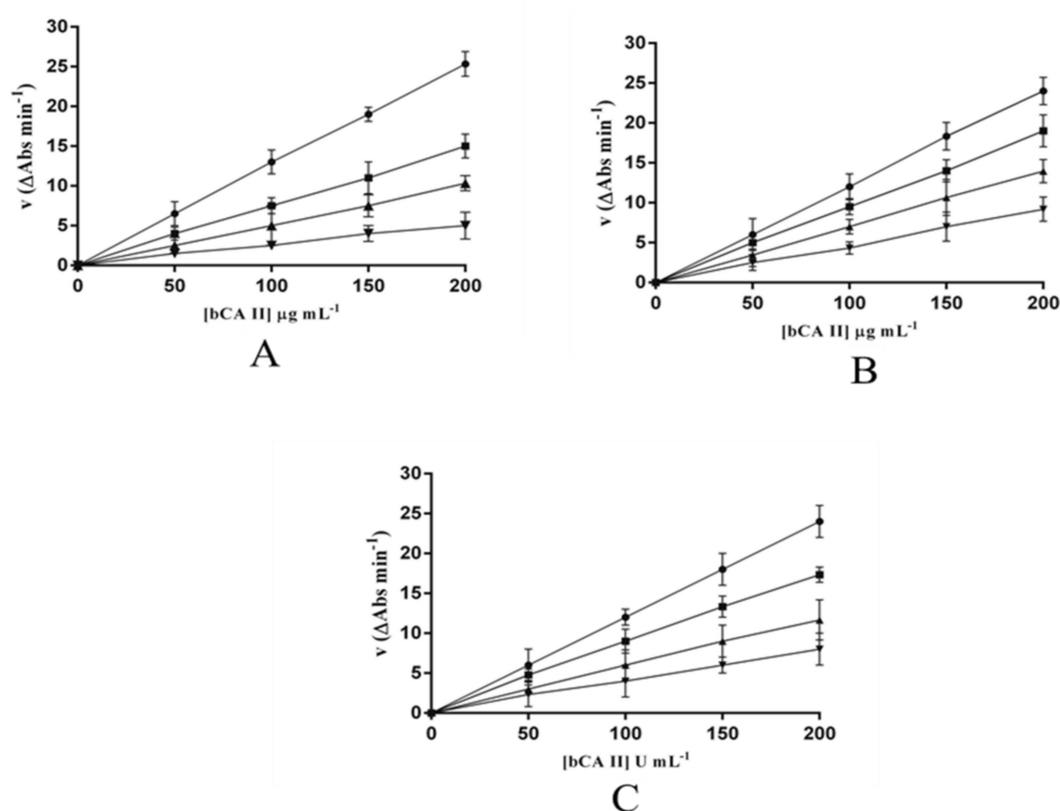


Figure 6.8. Reversible inhibition of inhibitors (A) coniferyl alcohol (B) ferulic acid (C) p-coumaric acid against carbonic anhydrase II [Plots of v vs. enzyme concentration]; The v value indicates the change in absorbance at 400 nm/min at inhibitor concentrations of 0 (●), 100 (■), 200 (▲), 300 (▼) mM. Data presented as mean ±SD (n = 3)].

6.4.2.4. Docking studies and binding mode analysis

A crystal structure of bCA II (PDB ID: 1V9E) was used for docking to reveal the binding modes of three compounds. The active site contains His-93, His-95, and His-118 amino acids coordinated with zinc atom which lies at the bottom of a deep cleft of bCA II. The nitrogen atoms of histidine directly coordinated with zinc, while Thr-198 and Glu-105 interact indirectly through the bound water (Saito et al., 2004). Molecular docking studies revealed the most favorable binding modes for these compounds as well as critical hydrogen bond interactions with the amino acid residues lining the active site. In addition, some hydrophobic interaction and *pi-pi* stacking between inhibitors and the

amino acid residues of the catalytic site were observed. The Glide XP scores were determined for each phenolic compound to estimate the free energy of binding at the active site cleft of bCA II. In molecular docking, higher the score means to lower the binding energy. The score of coniferyl alcohol was found highest at -5.054 (3 H bond), whereas the Glide XP score of p-coumaric acid was found as -3.108 (1 H bond). It was

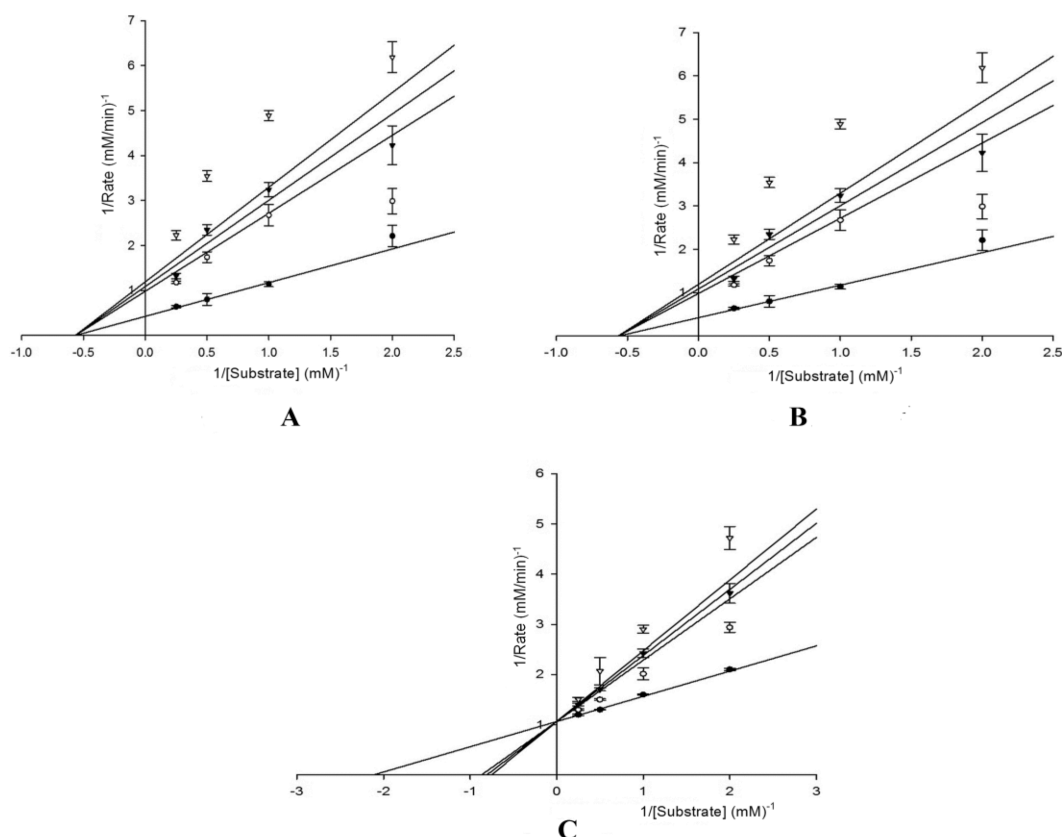


Figure 6.9. Lineweaver–Burk plot for kinetics analysis of (A) p-coumaric acid (B) Ferulic acid (C) Coniferyl alcohol [The v value indicates the change in absorbance at 400 nm min-1 at different concentrations of inhibitors].

noticed that three 'H' bonds were formed between coniferyl alcohol and the amino acid residues Gln91, Pro199, and Trp4 of the active site of the enzyme. Only one hydrogen bond was formed between p-coumaric acid and Thr198. These results suggested that binding capacity of CFA was found highest whereas p CA offered lower interaction capacity with active site. The details of Glide XP scores of the ligands, H bonds,

hydrophobic and pi-pi interactions among the inhibitors and the amino acid residues have been represented in Table 6.5.

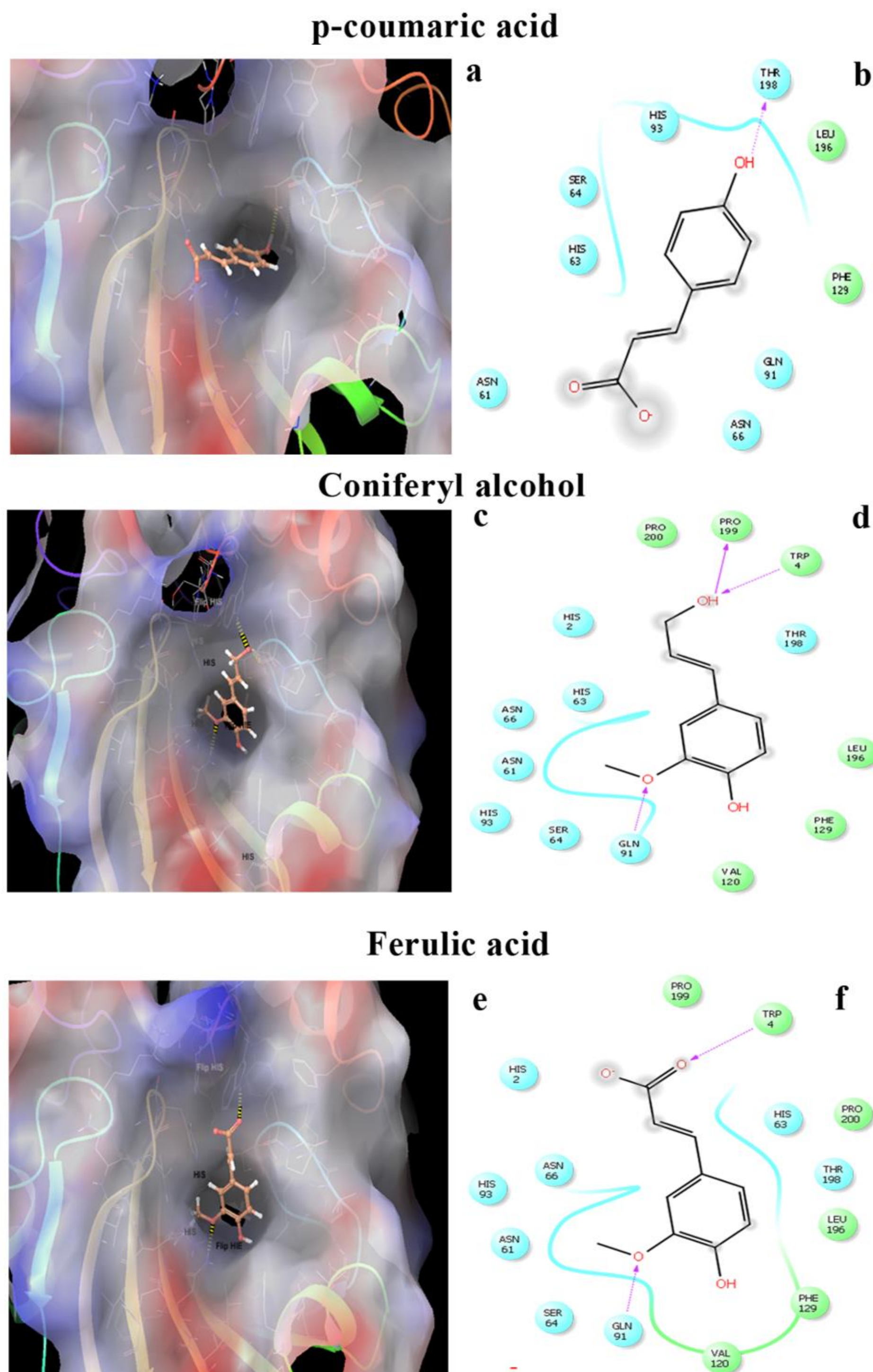


Figure 6.10. Molecular docking analysis (a, b) p-coumaric acid (c, d) coniferyl alcohol (e, f) ferulic acid

Graphical presentation of binding modes of interaction of molecular docking analysis showed in Figure 6.10. These results suggested that the binding capacity of CFA was found highest whereas 4-CA offered lower interaction capacity with the active site. The details of Glide XP scores of study reported that both the carboxyl and OH group present in CFA and FA bind to the enzyme by the formation of an H-bond with Gln-91, Trp-4 located at different regions of the active site cavity without any competition with the substrate. In addition, an ethynyl- hydroxy moiety of CFA forms a hydrogen bond with Pro-199 which reflected in its higher inhibition potential. On the other hand, 4-CA showed weak inhibition as it could form only one H-bond with a Thr-198 present in the outer shell of the active site (Saito et al., 2004). This finding reported the interaction mechanism of the hydroxyl group with the amino acid residues and water molecules from the active site of carbonic anhydrase (Chaturvedi et al., 2016). All of the above observations confirmed the inhibition type and mechanism of CFA, 4-CA, FA as well as their binding interaction in the active site of bCA II.

Table 6.5. Interactions involved in anchoring of coniferyl alcohol, ferulic acid and p-coumaric acid within bCA II

Name	**Glide XP Score**	**Interacting amino acid residues**	
		H-bond	Hydrophobic and pi-pi stacking
Coniferyl alcohol	-5.054	Gln91, Pro199, Trp4	Val120, Phe129, Leu196 Pro200
Ferulic acid	-4.203	Gln91, Trp4	Val120, Phe129, Leu196, Pro200, Pro199
p-coumaric acid	-3.108	Thr198	Leu196, Phe129

The current study identified coniferyl alcohol, ferulic acid, and p-coumaric acid as major carbonic anhydrase inhibitory constituents from *L. siceraria* fruit, offering reversible, dose-dependent inhibition. The results of the enzyme inhibition assay revealed that K_i values (inhibitory constatnt) of coniferyl alcohol, ferulic acid and p-coumaric acid as 68, 116 and 132 µM respectively. Enzyme kinetics study revealed that both CFA and FA inhibit carbonic anhydrase in a non-competitive manner, whereas 4-CA was found to be

competitive. Moreover, the molecular docking study showed that CFA, FA, and 4-CA can strongly interact with the amino acids near the catalytic site of bCA II through hydrogen bond as well as hydrophobic interaction. The distinct binding mechanism of the phenolic compounds will further address the development of novel inhibitors from natural sources against carbonic anhydrase. Thus the present research led us to conclude that, the phenolic compounds from *L. siceraria* could play a useful role in the management of edema, hypertension, obesity and related metabolic disorders linked with carbonic anhydrase inhibition.

6.5. Publication

- UPLC-QTOF-MS analysis of a carbonic anhydrase inhibiting extract and fractions of *Luffa acutangula* (L.) Roxb (ridge gourd). Phytochemical Analysis. 2018; 30(2):148-155. (John & Wiley).
- Study of carbonic anhydrase inhibition kinetics of bioactive constituents of *Lagenaria siceraria*: A LC-Q-TOF-MS based approach. Food Chemistry. (Elsevier Science, USA). Communicated.

Chapter 7

7. Pancreatic lipase inhibitory activity of *Lagenaria siceraria*, *Luffa acutangula*, *Momordica charantia* and *Coccinia grandis*

7.1. Role of pancreatic lipase inhibition

7.2. Evaluation of *in vitro* pancreatic lipase inhibitory activity of four plants

7.3. Pancreatic lipase inhibitiory kinetics of *Momordica charantia*

7.4. Publication

7.1. Role of pancreatic lipase inhibition

Pancreatic lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) (PL) are carboxylesterases, ubiquitous enzymes required for fat metabolism in the human body. The PL is a single chain glycoprotein consists of 465 amino acid with the molecular weight of 51,156 Da. The lipolytic action of PL exerts through the presence of His 263, Asp-176 and Ser-152 in their catalytic site. There are several lipolytic genes identified which encodes the primary amino acid sequence of lipase. Based on amino acid sequence and gene organization, the lipase gene family are categorized into three types *viz.* pancreatic lipase (PL), lipoprotein lipase, and hepatic lipase. There are two types of pancreatic lipase–related proteins, (PLRP1 and PLRP2), was isolated from human pancreas. The tertiary structure of PT shows two domains: a globular N-terminal domain formed by a central sheet core extending from amino acids 1–335, considered as catalytic domain and a C-terminal domain consisting of a ß-sheet sandwich structure which binds to colipase (as a cofactor) for essential lipolytic activity. Here the procolipase acts as a precursor molecule which converts to colipase by cleavage of the procolipase propeptide (APGPR) and subsequently bind to the C-terminal of the PL molecule (Tilbeurgh, 1992; Birari & Bhutani, 2007). The structure of pancreatic lipase-colipase complex 1N8S has been depicted in Figure 7.1.

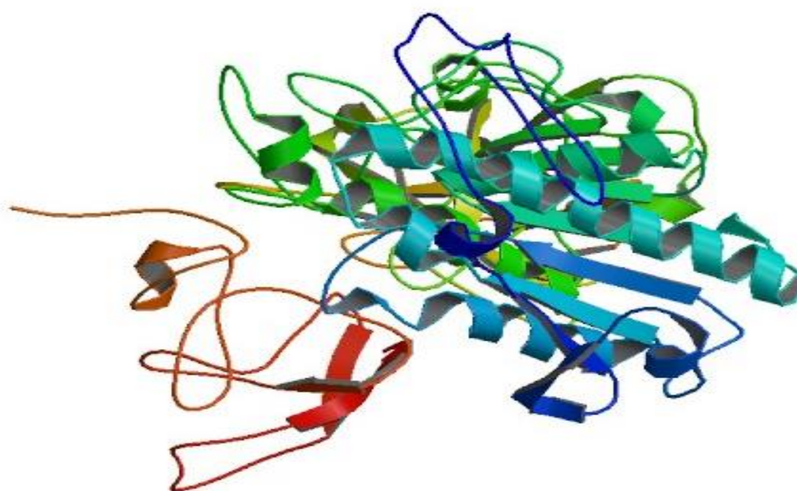


Figure 7.1. Pancreatic lipase-colipase complex 1N8S

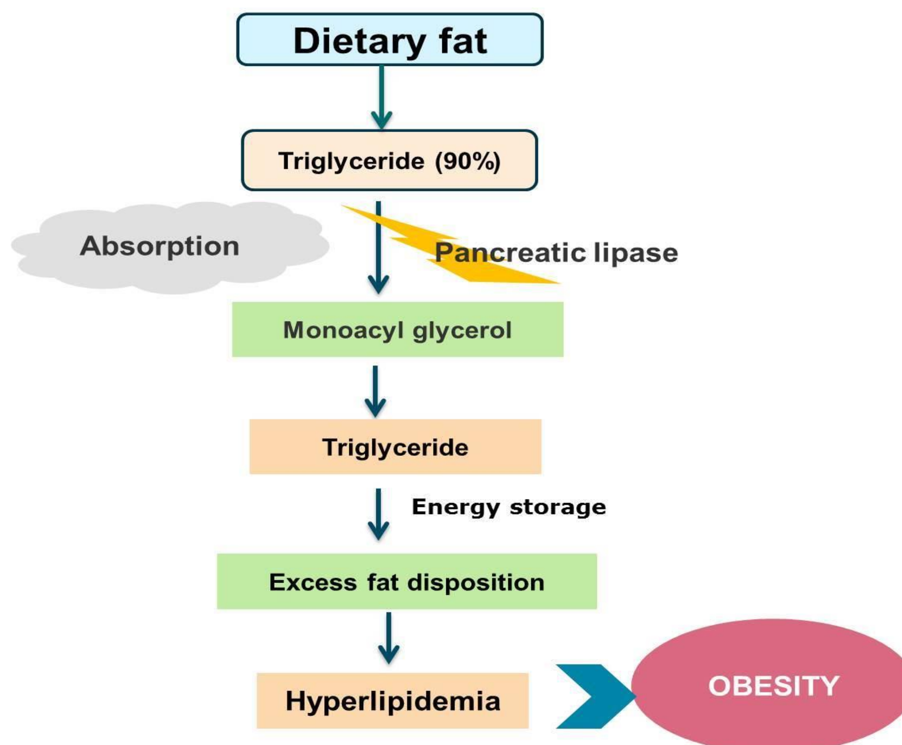


Figure 7.2. Physiological function of pancreatic lipase inhibitor

PL is responsible for the hydrolysis of 50–70% of total dietary fats. It plays a key role in the conversion of triglyceride into monoacylglycerol and free fatty acids which are converted to mixed micelle in presence of cholesterol and bile acids. It is then absorbed as monoacylglycerol and further transformed to triglyceride where energy is being stored (Mukherjee, 2003). The free fatty acids (FFA) are incorporated into bile acid-phospholipid micelles, absorbed at the level of the brush border of the small intestine, and finally entered into the peripheral circulation as chylomicrons. Thus FFA is responsible in the regulation of very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), collectively called as total lipid content. The excess disposition of triglyceride leads to hyperlipidemia which is a major causative factor of developing cardiovascular diseases, atherosclerosis, hypertension, diabetes and other functional depression of certain organs. Among several causative factors, pancreatic lipase is proposed to be an important target of developing anti-obesity, hypolipidemic agents. By inhibiting the pancreatic lipase action, conversion of triglyceride is being blocked which further results in decrease triglyceride reabsorption in adipose tissue and finally lead to hypolipidemia (Birari & Bhutani, 2007). By inhibiting the

pancreatic lipase action, conversion of triglyceride is being blocked which further results in decrease triglyceride reabsorption in adipose tissue and finally lead to hypolipidemia. The mechanism of pancreatic lipase inhibition has been explained further in Figure 7.2.

7.2. Evaluation of *in vitro* pancreatic lipase inhibitory activity of four plants

The anti-obesity activity of several medicinal plants has been extensively studied due to its less toxicity and side effects. A large number of naturally derived products (plant extracts and isolated compounds) have been reported to possess pancreatic lipase inhibition property due to their high phenolic and flavonoid content and multiple/synergetic effects to the substrate. It has been noticed that polyphenolic compounds (phenolic acids, hydroxycinnamic acids, hydroxybenzoic acids, lignans, flavonoids etc.) are present ubiquitously in the cucurbitaceae family plants. Moreover, there are reports found on the anti-obesity activity of cucurbitaceae plants viz. *Lagenaria siceraria*, *Momordica charantia* etc. Based on the ethnopharmacological evidence our study was designed to screen the pancreatic lipase inhibitory activity of fruits parts of some cucurbitaceae plants *viz. Lagenaria siceraria*, *Momordica charantia*, *Luffa acutangula* and *Coccinia grandis* linked with anti-obesity activity.

7.2.1. Materials and methods

7.2.1.1. Reagents and chemicals

Lipase from Porcine pancreas Type II (activity 59 units/mg protein), p-nitrophenyl caprylate (pNPC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Orlistat was procured as a gift sample from Central Drugs Laboratory (CDL), Kolkata, India. Hexane, ethyl acetate, ethanol were purchased from Merk, Mumbai, India. All aqueous solutions were prepared using purified water (resistivity of 18.2 MΩ.cm at 25°C) from Mili-Q filtration system.

7.2.1.2. Collection and authentication of plant materials

The fresh fruits of the selected curubitacaeae plants were collected from the local market of Jadavpur, Kolkata and authenticated by the field botanist. The voucher specimen refrence was given to each sample for future refrence. The details of collection and authentication of plant materials have been discussed in Chapter 3.

7.2.1.3. Extraction of the plant material

The shade dried plant materials were extracted with the method described in chapter 3, section 3.6. After extraction, the extract was dried under rotary evaporator and the yield was calculated.

7.2.1.4. Pancreatic lipase inhibitory activity assay

The *in vitro* pancreatic lipase inhibition assay was performed with the aqueous ethanol extract and fractions of *M. charantia* fruits, based on the method described by Gonçalves and his co-workers with minor modification (Gonçalves et al., 2010). The porcine pancreatic lipase was dissolved in 50 mM phosphate buffer at pH 7.0 and finally the enzyme concentration was made at 5.67 U/ml. p-nitrophenyl caprylate (pNPC) was used as a substrate in a concentration of 200 µM (in phosphate buffer). The assay was initiated by adding pNPC in the enzyme solution, in presence and absence of inhibitors. The rate of change of absorbance of the reaction mixture in each well was determined at 405 nm using a UV–visible spectrophotometer (SpectraMax Plus, Molecular Devices LLC, USA). The assay process was carried out in triplicate. Orlistat was used as a positive control. The PL inhibitory activity assayed without inhibitor was calculated as 100%. Relative enzymatic activity (%) and IC_{50} value was determined based on the following equation. Relative enzymatic activity (%) = (catalytic rate of enzymatic reaction with inhibitor)/ (catalytic rate of enzymatic reaction without inhibitor) × 100. IC_{50} values of inhibitors were determined by plotting the percentage of relative activities against the inhibitor concentration in logarithmic scale.

7.2.2. Results and discussion

The *in vitro* pancreatic lipase inhibition assay was performed with the aqueous ethanol extract of the selected plants (*L.siceraria*, *L.acutangula*, *M. charantia*, *C.grandis*). the results of the study indicated that *M. charantia* posseses highest inhibitory activity (655.24±21.88 µg/ml) wheres the IC_{50} values of other extract was found at 1183.19±7.70 µg/ml (*L. acutangula*), 256.03±3.28 µg/ml (*M. charantia*) and 893.19± 7.9 µg/ml (*C. grandis*) respectively. No significant difference (P value < 0.05) was observed between *M. charantia* extract and the positive control Orlistat (IC_{50} value 149.09±1.39 µg/ml). The data was represented in Figure 7.3. Based on the above results *M. charantia was* furthered considered for dose response and enzyme kinetics study.

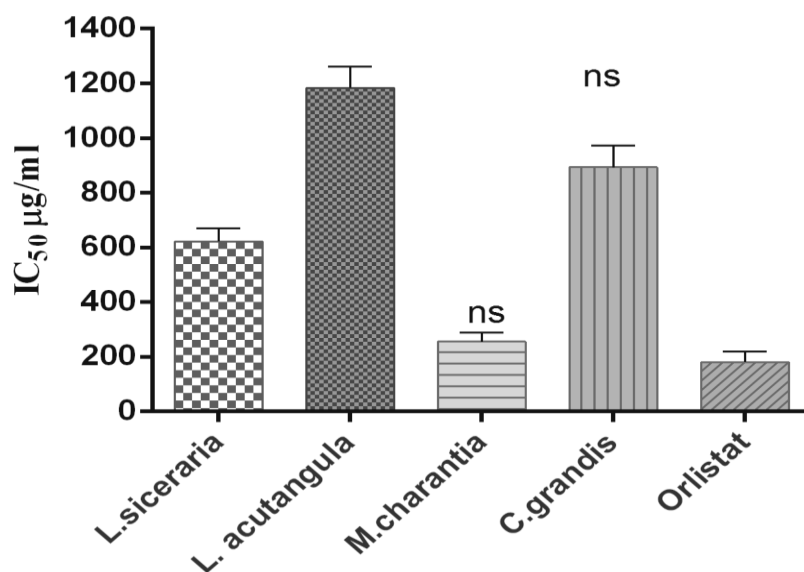


Figure 7.3. Half maximal inhibitory concentration (IC_{50} value) of *L. siceraria*, *L. acutangula*, *M. charantia*, *C. grandis* against pancreatic lipase [Orlistat—positive control; results are represented as mean ± SD (n = 3); One-way ANOVA with Dunnett's multiple comparisons, at P value 0.05].

7.3. Pancreatic lipase inhibitiory kinetics of *Momordica charantia*

There are several food plants of cucurbitaceae family reported to have anti-obesity property by lowering the lipid level (Sharma et al., 2005; de la Garza et al., 2011). In particular, *M. charantia* extract possesses potential hypocholesterolemic activity (Jayasooriya et al., 2000; Nerurkar et al., 2006). The whole fruit of bitter gourd have been reported to possess potential therapeutic activity against hyperglycemia and hyperlipidemia (Mahwish et al., 2017). The anti-obesity activity of *M. charantia* was reported in 3T3-L1 preadipocyte cell line (Sahib et al., 2011). In addition, the fruit extract of *M. charantia* was also reported to have potential therapeutic effect on ß-cells of isolated pancreatic islets of obese hyperglycemic rats (Singh et al., 2008).

In search of pancreatic lipase PL inhibitors from natural resources, identification and characterization of the active phyto-constituents are necessary as therapeutic alternatives against obesity. Although the anti-pancreatic lipase activity of *M. charantia* fruit has been reported earlier (Sahib et al., 2012), the mode and type of inhibition are unknown to date. However, there is still an increasing interest in identifying the bioactive

constituents present in the fraction of fruits of *M. charantia*. With this background, the objective of the present study was to evaluate pancreatic lipase inhibition kinetics to explore the inhibition mechanism of the bioactive fraction of fruits of *M. charantia* against PL; correlation analysis between PL inhibitory activity and total phenolic and flavonoid content of the extract and fractions of fruits of *M. charantia.* The phytochemical profiling of the bioactive frcation of *M. charantia* fruits was performed through LC-QTOF-MS (discussed in Chapter 9)

7.3.1. Methodology

7.3.1.1. Fractionation of *M. charantia aqueous ethanol extract*

The fresh fruits of *M. charantia* were collected from the local market of Jadavpur, Kolkata, India and extracted with aqueous ethanol based on the method described in Chapter 3, (section 3.6.2). 70 g aliquot of aqueous ethanol frcation was suspended in water and partitioned successively with hexane and ethyl acetate (100 ml each). All of the fractions were collected, filtered and evaporated under vacuum at 45–50°C using a Eyela rotary evaporator (Japan). The yields of the hexane (HFMC), ethyl acetate (EFMC) and aqueous (AFMC) fractions were calculated as 3.5, 12.7, and 14.2% (w/w), respectively. The samples were stored in borosilicate glass vials at 4°C prior to analysis.

7.3.1.2. Estimation of total phenolic and flavonoid content

The estimation of total phenolic and flavonoid content of aqueous ethanol extract of *M. charantia* fruits and partitioned fractions was performed based on the earlier method described from our laboratory with some modification (Biswas et al., 2016a). The details of the method has been described in Chapter 6, section 6.3.1.4. The content of total flavonoid was calculated as milligrams of quercetin equivalents (QE)/g of sample. The results were represented as mean±standard deviation (SD) (n = 3). In addition, to understand the interrelationship between pancreatic lipase inhibitory activity (IC_{50} value) and the total phenol and flavonoid contents of *M .charantia* fractions, Pearson's correlation coefficient was calculated.

7.3.1.3. Inhibition kinetics study on pancreatic lipase

In vitro pancreatic lipase inhibition assay was performed with the aqueous ethanol extract and fractions of fruits of *M. charantia,* based on the method described in section

7.2.1.4. To explore the inhibitory mechanism, the reversibility of the enzyme inhibitor reaction was studied. The plots of residual activity (expressed in ΔAbs/min) vs. PL concentrations (0–50 U/ml) (E) at different inhibitor concentrations (100–400 μg/ml) were constructed (Biswas et al., 2017). Additionally, in order to determine kinetic parameters, an enzyme inhibition study was performed with the above spectroscopic method using different concentrations of substrate (50–200 μM) and the inhibitors (100–400 μg/ml). The catalytic effect of the active fraction was monitored as the function of initial reaction velocity and incubation time (from 0 to 30 min). The kinetic parameters, viz. Km and V_{max} values, were estimated using the Lineweaver–Burk double reciprocal plot based on the equation: $1/V = K_m/V_{max} \times (1 + [I]/K_i) \times 1/[S] + 1/V_{max} \times (1 + [I]/\alpha K_i)$, where V is the reaction velocity, K_m is the Michaelis constant, V_{max} is the maximal velocity, [I] is the concentration of inhibitor, K_i is the inhibitor constant, [S] is the concentration of substrate and α is the apparent coefficient. The values were calculated using the software SigmaPlot 12.5. The K_i and αK_i values were determined by constructing the secondary plot of the slope (K_m/V_{max}) and y-intercept ($1/V_{max}$) vs. inhibitor concentration [I].

7.3.2. Results and discussion

7.3.2.1. Pancreatic lipase inhibitory activity

The extract and fractions of fruits of *M. charantia* were tested for their ability to inhibit pancreatic lipase activity in vitro, expressed as half-maximal inhibitory concentration (IC_{50}). The IC_{50} values of the aqueous ethanol extract, three subsequent partitioned fractions and Orlistat (positive control) were estimated to be 167.53±12.45 (EFMC), 266.47±24.58 (AFMC), 282.6±20.44 (EEMC), 342.7±37.12 (HFMC) and 146.33±20.88 (Orlistat) μg/ml (Figure 7.4). No significant difference ($p < 0.05$) was observed in the case of EFMC compared with standard inhibitor Orlistat. The inhibitory action of EFMC was found to be dose dependent in a range of inhibitor concentrations of 100–400 μg/ml (Figure 7.5). The results indicated that the ethyl acetate fraction exhibited better PL inhibitory activity among the other fractions. The increase in EFMC concentration resulted in the decrease in relative enzyme activity, which confirmed that EFMC inhibits PL in a dose-dependent manner.

7.3.2.2. Estimation of inhibition kinetics parameters

The plot of the reaction velocity (v) vs. enzyme concentration at different inhibitor concentrations gave a group of straight lines. It was observed that all of the straight lines passed through the origin and the slope of the line decreased inversely with the increasing concentration of the inhibitors (Figure 7.6). The data indicated that the enzymatic reaction followed first-order kinetics as the reaction rate (velocity) was dependent on the concentration of the reactant (enzyme). This result confirmed the reversible type of inhibition of EFMC against PL activity. The kinetics mode of pancreatic lipase inhibition was studied with respect to different substrate concentrations. It was observed that both of the values of apparent V_{max} and K_m were changed with an increasing concentration of the inhibitor. This indicated a probability of a mixed type of inhibition on lipase with the test sample. From the Lineweaver–Burk plot, the K_m and Vmax values were 2.0 mM and 271.9 µM/min (Figure 7.7). The inhibition constants (K_i) and αK_i were also calculated from the secondary plot as 123.60±0.09 and 4.24±1.12 µg/ml, respectively (Figure 7.8 a-b). The two different K_i values obtained from Lineweaver–Burk plot indicated that inhibitor can bind to both enzyme (K_i and αK_i values) as well as ES complex (αK_i), hence it implied a mixed type of inhibition (Biswas et al., 2016b). A higher value of K_i implies that the inhibitor binds to the enzyme alone, rather than the ES complex.

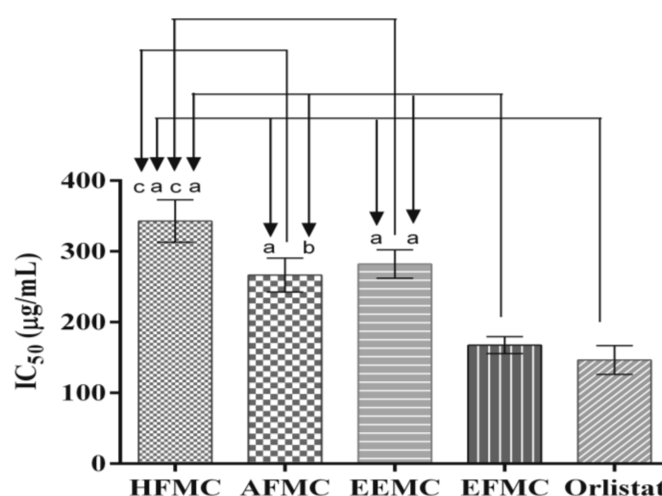


Figure. 7.4. Half maximal inhibitory concentration (IC_{50} value) of fractions of *M. charantia* extract. [Orlistat – Possitive control; Results are represented as mean ± SD (n=3); One way ANOVA with Tukey's multiple comparison test ; Significant value are represented as a = $p < 0.001$; b = $p < 0.01$; c = $p < 0.05$]

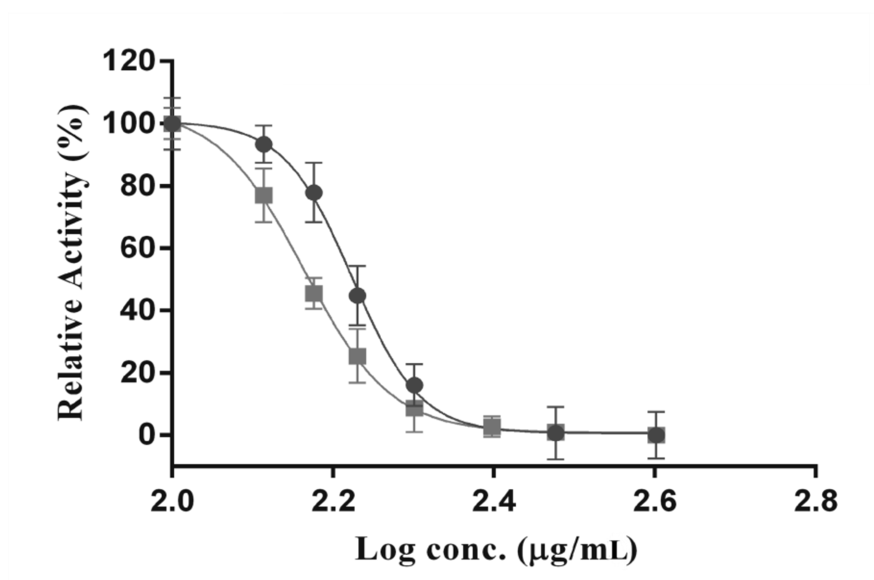


Figure 7.5. Log-dose response curve of EFMC and Orlistat on pancreatic lipase inhibitory activity [(●) EFMC– Ethyl acetate fraction of *M. charantia* fruits; (■) Orlistat; Data are presented as mean ± SD (n = 3)].

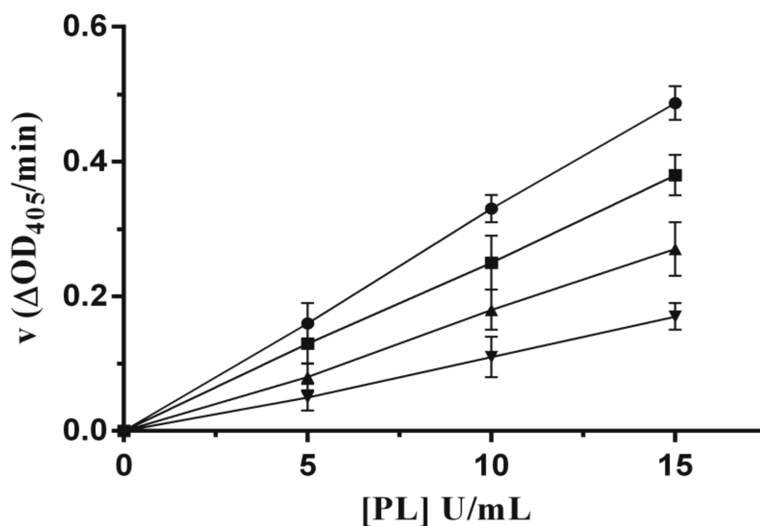


Figure 7.6. Reversible inhibition of EFMC against pancreatic lipase [Plots of velocity vs. enzyme concentration]. [The v value indicates the change in absorbance at 405 nm/min at EFMC concentrations of 0 (●), 100 (■), 200 (▲), 400 (▼) µg/ml. Data presented as mean ± SD (n = 3)].

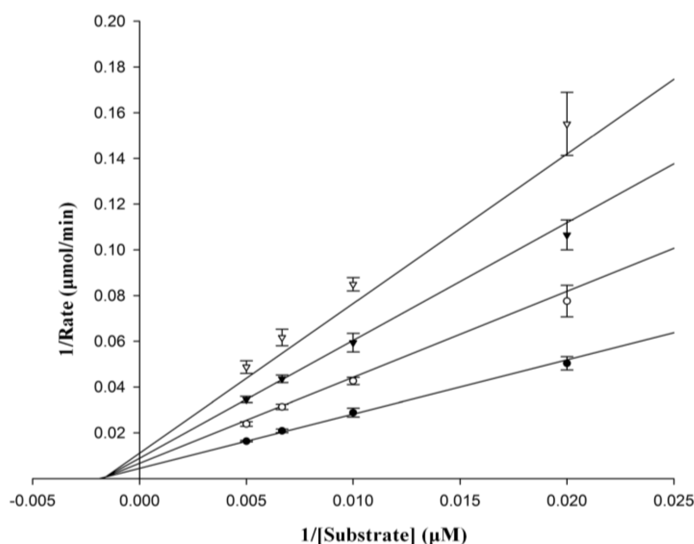


Figure 7.7. Enzyme kinetics study (LB plot) of EFMC against pancreatic lipase inhibitory activity. [The v value indicates the change in absorbance at 405 nm/min at different concentrations of inhibitors: (●) 100, (■) 200, (▲) 300 and (▼) 400 µg/ml. Data presented as means ± SD (n=3)].

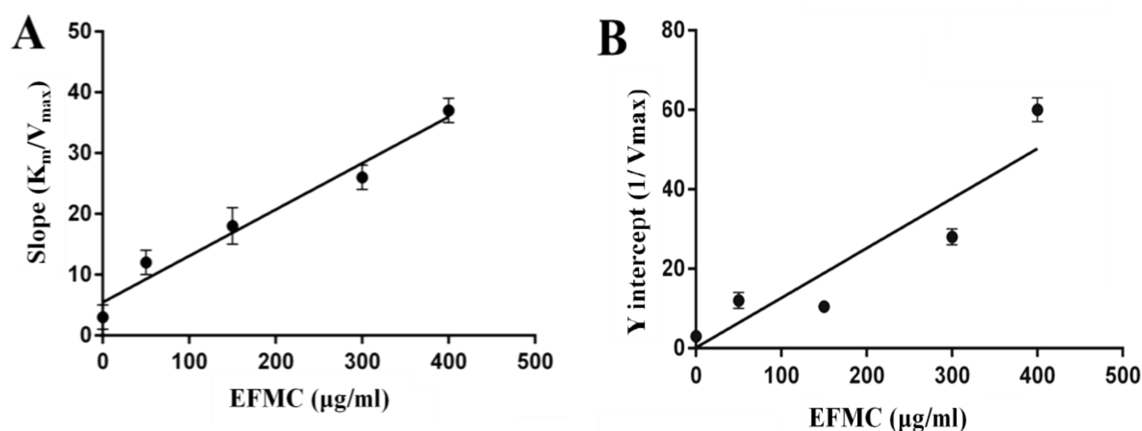


Figure 7.8. (a) Plot of slope (K_m/V_{max}) vs. EFMC concentration. (b) Plot of y-intercept ($1/V_{max}$) vs. EFMC concentration; [Secondary replots were constructed from the data, collected from the Lineweaver–Burk plot].

7.3.2.3. Correlation between pancreatic lipase inhibitory activity and total phenolic and flavonoid content

The TPC and TFC were the highest in EFMC at 3.79 ± 0.28 mg GAE/g and 2.44±0.48 mg QE/g of sample, respectively. The TPC and TFC of other fractions were in the range of EEMC > AFMC > HFMC. A correlation between PL inhibitory activity and TPC and

TFC of all of the fractions was studied through Pearson's correlation coefficient (R^2 value). A statistically significant correlation (R^2 value 0.9261) between the TPC of ethyl acetate fraction and its PL inhibition potential (IC_{50} value) was observed (Table 7.1).

Table 7.1. Correlation analysis (R^2) between lipase inhibitory action (IC_{50} value) of *M. charantia* fractions and total phenol and flavonoid content

R^2	EFMC	EEMC	AFMC	HFMC
Total phenol content	0.9261**	0.2361 ^{ns}	0.3214 ^{ns}	0.1017 ^{ns}
Total flavonoid content	0.4560^{ns}	0.4417 ^{ns}	0.4894 ^{ns}	0.3243 ^{ns}

A lower correlation value was observed with other fractions and their phenolic and flavonoid content. The positive correlation value between TPC and inhibition rate further confirmed that higher PL inhibition properties may be contributed by the phenolic compounds present in EFMC. It can be postulated that the anti-lipase activity of the fraction of *M. charantia* extract seems to be related to the phenolic hydroxyl groups present in their active pharmacophore (Buchholz & Melzig, 2015).

This study explored the inhibitory potential of fruits of *M. charantia* against pancreatic lipase and its mode of inhibition. The results indicated that ethyl acetate fraction of fruits of *M. charantia* offered reversible, dose dependent inhibition among the other fractions. The enzyme kinetics study revealed that the inhibition of PL was mixed type in nature. A significant correlation was observed between total phenol content of ethyl acetate fraction of *M. charantia* and pancreatic lipase inhibitory activity. Further, LC-QTOF-MS study was performed to identify to major phytoconstituents present in the active fraction which has been discussed in Chapter 9.

7.4. Publication

- Study of pancreatic lipase inhibition kinetics and LC-QTOF-MS based identification of bioactive constituents of *Momordica charantia* fruits, Biomedical Chromatography. 2019, 33(4), e4463.

Chapter 8

8. α-glucosidase inhibitory activity of *Lagenaria siceraria*, *Luffa acutangula*, *Momordica charantia* and *Coccinia grandis*

8.1.Role of α-glucosidase inhibition
8.2.Screening of four plants of cucurbitaceae family against α-glucosidase
8.3.Evaluation of α-glucosidase inhibitory kinetics of active fraction of *Coccinia grandis*
8.4. Publication

8.1. Role of α-glucosidase inhibition

Diabetes mellitus (Type-II) is a metabolism-related disorder, which affects nearly 300 million people yearly worldwide (Sim et al, 2010). This disease is associated with impaired glucose tolerance, which is followed by a set of secondary disorders, like diabetic nephropathy and diabetic neuropathy. It has been observed that different phenyl-propanoids and flavonoids, such as chlorogenic acid (Ishikawa et al, 2007) present in plant extracts have significant antioxidant, anti-diabetic and inhibitory activities on carbohydrate metabolizing enzymes. The plants of cucurbitaceae family have been found to be rich in such bioactive phyto-constituents, besides vitamins and minerals (Longvah et al, 2017). In some *in-vivo* studies these plants have been found to improve the glucose tolerance and also pancreatic β-cell histology in diabetic mice. Inhibition of the intestinal brush-border carbohydrate-metabolizing enzymes, such as the **α**-glucosidases and **α**-amylases has been proved to be quite effective in overcoming the post-prandial hyperglycemia in diabetic patients. Due to its inhibition, absorption of the glucose from the intestinal epithelia is delayed and the post-prandial hyperglycemia can be avoided (Lebovitz, 1997).

Glucosidase, a calcium containing enzyme belongs to glycosidases family which catalyzes the cleavage of glycosidic bonds of oligosaccharides or glycoconjugates. Glucosidases differ in the breaking of glycosidic bonds depending on the number, position, or configuration of the hydroxyl groups in the sugar molecule (Azam et al., 2012). Thus, α-and β-glucosidases are able to break the glycosidic bonds involving terminal glucose connected at the site of cleavage, respectively, through α - or β - linkages at the anomeric centre. The active site of **α** -glucosidase consists of Trp-516 and Asp-518 residues as enzyme's catalytic functionality (Hermans et al., 1991). The structure of α-glucosidase has been presented as Figure 8.1. The catalytic activity of **α**-glucosidase enzyme lead to reduction of polysaccharides to monosaccharide, biosynthesis of oligosaccharide units in glycoproteins or glycolipids and catabolism of lysosomal glycoconjugate and processing of glycoprotein. **α**-glucosidase inhibitors inhibit intestinal **α**-glucosidase enzyme and slow down the digestion and absorption of carbohydrates by competitively blocking the activity of glucosidase. As a result, the peak concentration of postprandial blood glucose is reduced and the blood sugar level comes under control (de Melo et al., 2006). Inhibition of α-glucosidase enzyme in the gut results

in delaying of the carbohydrate absorption from the intestinal epithelia, decreasing the postprandial hyperglycemia in diabetic individuals (de Melo et al., 2006). As a result, they offer a potential therapeutic benefit to the diabetic patients by reducing insulin independent hyperglycemic level. The α-glucosidase inhibitors effectively decrease postprandial hyperglycemia and thus it has been clinically used in the treatment of diabetes mellitus (Azam et al., 2012) and obesity. The role of **α**-glucosidase in glucose absorption has been depicted in Figure 8.2.

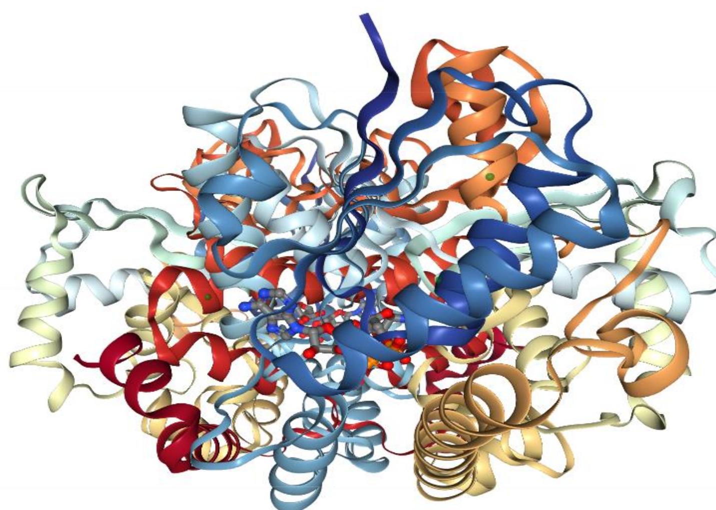


Figure 8.1. Crystal structure of α -glucosidase 1VJT

Inhibition of **α**-glucosidase affects the glycan structure by altering the maturation, transport, secretion, and function of glycoproteins (Azam et al., 2012). In addition, α-glucosidase inhibitor also posseses some therapeutic benefits against cancer,viral infections, and hepatitis (Park et al., 2008). In spite of the significant therapeutic potentials of α-glucosidase inhibitors (*viz.* acarbose and voglibose), clinically they have been associated with serious gastrointestinal side effects, mainly due to passage of undigested sugar into colon.

Therefore, attention needs to be given in order to explore traditional medicinal plants which can provide valuable leads for the development of alternative α-glucosidase inhibitors with lower toxicity and fewer side effects than synthetic drugs (Yin et al., 2014). The phytoconstituents obtained from medicinal plants (eg. terpenes, alkaloids, quinines,

flavonoids, phenols, phenylpropanoids, steroids etc. are considered to be a potential source of α-glucosidase inhibitors as safer alternatives of the synthetic drug.

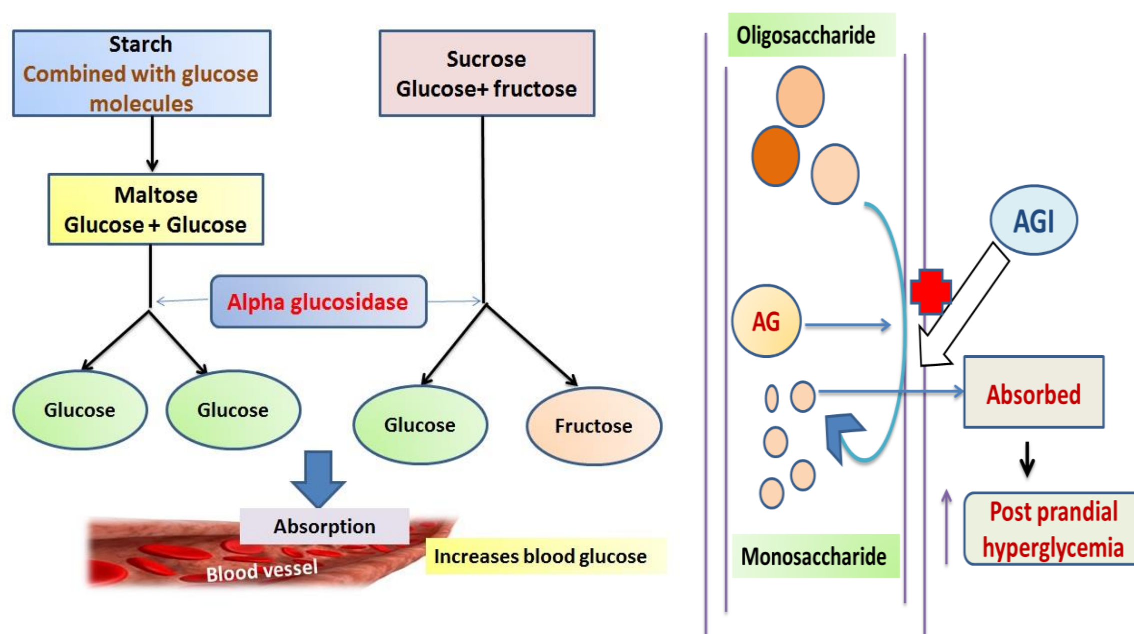


Figure 8.2. Role of α-glucosidase in glucose absorption [AG: α-glucosidase; AGI: α-glucosidase inhibitor]

8.2. Screening of four plants (*L. siceraria, L. acutangula, M. charantia, C. grandis*) of cucurbitaceae family against α -glucosidase

α-glucosidase inhibitors competitively inhibit glycosidase activity and thus to prevent the fast breakdown of sugars and thereby control the blood sugar level. Several α-glucosidase inhibitors have been isolated from medicinal plants till date. It has been noticed that, the development of α-glucosidase inhibitors isolated from medicinal plants serve as a potential source of antidiabetic agents. Several plants of cucurbitaceae plants have been reported to possess potential anti-diabetic activity by delaying glucose absorption or by stimulating insulin secretion. Moreover, due to the presence of large number of phenolic compounds in cucurbitaceous plants, they can counteract with the oxidative stress caused by hypoglycemia-generated free radicals (Sulaiman et al., 2013). In this chapter, the screening of four plants of cuucrbitaceae family was carried out.

8.2.1. Reagents and chemicals

α-glucosidase (from *Saccharomyces cerevisiae*) Type I (≥10 units/mg protein) and 4-Nitrophenyl α-D-glucopyranoside (p-NPG, purity ≥99%) were purchased from Sigma-Aldrich. Acarbose extrapure, 95% was purchased from Sisco Research Laboratories Pvt. Ltd., India. Disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were procured from Merk, Mumbai. n-Hexane, chloroform, ethyl acetate and ethanol (synthesis grade) were bought from Merck, Mumbai. Acetonitrile, anhydrous acetonitrile (99.8%) and trifluoroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions were prepared using purified water from a Mili-Q filtration system.

8.2.2. α-glucosidase inhibition assay

In vitro α-glucosidase inhibition assay of *C. grandis* fractions were performed in a 96 well plate based on the previously reported methods with some minor modifications (Chen et al, 2017). The enzyme, substrate and the sample solutions were prepared in 50 mM phosphate buffer (at pH 6.8). The reaction mixture consisted of 20 µl of α-glucosidase (0.5 U/ml), 10 µl of sample solutions (varying concentrations, 0.5-7 mg/ml). Thereafter the microplate was pre-incubated for 10 min at 37°C prior to the addition of 20µl of 5.0 mM p-nitrophenyl-α-D-glucopyranoside (p-NPG) as substrate. Then the reaction mixture was incubated for 5 minutes at 37°C and the absorbance were taken in a kinetic mode at λ_{max} 405 nm using a spectrophotometer (SpectraMax Plus, Molecular Devices LLC, USA). The change of absorbance was monitored (1 min intervals for 15 mins) as p-nitrophenol was liberated due to hydrolysis of p-NPG. The % relative activity was calculated using the following formula: % relative activity = catalytic rate of hydrolase reaction with inhibitor)/(catalytic rate of hydrolase reaction without inhibitor)×100%. The negative control well (without inhibitor) was referred to as 100% relative activity of the enzyme. The assay was done in triplicate, and the results were presented as half-maximal inhibitory concentration value (IC_{50} value).

8.2.3. Results and discussion

The *in vitro* α-glucosidase inhibition assay was performed with the aqueous ethanol extract of the selected plants (*L. siceraria*, *L. acutangula*, *M. charantia*, *C. grandis*). The results of the study indicated that *C. grandis* exhibited highest inhibitory activity (3.32 ±

0.43 mg/ml) wheres the IC_{50} values of other extract was found at 10.41±0.36 mg/ml (*L. acutangula*), 5.43±0.28 mg/ml (*M. charantia*) and 8.46±0.45 mg/ml (*L. siceraria*) respectively. No significant difference (P value < 0.05) was observed between *C. grandis* extract and the positive control Acarbose (IC_{50} value 2.08±0.19 mg/ml). The data was represented in Figure 8.3. Based on the above results *C. grandis was* furthered considered for dose response and enzyme kinetics study.

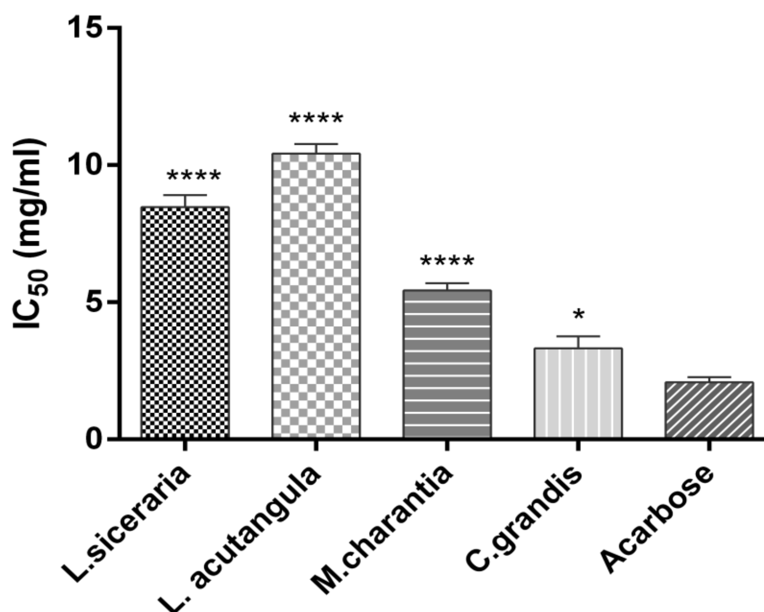


Figure 8.3. Half maximal inhibitory concentration (IC_{50} value) of *L. siceraria*, *L. acutangula*, *M. charantia*, *C. grandis*. [Acarbose—positive control; results are represented as mean ± SD (n = 3); One-way ANOVA with Dunnett's multiple comparisons, at P value 0.05].

8.3. Evaluation of α-glucosidase inhibitory kinetics of active fraction of *Coccinia grandis*

Various parts of *C. grandis* have been reported to possess potential hypoglycemic (Ajay, 2009), antidyslipidemic activity (Singh et al., 2007). The chloroform extract of *C. grandis* has been reported to have significant hypolipidemic activity followed by increasing HDL/LDL ratio (Tamilselvan et al., 2011). The fruits of the plants also reduce the risk of progression of diabetes mellitus by inhibiting the α-amylase enzyme (Sudha et al., 2011). In recent years, Meenatchi and his group reported antioxidant, antiglycation and insulinotropic properties of *C. grandis* in relation to its anti-diabetic activity (Meenatchi et

al., 2017). With this background, our study was aimed to explore the kinetics behavior of α-glucosidase inhibition of fractions of *C. grandis* fruit; the inter-relationship between total antioxidant and α-glucosidase inhibitory activity; the tentative identification of phytoconstituents present in active fraction through high-resolution liquid chromatography-quadrupole time-of-flight mass spectrometry (LC–QTOF–MS) analysis.

8.3.1. Methodology

8.3.1.1. Extraction and fractionation

The fresh fruits of *C. grandis* were collected from the local market of Jadavpur, Kolkata, India and extracted with aqueous ethanol based on the method described in Chapter 3. 100 g aliquot of aqueous ethanol frcation was suspended in water and partitioned successively with hexane, ethyl acetate and chloroform (150 ml each). All of the fractions were collected, filtered and evaporated under vacuum at 45–50°C using a Eyela rotary evaporator (Japan).The % yield of each fractions *viz.* hexane (HFCG), chloroform (CFCG), ethyl acetate (EFCG) and aqueous ethanol fraction was calculated as 1.62, 3.18, 9.25 and 15.27 % (w/w) respectively. The samples were stored in borosilicate glass vials at 4°C prior to analysis.

8.3.1.2. *In vitro* α-glucosidase inhibition assay

The *in vitro* α-glucosidase inhibition assay was carried out based on the method described in section 8.2.1.2. The % relative activity was calculated using the following formula: % relative activity= catalytic rate of hydrolase reaction with inhibitor)/ (catalytic rate of hydrolase reaction without inhibitor) × 100%.

8.3.1.3. Estimation of total phenolic and flavonoid content

The estimation of total phenolic and flavonoid content of four fractions obtained from *C. grandis* was performed based on the earlier method described from our laboratory with some modification. The method has been described in Chapter 6.

8.3.1.4. Inhibition kinetics study on α-glucosidase

The plots of reaction velocity (∆OD/min) vs. enzyme concentrations [E] (at different sample concentrations) were constructed to explore the inhibitory mechanism of EFCG against α-glucosidase (Biswas et al., 2017). The kinetics behaviour of α-glucosidase

inhibition was studied by using the above method with different concentrations of substrates (1.5- 5.0 mM) and the inhibitors (1500-3500 µg/ml). The inhibitory effect was monitored as the function of initial reaction velocity and incubation time (from 0 to 30 mins). The initial reaction rates were determined from the slope of the calibration curve obtained from the change of the absorbance due to the liberation of p-nitrophenol with respect to time. The kinetics parameters *viz.* K_m and V_{max} value were estimated by using Lineweaver–Burk double reciprocal plot based on the equation: $1/V = K_m/V_{max} \times (1+[I]/K_i) \times 1/[S] + 1/V_{max} \times (1+[I]/\alpha K_i)$; where v is the reaction velocity; K_m is the Michaelis constant; V_{max} is the maximal velocity; [I] is the concentration of inhibitor; K_i is the inhibitor constant; [S] is the concentration of substrate; α is the apparent coefficient. The K_i and αK_i values were determined by constructing the secondary plot of the slope (K_m/V_{max}) and Y-intercept ($1/V_{max}$) vs. inhibitor concentration [I], respectively. The values were calculated by using the software SigmaPlot 12.5.

8.3.1.5. Evaluation of the total antioxidant activity

The antioxidant activities of the fractions of *C. grandis* fruits were evaluated using DPPH free radical scavenging assay based on the method developed in our laboratory (Biswas et al., 2016). In this assay, 100 µl of both the sample and DPPH solution (0.2 mg/ml) was mixed in a 96-well microplate and allowed to stand at room temperature in the dark for 10 min. The decrease in absorbance of the sample solution was measured at 517 nm using spectrophotometer (SpectraMax Plus, Molecular Devices LLC, USA). Butylated hydro-xyanisole (BHA) was used as a positive control. The free radical scavenging activity was expressed in terms of IC_{50} value (µg/ml). To understand the association between antioxidant and α-glucosidase inhibitory activity, Pearson correlation analysis was performed.

8.3.2. Results and discussion

8.3.2.1. α- glucosidase inhibitory activity

The α-glucosidase inhibitory activity of different fractions of *C. grandis* fruits was studied in-vitro, expressed as half-maximal inhibitory concentration (IC_{50}). Among the four fractions, the inhibitory activity (IC_{50} value) of EFCG was found highest as 2.43±0.27 mg/ml, whereas the IC_{50} values of acarbose and chlorogenic acid were determined at

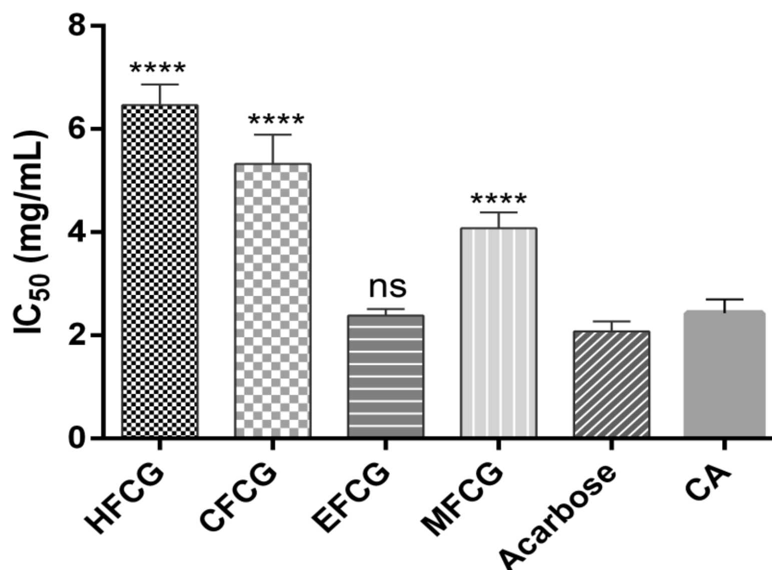


Figure 8.4. IC_{50} value of fractions of *C. grandis* fruit extract. Acarbose and chlorogenic acid —positive control; [Results are represented as mean ± SD (n = 3); one-way ANOVA with Tukey's multiple comparison test; Significant value are represented as **** = $p < 0.0001$; ns = $p < 0.05$]

2.08±0.19 and 2.54±0.17 mg/ml respectively. The IC_{50} values of the other sub-fractions were found in the range of 6.46±0.44 (HFCG), 5.32±0.57 (CFCG), 4.08±0.3 (MFCG) mg/ml respectively (Figure 8.4). No significant difference ($p < 0.05$) was observed in the case of EFCG compared with standard inhibitor acarbose. The inhibition was found to be dose dependent in a range of inhibitor concentration (1500-3500 µg/ml) (Figure 8.5).

8.3.2.2. Determination of total phenolic and flavonoid content

The total phenolic (TPC) and flavonoid content (TFC) in the extract and fractions of *C. grandis* fruits were determined from the calibration curves of gallic acid (y = y= 0.008x + 0.005, R2 = 0.924) and quercetin (y = 0.002x-0.005, R^2 0.979), respectively. The highest TPC was exhibited by the ethyl acetate fraction (EFCG) as 15.17 mg GAE/g whereas the lowest TPC was exhibited by the n-hexane fraction. The highest TFC was exhibited by the ethyl acetate fraction (14.68 ± 0.21 mg QE/g extract), followed by aqueous ethanol and hexane fraction (Table 8.1). The Pearson's correlation coefficients between the α-glucosidase inhibition potential and the TPC and TFC values of the fractions were calculated. It was observed that both the phenolic and flavonoid components of EFCG

affects α-glucosidase inhibitory potential in a higher extent, which in terms reflected in R^2 value. Table 8.2 summarizes the correlations analysis between the TPC/TFC values of fractions of *C. grandis* fruits and their α- glucosidase and antioxidant activity.

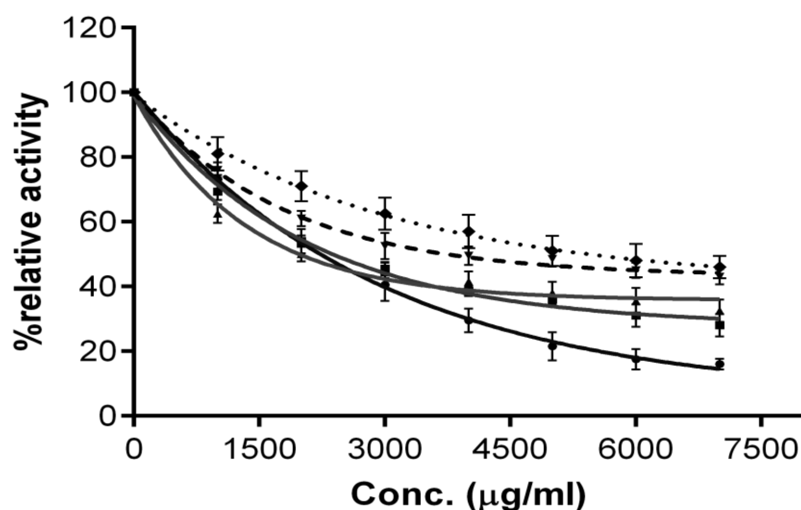


Figure 8.5 Dose-response curve of ethyl acetate fraction, acarbose and chlorogenic acid against α- glucosidase [(▲) EFCG– Ethyl acetate fraction of *C. grandis*; (●) Acarbose; (■) Chlorogenic acid; (•) Ethanol (▼)Hexane fraction [Data are presented as mean ± SD (n = 3)].

8.3.2.3. Effect of anti-oxidant property on α-glucosidase inhibition

The antioxidant capacity of the extract and fractions of *C. grandis* fruits was determined on the basis of DPPH free radical scavenging potential, represented as IC_{50} value of the samples (Table 8.1). The antioxidant property was found maximum in case of EFCG, with compared to ascorbic acid in terms of their free radical scavenging activity. The correlation between theα-glucosidase inhibitory and antioxidant property of the ethyl acetate fraction was determined. The highest positive linear correlation (r = 0.89) was observed between the α-glucosidase inhibitory and DPPH free radical scavenging activity of EFCG. From the correlation analysis, it was found that, α-glucosidase inhibitory activity of *C. grandis* fruits positively correlated with TPC and anti-oxidant property. These findings strongly suggested that higher anti-oxidant property of EFCG does confer with the higher α-glucosidase inhibitory activity linked to its total phenolic content.

Table 8.1. TPC, TFC and DPPH free radical scavenging capacity of fractions of *C. grandis* fruits

Parameters	n-Hexane fraction (Mean±SD)	Chloroform fraction (Mean±SD)	Ethyl acetate fraction (Mean±SD)	Aqueous ethanol (Mean±SD)
Total phenol content (mg/g)	5.43±1.13	10.21±0.32	15.17±1.21	12.79±2.24
Total flavonoid content (mg/g)	10.13±2.09	8.32±3.89	14.68 ±1.81	34.83±1.89
DPPH free radical scavenging capacity (IC_{50} mg/ml)	152.13±2.65	146.76±5.14	101.74±1.95	128.34±8.94

Table 8.2. Correlations analysis between the TPC/TFC values of *C. grandis* fractions and their α- glucosidase and antioxidant activity (IC_{50} value)

Parameters	Pearson's correlation (R^2) coefficient (IC_{50} value of α-glucosidase inhibition)			
	n-Hexane	Chloroform	Ethyl acetate	Aqueous ethanol
Total phenol content (mg/g)	0.72	0.64	0.92	0.81
Total flavonoid content (mg/g)	0.68	0.59	0.93	0.78
Antioxidant (DPPH free radical scavenging capacity) (IC_{50} mg/ml)	0.61	0.71	0.89	0.69

8.3.2.4. Estimation of inhibition kinetics parameters

The kinetic mode of α-glucosidase inhibition was studied with various concentrations of EFCG. The plots of the reaction velocity (v) vs. enzyme concentration at different inhibitor concentrations gave a group of straight lines. It was noted that all the straight lines passed through the origin and the slope of the line decreased inversely with the increasing concentration of the inhibitors (Figure 8.6). This result suggested that EFCG inhibited α- glucosidase, reversibly.

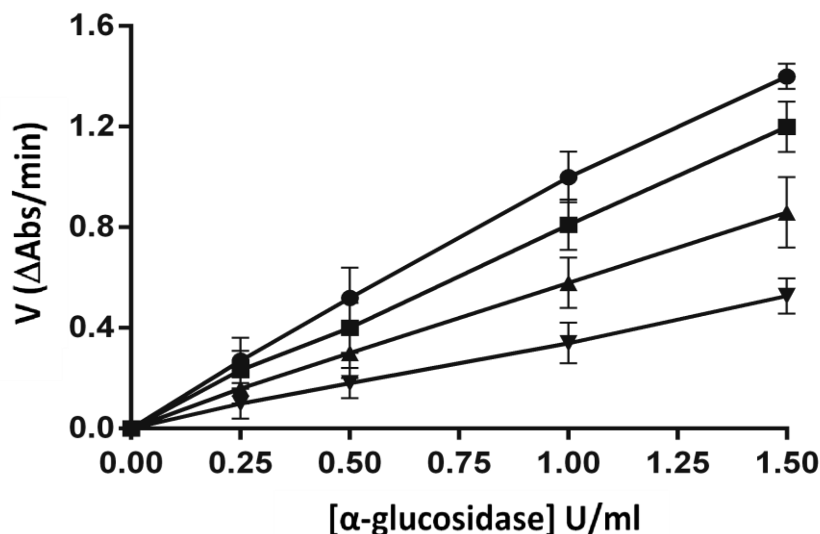


Figure 8.6. Reversible inhibition of EFCG against α-glucosidase [Plots of velocity vs. enzyme concentration]. [The v value indicates the change in absorbance at 405 nm/min at EFCG concentrations of 0 (●), 1000 (■), 2000 (▲), 4000 (▼) µg/ml. Data presented as mean ± SD (n = 3)].

The results of the kinetics study revealed that, both the values of apparent V_{max} and K_m were changed with an increasing concentration of EFCG. The apparent V_{max} value decreased with increase in K_m value (obtained from LB double-reciprocal plots) indicating that the EFCG induced mixed type of inhibition on α-glucosidase (Figure 8.7). The inhibition constants (K_i) and αK_i were calculated from the secondary plot as 2.4 mg/ml and 9.1 mg/ml respectively (Figure. 8.8 A-B). In a subsequent analysis, it was observed that the secondary replot of Slope vs. [I] and Y-intercept vs. [I] was linearly fitted indicating that EFCG induces typical pure non-competitive inhibition.The difference in K_i values (K_i and αK_i values) indicated that the phenolic compounds present in EFCG may bind to both free enzyme (K_i) as well as ES complex (αK_i) implied mixed type of inhibition. The higher αK_i value indicates that the inhibitor can tightly bind to the ES complex rather than enzyme alone.

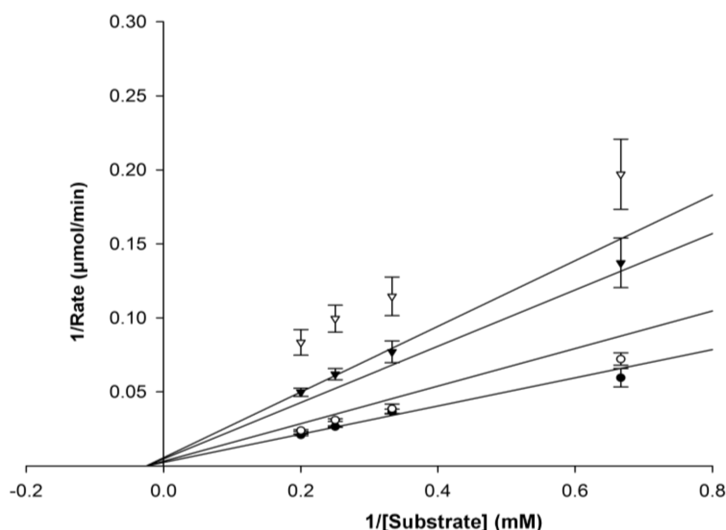


Figure 8.7. Enzyme kinetics study (LB plot) of EFCG against α-glucosidase inhibitory activity

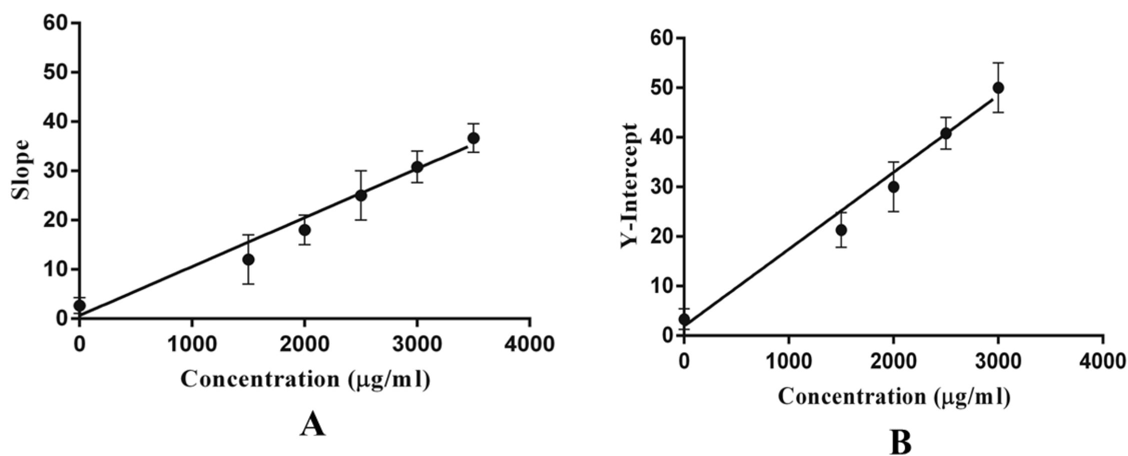


Figure 8.8.A. Plot of Slope (K_m/V_{max}) *vs.* EFCG concentration. B. Plot of Y-intercept ($1/V_{max}$) *vs.* EFCG concentration [Secondary replots were constructed from the data, collected from Lineweaver-Burk plot].

The outcome of the study indicated that the ethyl acetate fraction of aqueous ethanol extract of *C. grandis* inhibited α-glucosidase enzyme in a reversible, dose-dependent and mixed competitive in nature. The reported α-glucosidase inhibitory activity of the fraction of *C. grandis* fruit strongly correlated with total phenol content and high anti-oxidant property. Further LC-Q-TOF based analysis of active fraction was carried out to

derepliate the major constituents present in the bioactive frcation (described in Chapter 9). This finding may lead to develop functional food components from the fruits of *C. grandis* as α-glucosidase inhibitor, which can be used to control glucose level in post prandial hyperglycemia.

8.4. Publication

- LC-QTOF-MS based metabolite profiling and evaluation of α-glucosidase inhibitory kinetics of active fraction of *Cocccinia grandis* fruit. *Industrial Crops and Products*, Elsevier Science, Communicated.

.

Chapter 9

9. LC-QTOF-MS based identification of bioactive constituents of *L. acutangula, L. siceraria, M. charantia, C. grandis*

9.1. Role of LC-MS in medicinal plant analysis

9.2. Bioactivity guided identification, quantification of carbonic anhydrase inhibitory constituents of *Lagenaria siceraria*

9.3. UPLC-QTOF-MS analysis of a carbonic anhydrase-inhibiting extract and fractions of *Luffa acutangula*

9.4. LC–QTOF–MS-based identification of bioactive constituents of *Momordica charantia* fruits

9.5. LC-QTOF-MS based metabolite profiling of *Cocccinia grandis* fruit against α-glucosidase

9.6. Publications

9.1. Role of LC-MS in medicinal plant analysis

Medicinal plants consist of a large number of secondary metabolites including alkaloids, flavonoids, terpenoids, saponins and phenolic acids with potential therapeutic efficacies. The LC-MS based technology offers a versatile tool for plant metabolite profiling, metabolite target analysis as well as dereplication of major bioactive constituents and other aspects of medicinal plants research. In general, metabolite profiling helps in identification and quantitative estimation of marker compounds present in the plant (Wolfender et al., 2015) whereas dereplication is the process of exploring active constituents in order to differentiate the novel compounds from active substances that have already been studied. The major advantages of dereplication process are to avoid the isolation or purification process with the prior knowledge of the chemical compounds and thus offer a robust and time-saving approach in natural product-based drug discovery process (Guillarme et al., 2010).

The MS-based techniques are found suitable in studying structural information of the chemical constituents along with their qualitative and quantitative profiling. As the plant extracts are very complex in nature, it is very important to separate those compounds in the chromatographic platform based on their physiochemical nature. The introduction of HPLC offers a versatile technique for the separation of natural product. In recent advancement of separation sciences, ultra-high pressure liquid chromatography (UHPLC) is developed with higher chromatographic resolution in order to identify a large number of metabolites present in the plant samples (Wolfender et al., 2015). The combination of UHPLC with an MS detector appears to be a suitable approach that fulfills the key requirements of analysis in terms of sensitivity, selectivity, and peak-assignment certainty for the rapid determination of analytes at low concentrations in complex matrices. Moreover, the UHPLC-TOF-MS and UHPLC-QqTOF-MS platforms offer high resolution and increased sensitivity for analysis of targeted metabolites. It is also noted that triple quadrupole (in SRM mode) coupled with UHPLC is most suitable for target compound analysis, whereas TOF-MS analyzers are suitable for non-targeted analysis (Jiang et al., 2017). In this work, the LC–QTOF-MS-guided dereplication methodology was used for rapid identification of the bioactive constituents with major therapeutic activity linked with enzyme inhibition. This approach was found applicable for exploration of lead molecules with potential therapeutic activity from natural products and

also can be considered as a critical step for isolation, characterization of compounds in the natural product-based drug discovery process.

9.2. Bioactivity guided identification, quantification of carbonic anhydrase inhibitory constituents of *Lagenaria siceraria*

The extraction and bioassay guided fractionation was performed to identify the major active constituents of *L. siceraria* against carbonic anhydrase. It was observed that aqueous ethanol fraction exhibited maximum inhibition potential among the other fractions. The LC-QTOF-MS analysis was carried out to identify the phytoconstituents present in the active fraction. Further on flash chromatographic separation of aqueous ethanol fraction, total thirty sub-fractions (Fr. A.1- A.30) were obtained and their carbonic anhydrase inhibitory activity was determined, in terms of half maximal inhibitory concentration (IC_{50}). Based on the IC_{50} values, six sub-fractions (Fr.A.15-Fr. A.17 and Fr. A.27-Fr. A.29) were selected with higher carbonic anhydrase inhibition potential. Moreover, among the six major sub-fractions (from Fr. A), Fr.A.16 offered better inhibition profile, even higher than Fr. A. Further the Fr. A was directed to LC-QTOF-MS analysis to explore the major bioactive constituents in *L. siceraria*. The schematic diagram of extraction and bioactivity guided fractionation has been described in Figure 9.1.

9.2.1. LC-MS method

The HPLC system consisted of LC 800, GL Sciences equipped with a vacuum degasser, a binary pump, an auto-sampler, and a diode array detector (DAD). The chromatographic separation was achieved on Agilent Zorbax Eclipse C18 column (50mm × 2.1mm, 1.7μm). The mobile phases consisted of acetonitrile (A) and water (B) both containing 0.1% formic acid. The gradient profile was set to 10% B from 0 to 1 min, 30% B at 8 min, 40% B at 12 min, 80% B at 16 min, 95% B for 20-27 min, and finally 10% B at 28-35 min. The flow rate for all separations was set at 0.7 ml/min. The sample was prepared at a concentration of 100 μg/ml with acetonitrile and filtered through 0.45 μm filter before injection. The auto-sampler and column heater temperatures were maintained at 25°C, and the injection volume was 15 μl for all analyses. The HPLC system was hyphenated with Triple TOF 5600 System (AB Sciex, Concord, Canada) supported with a DuoSpray ion source.

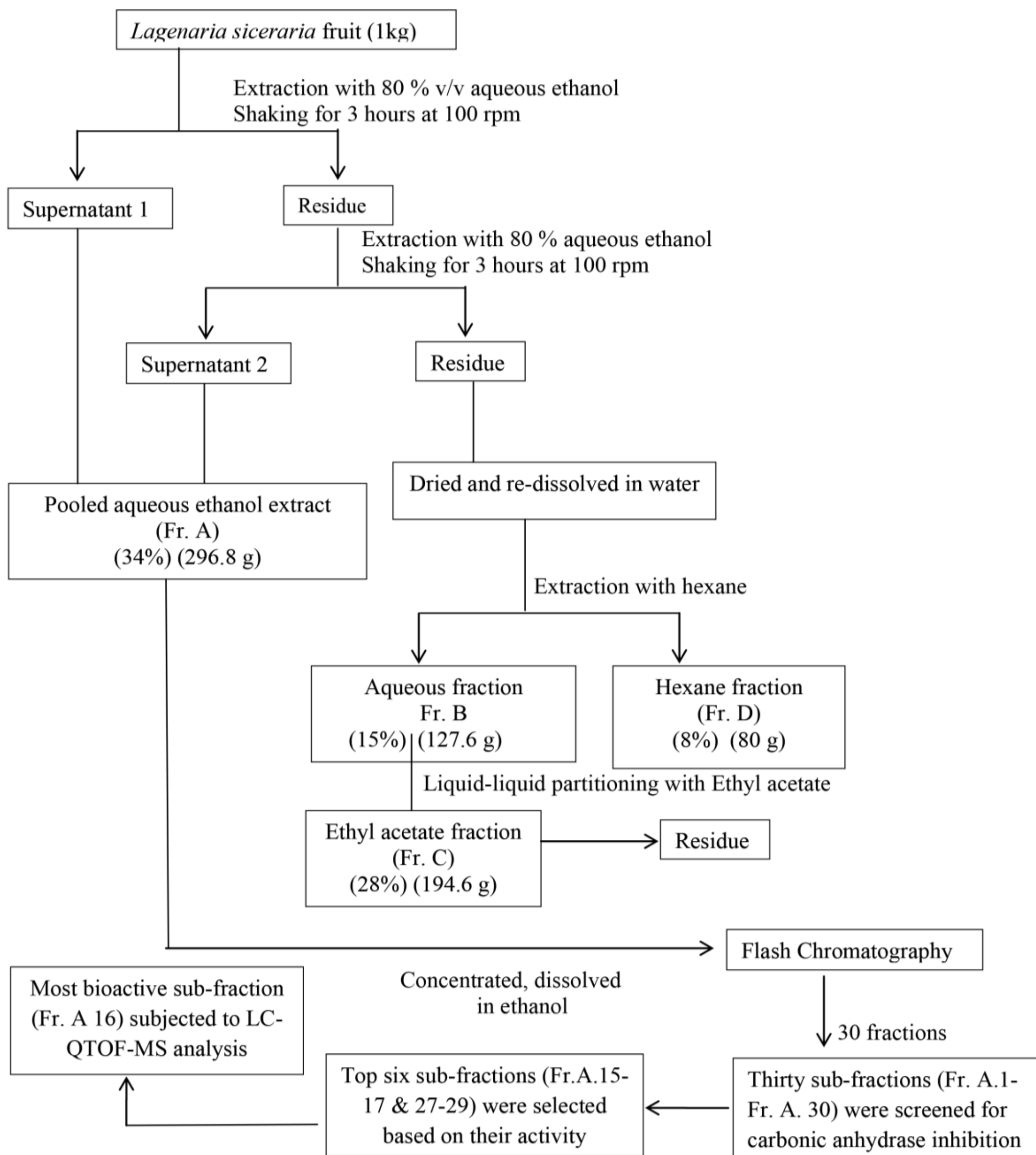


Figure 9.1. Schematic representation of extraction and fractionation of *L. siceraria* fruit. [Carbonic anhydrase inhibition activity represented as percentage (%) at sample concentration of 400 µg/ml; Yield of the fraction in (g)]

The spectra were acquired in negative ionization modes over a mass-to-charge (m/z) ranged between 50-1100 Da. The source voltage was set to 5.0 kV for positive and 4.0 kV for negative ionization mode. The declustering potential was 80 V, and source temperature was 550°C for both modes. The curtain gas flow, nebulizer, and heater gas were set to 35, 50, and 55 arbitrary units. The instrument was set to perform one TOF-MS survey scan (150 ms) and 20 MS/MS scans (50 ms each) with a total duty cycle time of 1.2 s. The mass range of both scan types was 50-1000 m/z. Acquisition of MS/MS spectra was controlled by Information dependent acquisition (IDA) IDA function of the Analyst TF software (AB Sciex, Concord, Canada). The data files were subjected to isotope pattern matched peak mining using the PeakView software with the Master View add-in.

9.2.2. RP-HPLC analysis

The RP-HPLC analysis was performed to quantify the bioactive compounds present in the active fraction. The analysis was performed using an HPLC system (Waters, Milford, MA, USA) consisted of a 600 controller pump, a dual-wavelength ultraviolet–visible (UV–VIS) detector equipped with a degasser AF 2489. Simultaneous estimation of 4-CA and FA was carried out based on the validated chromatographic method developed in our laboratory. The method consisted of isocratic separation with water (1% glacial acetic acid) and acetonitrile in the ratio of 80:20 (v/v) at λ_{max} of 320 nm. The content of CFA was estimated based on the method reported by dos Santos and his co-workers (dos Santos et al., 2006). The separation was achieved by the mobile phase consisted of methanol and water (2% glacial acetic acid) in the ratio of 20:80 (v/v) at the λ_{max} of 260 nm. The quantitative estimation of apigenin-7-glucoside was done at the λ_{max} of 335 nm, using methanol and water (1% ortho-phosphoric acid) in the ratio of 50:50 (v/v) as mobile phase (Chen et al., 2011). The content of hesperidin in the bioactive fraction was also estimated. A mixture of methanol-water (60:40, v/v) was used as mobile phase and detection was made under UV at λ_{max} of 345 nm (El-Shafae et al., 2001). In all cases, separations were carried out using Waters Spherisorb column (C18; 250×4.6 mm, 5 µm particle size), the flow rate was set at 1 ml/min, and the sample injection volume was 20 µl. The column temperature was set at 25ºC. The standard stock solutions (in methanol at 1 mg/ml concentration) of each compound were prepared by dissolving in the diluent at a concentration range of 100-1000 µg/ml. The sample solution was prepared level.

The compounds were identified by comparing their retention times with that of standards. The content of the bioactive compounds was determined using a calibration curve by plotting the mean peak area (y-axis) against the concentrations (x-axis) on its standard. The quantitative estimation was performed with Empower 2 software program.

9.2.3. Results and discussion

The LC-QTOF-MS analysis of the aqueous ethanol fraction tentatively identified the presence of phenolic compounds *viz.* phenolic acids, phenolic glycosides etc. The presence of the compounds was confirmed by their respective retention times, experimental and calculated m/z, molecular formula, error (in ppm), sigma value (an exact numerical comparison between the theoretical and measured isotope patterns) (Figure 9.2; Table 9.1). Further, LC-QTOF-MS analysis of bioactive, purified sub-fraction (Fr. A.16) identified six major polyphenolic compounds namely p-coumaric acid, ferulic acid (phenolic acids), coniferyl alcohol (monolignol), hesperidin and apigenin-7-glucoside (flavonoid glycosides). The compounds were identified with a retention time (RT) of 12.15, 15.14, 14.8, 8.63 and 10.86 min respectively. The *m/z* values were found at 163.0408, 193.0512, 179.0721, 609.1835 and 431.0979 respectively. The presence of these compounds was confirmed by their respective retention times, experimental and calculated m/z, molecular formula, error (in ppm), sigma value (an exact numerical comparison between the theoretical and measured isotope patterns), MS/MS fragmentation pattern. However, apigenin-7-glucoside and hesperidin showed poor fragmentation pattern and low intensity. The analysis was carried out in the mass error window of 5 ppm and thus confirming their authenticity in chemical composition. The Phenol-Explorer database was used to identify the polyphenolic compounds in *L. siceraria* (Neveu et al., 2010). The Extracted Ion Chromatogram (XIC) and the MS/MS spectra have been represented in Figure 9.3 & 9.4. Table 9.2 summarises the LC-QTOF-MS parameters of all the compounds. The quantitative estimation of the bioactive compounds was carried out by HPLC-DAD method. The content of CFA, 4-CA and FA was found to be 11.71, 8.24, and 7.67 % (w/w) whereas the amount of apigenin-7-glucoside and hesperidin was calculated as 4.11 and 3.73 % (w/w) respectively (Table 9.3). The chromatograms have been provided as Figure 9.5 (A-G).

Table 9.1. Compounds identified in aqueous ethanol extract of *L. siceraria*

Compound Name	Molecular formulae	Theoritical Mass	Found mass	Error ppm	Retention time	Isotopic diff.
4-O-glucosyl-3,4-dihydroxybenzyl alcohol	$C_{13}H_{18}O_8$	301.0929	301.0937	2.6	4.74	3.6
4-O-glucosyl-4-hydroxybenzoic acid	$C_{13}H_{16}O_8$	299.0772	299.0778	1.8	8.22	7.6
4-O-(6′-O-glucosylcaffeoyl)-4-hydroxybenzyl alcohol	$C_{22}H_{24}O_{10}$	477.1297	447.1294	-0.5	10.98	0.9
4-O-(6′-O-glucosyl-p-coumaroyl)-4-hydroxybenzyl alcohol	$C_{22}H_{24}O_9$	431.1348	431.1351	0.8	12.13	1.3
4-O-(6′-O-glucosyl-4″-hydroxybenzoyl)-4-hydroxybenzyl alcohol	$C_{20}H_{22}O_9$	405.1191	405.1188	-0.7	10.95	2.8
4-O-(6′-O-glucosylcaffeoyl)-4-hydroxybenzoic acid	$C_{22}H_{22}O_{11}$	461.1089	461.1089	0	13.19	1.1
4-O-(6′-O-glucosylferuloyl)-4-hydroxybenzyl alcohol	$C_{23}H_{26}O_{10}$	461.1453	461.1449	-0.9	12.37	0.4
4-O-(6′-O-glucosylcaffeoylglucosyl)-4-hydroxybenzyl alcohol	$C_{28}H_{34}O_{15}$	609.1825	609.1835	1.6	8.63	1.8
4-O-(6′-O-glucosylcaffeoylglucosylp-coumaroyl)-4-hydroxybenzyl alcohol	$C_{37}H_{40}O_{17}$	755.2193	755.2202	1.2	12.03	2
4-O-(6′-O-glucosylferuloylglucosylcaffeoyl)-4-hydroxybenzyl alcohol	$C_{38}H_{42}O_{18}$	785.2298	785.2335	4.6	12.01	4.9
4-O-(6′-O-glucosylcaffeoylglucosylferuloyl)-4-hydroxybenzyl alcohol	$C_{38}H_{42}O_{18}$	785.2298	785.2335	4.6	12.01	6.2

Ferulic acid	$C_{10}H_{10}O_4$	193.0506	193.0513	3.4	15.14	0.50
Caffeic acid	$C_9H_8O_4$	179.035	179.0355	3	12.29	0.50
p-Coumaric acid	$C_9H_8O_3$	163.0401	163.0408	4.7	12.15	1.10
Genistin	$C_{21}H_{20}O_{10}$	431.0984	431.0979	-1.1	10.86	4.1
Hesperidin	$C_{28}H_{34}O_{15}$	609.1825	609.1835	1.6	8.63	4.90
5-Hydroxy coniferaldehyde	$C_{10}H_{10}O_4$	193.0506	193.0513	3.4	15.14	1.70
Coniferyl alcohol	$C_{10}H_{12}O_3$	179.0714	179.0721	4.1	14.8	0.90
Caffeoyl aldehyde	$C_9H_8O_3$	163.0401	163.0408	4.7	12.15	1.10
Sinapaldehyde	$C_{11}H_{12}O_4$	207.0663	207.0666	1.5	14.5	5.90
7-Hydroxycoumarin	$C_9H_6O_3$	163.039	163.0388	-0.9	22.98	1.70

Table 9.2. QTOF-MS parameters

Name of the compounds	QTOF-MS parameters				Molecular formulae
	Theoretical mass	Found mass	Error (ppm)	MS/MS fragments	
Coniferyl alcohol	179.0714	179.0721	4.1	133.0656, 72.9945, 161.0602	$C_{10}H_{12}O_3$
p-coumaric acid	163.0401	163.0408	4.7	119.0507, 93.0353, 117.0358	$C_9H_8O_3$
Ferulic acid	193.0506	193.0513	3.4	133.0294, 161.0245	$C_{10}H_{10}O_4$
Apigenin -7-glucoside	431.0984	431.0979	-1.1	--	$C_{21}H_{20}O_{10}$
Hesperidin	609.1825	609.1835	1.6	--	$C_{28}H_{34}O_{15}$

Table 9.3. RP-HPLC parameters

Name of the compounds	RP-HPLC parameters		
	λ_{max}	RT[a]	Content (% w/w)
Coniferyl alcohol	260	7.231	11.71
p-coumaric acid	320	13.264	8.24
Ferulic acid	320	16.508	7.67
Apigenin -7-glucoside	335	8.619	4.11
Hesperidin	345	6.744	3.73

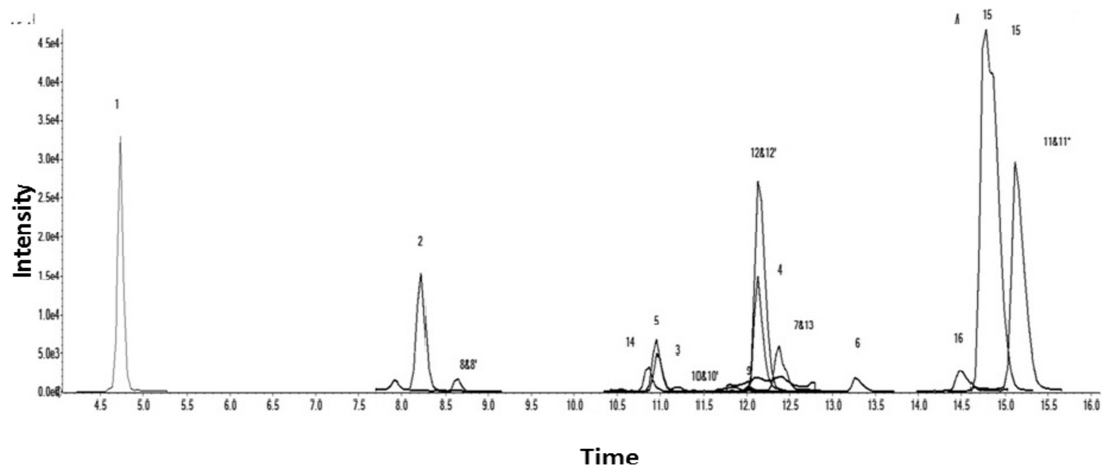


Figure 9.2. Total ion chromatogram of *L. siceraria* aqueous ethanol extract

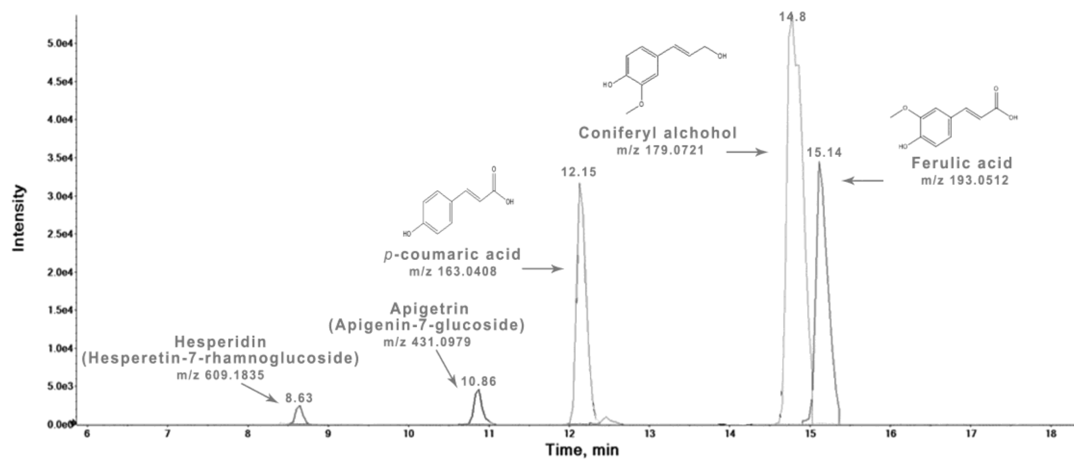
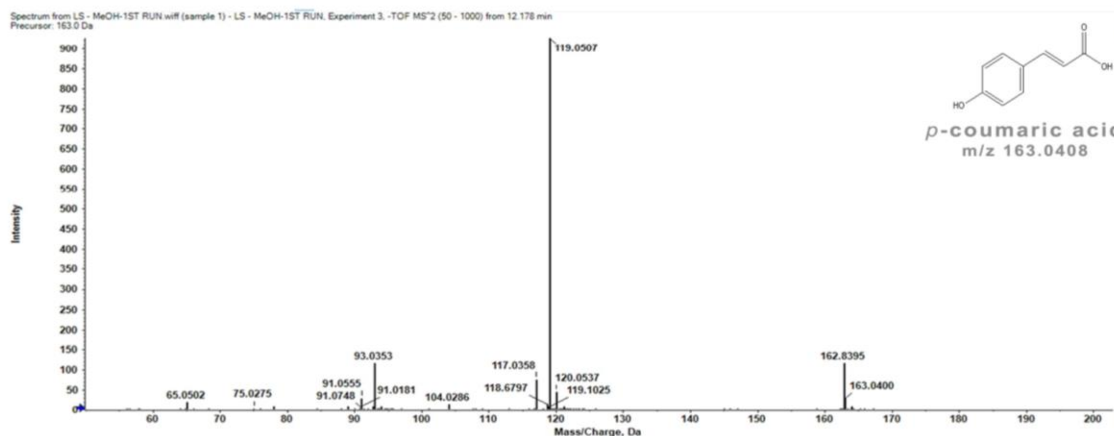


Figure 9.3. Total ion chromatogram of Fr. A. 16 of aqueous ethanol extract of *L. siceraria*



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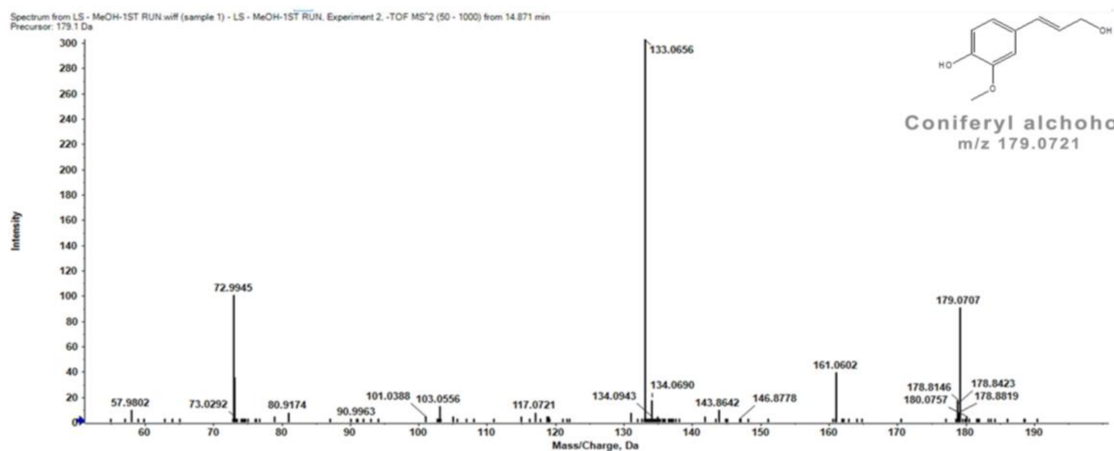

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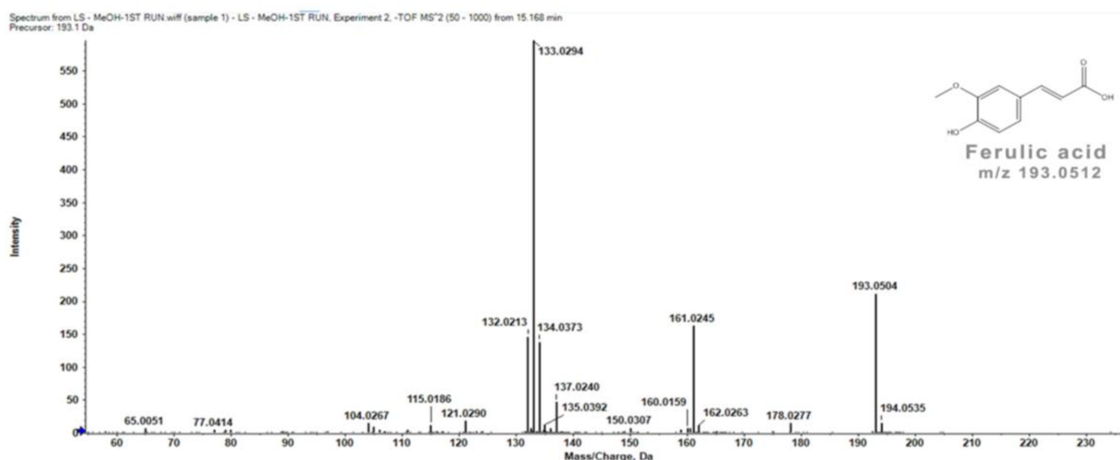

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Figure 9.4. (A-C) LC-MS/MS spectrum of A. p-coumaric acid; B. Conideryl alchohol; C. Ferulic acid

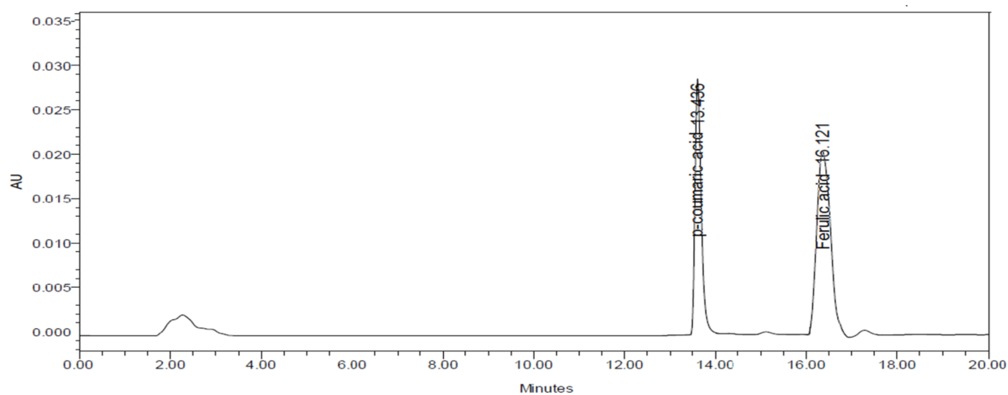


Figure 9.5.A. HPLC chromatogram of p-coumaric acid and ferulic acid standard

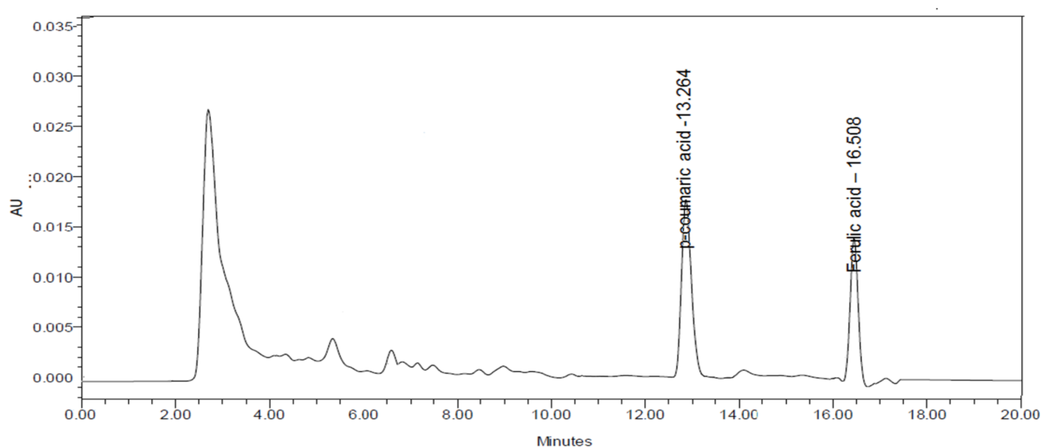


Figure 9.5.B. HPLC chromatogram of p-coumaric acid and ferulic acid standard

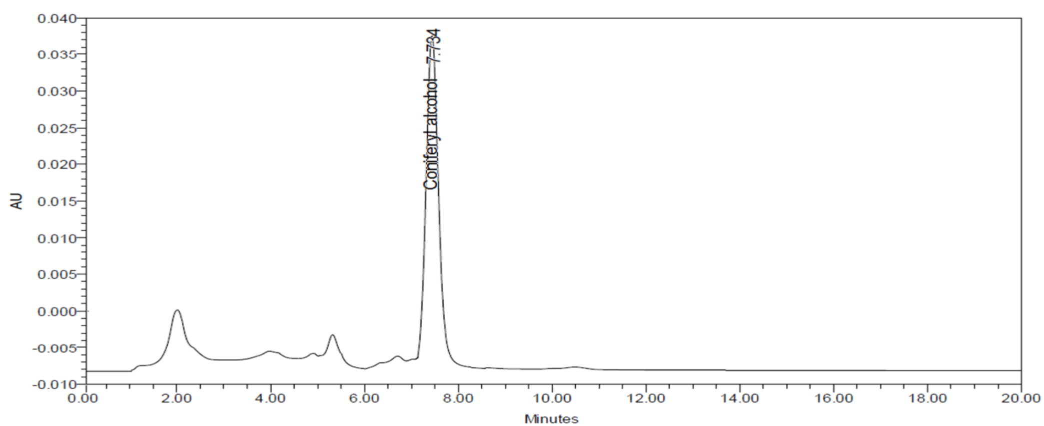


Figure 9.5.C. HPLC chromatogram of coniferyl alcohol standard

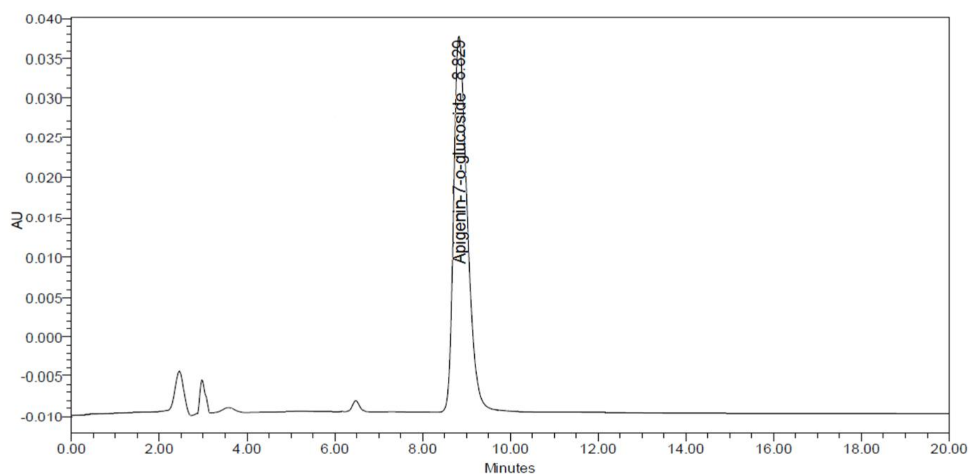


Figure 9.5.D. HPLC chromatogram of apigenin-7-o-glucoside standard

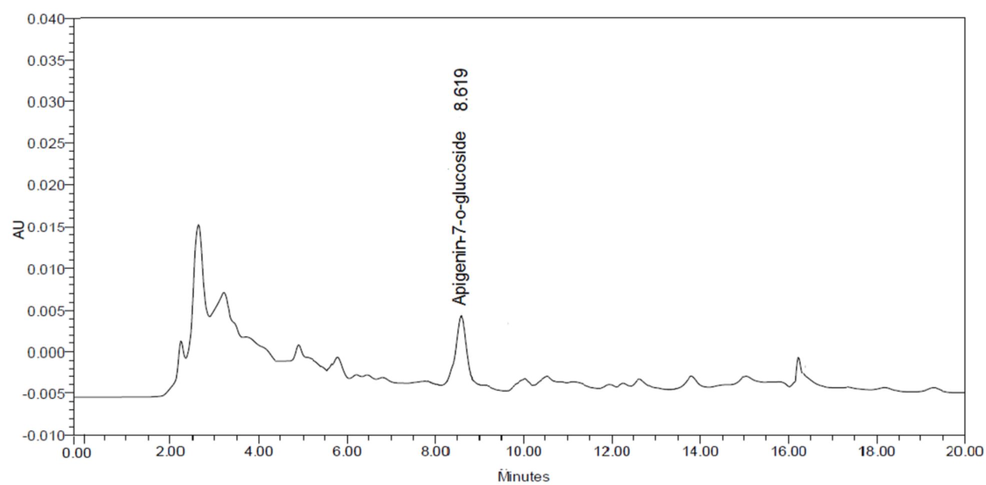


Figure 9.5.E. HPLC chromatogram of apigenin-7-o-glucoside in Fr. A. 16

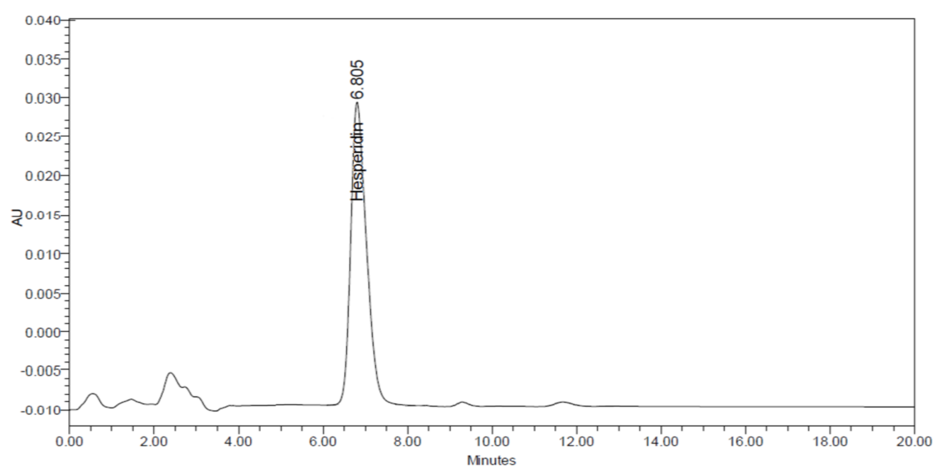


Figure 9.5.F. HPLC chromatogram of hesperidin standard

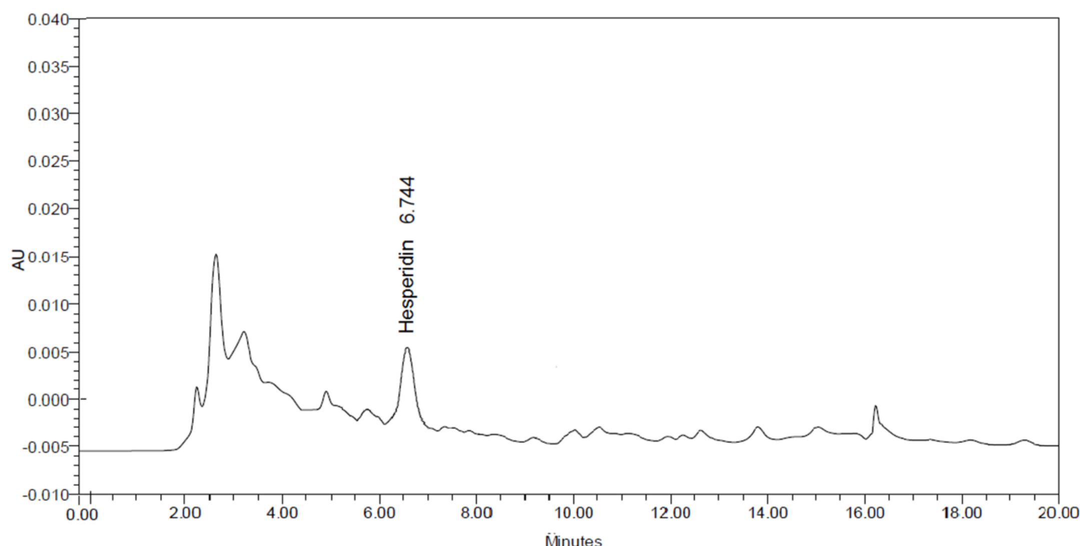


Figure 9.5.G. HPLC chromatogram of hesperidin in Fr. A. 16

Figure 9.5. (A-G). RP-HPLC chromtaograms of the major phytoconstituents present in Fr. A. 16 of aqueous ethanol extract of *L. siceraria*

Thus the flash chromatography based separation and purification techniques allowed us to obtain enriched fractions with higher amount of phenolic metabolites which was reflected in their bioactivity. The LC-QTOF-MS analysis of the Fr.A.16, identified coniferyl alcohol (CFA), ferulic acid (FA), p-coumaric acid (4-CA), hesperidin (hesperitin-7-rutinoside), and apigenin-7-glucoside as a major compounds. The HPLC-DAD analysis revealed that the presence of coniferyl alcohol was found maximum in the bioactive fraction whereas the content of flavonoids (hesperidin and apigenin-7-glucoside) was low. These phenolic compounds were evaluated for their carbonic anhydrase enzyme inhibition activity (Details in Chapter 6). The results suggested that CFA, FA and 4-CA inhibited carbonic anhydrase activity significantly (IC50 value range of 80 - 250 μM), whereas hesperidin and apigenin-7-glucoside (both are flavonoid glycoside) showed weak inhibition profile (IC50 value > 500 μM).

9.3. UPLC-QTOF-MS analysis of a carbonic anhydrase-inhibiting extract and fractions of *Luffa acutangula*

The ethyl acetate frcation of *L. acutangula* aqueous ethanol extract showed highest carbonic anhydrase inhibitory activity (as described in Chapter 6). The UPLC-QTOF-MS analysis of the ethyl acetate fraction was carried out for tentative identification of the bioactive constituents. The major phytoconstituents of the ethyl acetate fraction of *L. acutangula* were identified based on their elution order, measurement of accurate molecular mass and isotope peak pattern.

9.3.1. UPLC-QTOF-MS conditions

UPLC-QTOF-MS analysis was carried out in Agilent 1290 Infinity LC system coupled to Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) with Agilent Jet Stream Thermal Gradient Technology to identify the major constituents present in ethyl acetate fraction. The chromatographic method was refined based on the mobile phase composition, gradient, flow rate, and injection volume. The separation was achieved on Agilent Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 1.8 µm) as stationary phase. The mobile phase was consisted of 10 mM ammonium acetate in water (A) and acetonitrile (B). The gradient profile was set to 20% B from 0 to 8 min, 35% B at 12 min, 50% B at 15 min, 75% B at 20 min followed by a post time run of 5 min to equilibrate the column. The flow rate of all separations was set at 0.5 ml/min. The sample (1 mg) was prepared by dissolving in methanol (1 ml) and filtered through 0.45 µm filter. The stock solution was diluted to 100 µg/ml before injection. The phenolic compounds were identified based on the high resolution accurate mass analysis which corresponds to the score value (on a scale of zero to 100) of the measured mass (*m/z*) with respect to their theoretical formula. Considering the MS conditions, negative ion mode was used to obtain high-resolution mass spectra. The operating parameters were optimized as follows: drying gas (N_2) flow, 8 l/min; drying gas temperature, 150ºC. Other parameters were set as nebulizer gas, 35 psig; capillary voltage, 3500 V; skimmer voltage, 60 V; nozzle voltage 1500 V. Mass spectra were recorded across the range m/z 50–1500 with accurate mass measurement. The fragmentor voltage was set 150 V and sheath gas temperature was at 350° C, at a flow of 11 l/min. The data were acquired using the Extended Dynamic Range mode (2 GHz) and the mass range was set at 50–1000 Da. The data acquisition on the LC-Q-TOF was performed using Agilent MassHunter

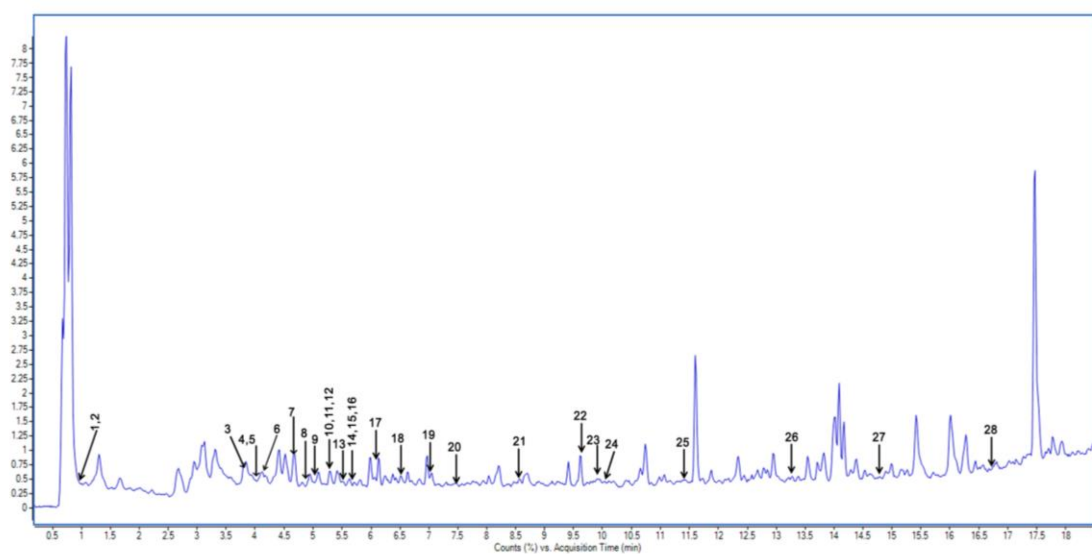
Acquisition B.06.01 software (Agilent Technologies, Santa Clara, CA, USA). The data were deconvoluted into individual chemical peaks with Agilent MassHunter Qualitative Analysis B.07.00 (MassHunterQual, Agilent Technologies, Santa Clara, CA, USA), using Molecular Feature Extractor (MFE).

9.3.2. Library searching

Agilent Personal Compound Database and Library (PCDL) was utilized to create the custom database with the phenolic compounds obtained from Phenol-Explorer database (Neveu et al., 2010). This customised database was uploaded into MassHunterQual software's algorithm for targeted data mining. The PCDL search process integrates the chromatogram within a specified mass window and generates match score, based on the combination of accurate mass, isotopic abundance and isotopic spacing of the identified compounds (DeTata et al., 2013).

9.3.3. Results and Discussion

The phenolic and flavonoid compounds of the ethyl acetate fraction of *L. acutangula* were tentatively identified based on their elution order, measurement of accurate molecular mass and isotope peak pattern. The total ion chromatogram and extracted ion chromatogram of ethyl acetate fraction were obtained by full scan mode under negative electrospray (ESI) condition as shown in Figure 9.6 (A-B) respectively. Table 9.4 summarizes the mass spectrometric data of the identified compounds, including experimental and calculated *m/z*, molecular formula, error in ppm, retention time as well as the classification of the proposed compounds. The high mass accuracy of the TOF-MS method was able to display a mass error of below 5 ppm, and thus supporting tentative identifications of various phenolic acids *viz.* ellagic acid, chlorogenic acid, gallic acid, hydroxycaffeic acid, gentisic acid, dihydroferulic acid, caffeic acid, coumaric acid. Different flavonones *viz.* sakuranetin, gardenin B, chrysin, scutellarein, geraldone E, hydroxycoumarins (scopoletin, mellein etc.), guaiacol (methoxyphenol) in ethyl acetate fraction of aqueous ethanol extract of *L. acutangula* fruits. The high resolution Q-TOF mass spectrometry coupled with custom Personal Compound Database and Librar searching provided a selective and highly sensitive procedure for rapid identification of phenolic compounds in ethyl acetate fraction. Thus this study reports LC-QTOF-MS based identification of phenolic compounds in carbonic anhydrase inhibiting extract and fractions of *L. acutangula* fruit.



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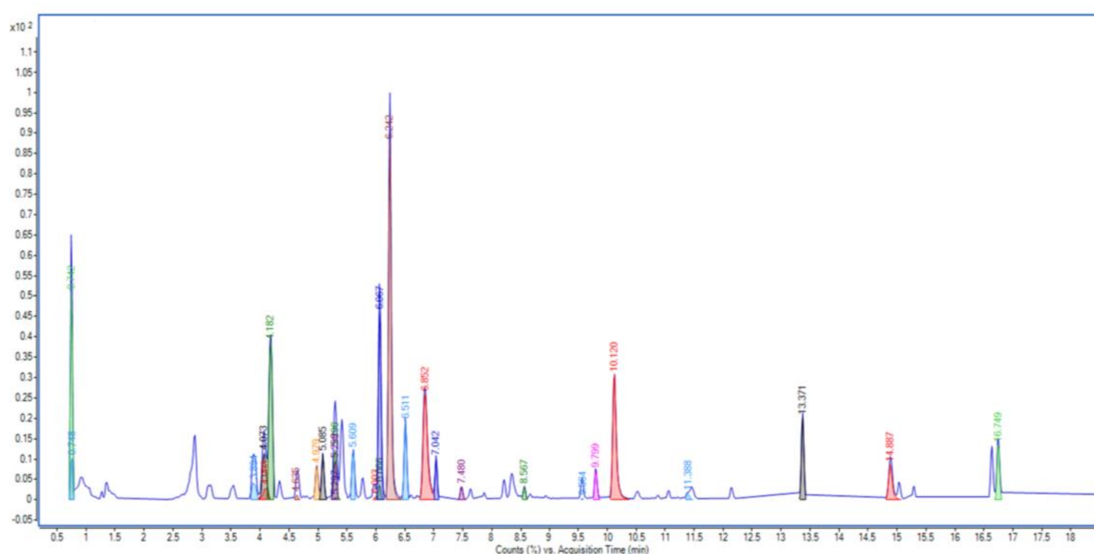


B

Figure 9.6. (A) Total ion chromatogram of ethyl acetate fraction of *L. acutangula* in negative ion mode. (B) Extracted ion chromatogram of ethyl acetate fraction in negative ion mode [Peak labels represent the identified compounds]

Table 9.4. Phenolic compounds identified in ethyl acetate fraction of *L. acutangula* by UPLC-QTOF-MS

Peak	Retention time	*m/z*	Mol. formulae	Mass (Exp.)	Mass (Theo.)	Error (ppm)	Proposed compounds
1.	0.742	302.194	$C_{14}H_6O_8$	302.0069	302.0063	2.01	Ellagic acid
2.	0.748	353.0863	$C_{16}H_{18}O_9$	354.0948	354.0951	-1.92	Chlorogenic acid
3.	3.894	207.0662	$C_{11}H_{12}O_4$	208.0732	208.0736	-1.82	Sinapaldehyde
4.	4.073	151.0398	$C_8H_8O_3$	152.0471	152.0473	-1.77	Hydroxy-phenyl acetic acid
5.	4.093	191.0353	$C_{10}H_8O_4$	192.0426	192.0423	1.89	Scopoletin
6.	4.182	137.0247	$C_7H_6O_3$	138.0323	138.0317	4.49	Salicylic Acid
7.	4.635	169.0146	$C_7H_6O_5$	170.0218	170.0215	1.92	Gallic acid
8.	4.979	161.0248	$C_9H_6O_3$	162.0321	162.0317	2.73	4-Hydroxy coumarin
9.	5.296	135.0449	$C_8H_8O_2$	136.0521	136.0524	-2.25	Phenylacetic acid
10.	5.297	123.0455	$C_7H_8O_2$	124.0528	124.0524	2.95	Guaiacol
11.	5.298	167.0347	$C_8H_8O_4$	168.042	168.0423	-1.41	Dihydroxy phenylacetic acid
12.	5.609	195.0302	$C_9H_8O_5$	196.0375	196.0372	1.66	Hydroxy caffeic acid
13.	6.003	153.0199	$C_7H_6O_4$	154.0272	154.0266	3.84	Gentisic acid
14.	6.066	151.0758	$C_9H_{12}O_2$	152.0832	152.0837	-4.73	4-Ethyl-guaiacol
15.	6.067	195.0669	$C_{10}H_{12}O_4$	196.0742	196.0736	3.46	Dihydro ferulic acid
16.	6.242	179.0356	$C_9H_8O_4$	180.0428	180.0423	3.0	Caffeic acid

17.	6.511	255.0671	$C_{15}H_{12}O_4$	256.0745	256.0736	3.68	Pinocembrin
18.	6.852	121.0296	$C_7H_6O_2$	122.0368	122.0368	0.1	Benzoic acid
19.	7.042	313.0731	$C_{17}H_{14}O_6$	314.0805	314.079	4.71	Cirsimaritin
20.	7.48	163.0769	$C_{10}H_{12}O_2$	164.0842	164.0837	2.95	Eugenol
21.	8.567	163.0395	$C_9 H_8 O_3$	164.0467	164.0473	-3.67	Coumaric acid
22.	9.564	285.0768	$C_{16}H_{14}O_5$	286.0841	286.0841	-0.03	Sakuranetin
23.	9.799	357.0994	$C_{19}H_{18}O_7$	358.1067	358.1052	4.03	Gardenin B
24.	10.12	253.0513	$C_{15}H_{10}O_4$	254.0585	254.0579	2.35	Chrysin
25.	11.388	285.0407	$C_{15}H_{10}O_6$	286.048	286.0477	0.79	Scutellarein
26.	13.371	341.1041	$C_{19}H_{18}O_6$	342.1111	342.1103	2.27	Tetramethyl scutellarein
27.	14.887	177.0555	$C_{10}H_{10}O_3$	178.0628	178.063	-1.31	Mellein
28.	16.749	283.0621	$C_{16}H_{12}O_5$	284.0694	284.0685	3.35	Geraldone

9.4. LC–QTOF–MS based identification of bioactive constituents of *Momordica charantia* fruits

The ethyl acetate fraction of *M. charantia* fruit exhibited highest inhibition capacility with compared to others (Described in Chapter 7). The LC–QTOF–MS analysis was performed to identify the phytoconstituents present in the bioactive fraction of *M. charantia* fruits.

9.4.1. LC-QTOF-MS conditions

Chromatographic separation was carried out using an LC800, GL Sciences equipped with a vacuum degasser, a binary pump, an autosampler and a diode array detector. The chromatographic separation was achieved on Agilent Zorbax Eclipse C18 column (50 × 2.1 mm, 1.7 μm). The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. The gradient profile was set to 10% B from 0 to 1 min, 30% B at 8 min, 40% B at 12 min, 80% B at 16 min, 95% B for 20–27 min, and finally 10% B at 28–45 min. The flow rate was set at 0.7 ml/min. The sample was prepared at a concentration of 100 μg/ml with acetonitrile and filtered through 0.45 μm filter before injection. The auto-sampler and column heater temperatures were maintained at 25°C, and the injection volume was 15 μl for all analyses. The HPLC system was hyphenated with a Triple TOF 5600 System (AB Sciex, Concord, Canada) supported with a

DuoSpray ion source. The spectra were acquired in negative ionization modes over a mass-to-charge (*m/z*) range between 50 and 1100 Da. The source voltage was set to 4.0 kV for negative ionization mode. The declustering potential and source temperature were maintained at 80 V and 550°C respectively. The curtain gas flow, nebulizer and heater gas were set to 35, 50 and 55 arbitrary units (AU). The instrument was configured to perform one TOF–MS survey scan (150 ms) and 20 MS/MS scans (50 ms each) with a total duty cycle time of 1.2 s. The mass range of both scan types was 50–1000 *m/z*. The MS–MS spectra were acquired using the IDA function of the Analyst TF software (AB Sciex, Concord, Canada). The data files were subjected to isotope pattern matched peak mining using the extracted ion chromatogram manager add-on for PeakView (AB Sciex, Concord, Canada) and reported the most intense peak across the chromatogram. The metabolites were putatively identified by utilizing the custom database with the phenolic compounds obtained from Phenol-Explorer database (Neveu et al., 2010).

9.4.2. Results and discussion

LC–QTOF–MS-based fingerprinting of the ethyl acetate fraction led to the identification of phenolic compounds based on their accurate mass, experimental and calculated *m/z*, molecular formula, error (in ppm), isotopic ratio and MS fragmentation pattern (matched within 25 ppm error tolerance). Table 9.5 summarizes 22 phenolic compounds with their respective retention time, molecular formulae, exact and deprotonated mass, isotopic difference and MS/MS fragment ions. The total ion chromatogram and the representative MS/MS spectra of the identified compounds have been represented in Figure 9.7. and Figure 9.8. The results demonstrated that ethyl acetate fraction contains a large number of phenolic compounds, including hydroxybenzoic acids, hydroxycinnamic acid, flavonol, isoflavonoid, flavanone and hydroxycoumarin. The high mass accuracy of the TOF–MS method was able to display a mass error of < 5 ppm, and thus confirmed the authenticity of the chemical composition.

The LC–QTOF–MS analysis of the ethyl acetate frcation of *M. charantia* fruits revealed the the presence of phenolic compounds *viz.* feruloylquinic acid (RT 3.53 min), vanillic acid (RT 4.29 min), gentisic acid (RT 5.96 min), esculetin (RT 20.68 min), scopoletin (RT 21.17 min), coumaric acid (RT 24.30 min), epigallocatechin glucuronide (RT 28.01 min), glycitin (RT 30.69 min), naringenin-7-O-glucoside (RT 33.20 min), hersperidin (RT 34.34 min), 1-acetoxypinoresinol (liganan derivative) (RT 35.37 min) and methylgallic acid (RT

36.30 min). It has to be noted that, owing to its faster acquisition rate and accurate mass acquisition capacity, high-resolution mass spectrometric analysis offered a reliable technique for putative identification of the polyphenolic compounds in *M. charantia* fraction.

A large number of studies have suggested that phytochemicals present in vegetables offer some prophylactic benefits against several lifestyle-related disorders, including obesity, owing to their synergistic interaction (Williams et al., 2013). Many polyphenolic compounds such as phenolic acids, flavones, flavonols, tannins and chalcones are found to be active against pancreatic lipase (Birari & Bhutani, 2007).

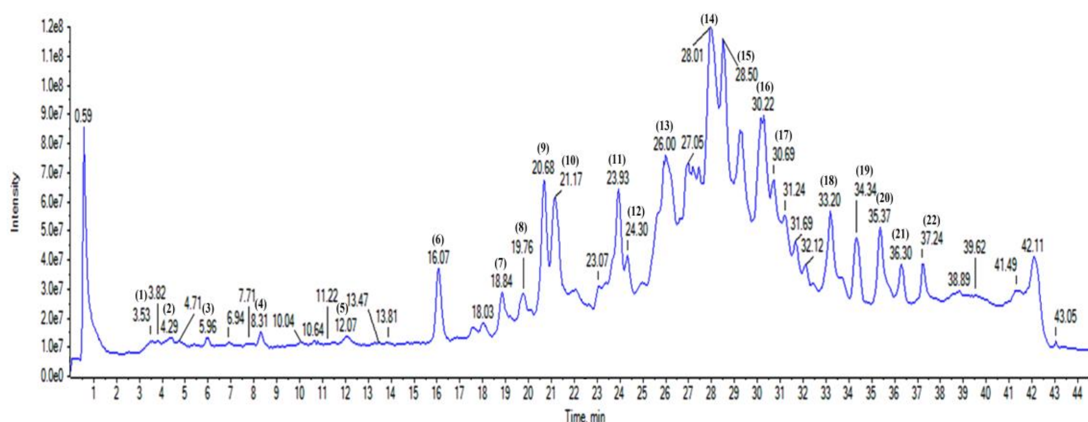


Figure 9.7. LC-ESI-MS total ion chromatogram (TIC) of bioactive fraction of *M. charantia* fruit

A variety of hydroxycinnamic acids (viz. vanillic acid, gentisic acid, gallic acid, feruloylquinic acid, coumaric acid and benzoic acid derivatives) found in fruits and different parts of plants have shown promising lipase inhibitory activity (Buchholz & Melzig, 2015; Tan et al., 2017). In 2011, Kim and co-workers reported moderate pancreatic lipase inhibition activity of vanillin, obtained from radiolytic transformation of curcumin (Kim et al.,2011). The inhibitory effect of catechol and related hydroxyl phenol compounds on pancreatic lipase was exhibited earlier (Weinstein & Wynne, 1936). Two coumarin derivatives, esculetin and scopoletin, were reported to have potential anti-obesity activity by inhibiting adipocyte differentiation in three T3-L1 cells (Shin et al., 2010). Another phenolic metabolite, protocatechuic aldehyde (isolated from *Taraxacum*

ohwianum), was reported to exhibit pancreatic lipase inhibitory activity (Kim & Kim, 2011). Some catechins and their metabolites/derivative (viz. epigallocatechin) lower cholesterol level by inhibiting pancreatic lipase (Juhel et al., 2000). The efficacy of quercetin-3-xyloside and quercetin-3-glucoside in inhibiting the catalytic activity of PL was reported in the literature (Martinez-Gonzalez et al., 2017). The dihydroflavonol derivatives, *viz.* quercetin-3-O-rhamnoside, glycitin (isoflavone glycoside) were reported to inhibit pancreatic lipase in a dose-dependent manner (Choi et al., 2007, Zhang et al., 2018). Another study showed that flavonoids (*viz.* naringenin, hesperidin) and lignans (*viz.* pinoresinol derivative) offer promising lipid-lowering activity by inhibiting pancreatic lipase (Ahn et al., 2012; Assini et al., 1997).

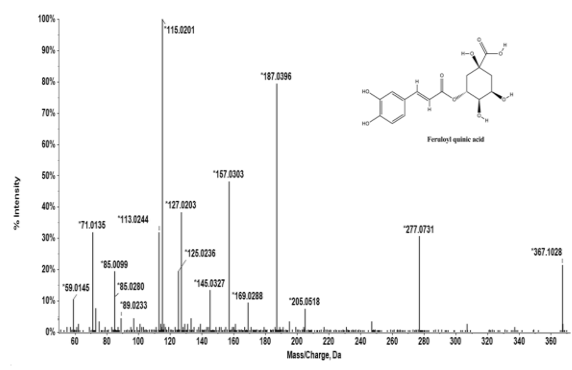
Table 9.5. Phenolic compounds identified in ethyl acetate fraction of *M. charantia* by LC–QTOF–MS

Peak	Rete-ntion time	Mol. formulae	Exact mass	Deproto-nated mass	Error (ppm)	Isotopic diff.	MS/MS fragments	Proposed com-pounds
1	3.53	$C_{17}H_{20}O_9$	368.11073	367.10346	1.3	7.5	115.0201 187.0396 157.0303 127.0203	Feruloyl quinic acid
2	4.29	$C_8H_8O_4$	168.04226	167.03498	1.3	7.5	108.0221 152.0096 80.0265	Vanillic Acid
3	5.96	$C_7H_6O_4$	154.02661	153.01933	0	3.1	108.0205 78.9590	Gentisic acid
4	8.31	$C_{13}H_{16}O_8$	300.08452	299.07724	-1	3.1	93.0340, 137.0232	4-Hydroxy benzoic acid 4-O-glucoside
5	12.07	$C_8H_8O_3$	152.04734	151.04007	0.2	9.1	108.0215 92.0278, 136.0166 95.0145	Vanillin
6	16.07	$C_8H_8O_2$	136.05243	135.04515	-0.4	9	92.0260, 121.0293	P-Anisaldehyde
7	18.84	$C_8H_8O_2$	136.05243	135.04515	-0.4	9	92.0260 121.0293	Phenylacetic acid
8	19.76	$C_7H_8O_2$	124.05243	123.04515	-5	7.9	93.0341 65.0393	Methyl catechol
9	20.68	$C_9H_6O_4$	178.02661	177.01933	0.7	1.7	133.0294 121.0272 93.0334 71.0138	Esculetin
10	21.17	$C_{10}H_8O_4$	192.04226	191.03498	0	6.8	93.0334	Scopoletin

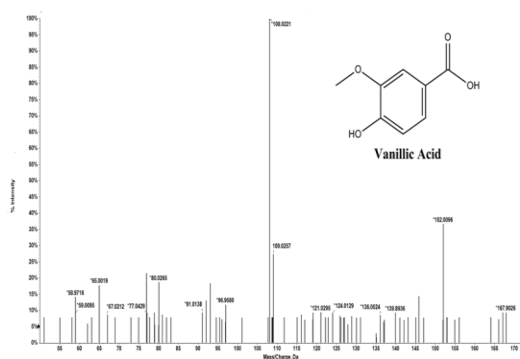
11	23.93	$C_7H_6O_2$	122.03678	121.0295	0.6		92.0263	Benzoic acid
12	24.30	$C_9H_8O_3$	164.04734	163.04007	-1	2.6	119.0498 93.0350	Coumaric Acid
13	26.00	$C_7H_6O_3$	138.03169	137.02442	0.7	1.1	93.0352 65.0398	Protocatechuic aldehyde
14	28.01	$C_{22}H_{24}O_{13}$	496.12169	495.11441	2.5	6.9	449.1100 431.0981	4'-O-Methyl-(-)-epigallo catechin 3'-O-glucuronide
15	28.50	$C_{26}H_{28}O_{16}$	596.13774	595.13046	-2.2	9.6	549.0949 , 300.0250	Quercetin 3-O-glucosyl-xyloside
16	30.22	$C_{21}H_{22}O_{11}$	450.11621	449.10894	-2.2	2.6	167.0334 137.0231 152.0102 281.0650	Dihydro quercetin 3-O-rhamnoside
17	30.69	$C_{22}H_{22}O_{10}$	446.1213	445.11402	-2.8	1.9	145.0287 137.0236 163.0387 119.0492	Glycitin
18	33.20	$C_{21}H_{22}O_{10}$	434.1213	433.11402	-3	8.6	152.0099 167.0330 108.0207 123.0443	Naringenin 7-O-glucoside
19	34.34	$C_{28}H_{34}O_{15}$	610.18977	609.18249	-0.5	7.7	281.0633 137.0267 579.1946 495.1208	Hesperidin
20	35.37	$C_{22}H_{24}O_8$	416.14712	415.13984	-1.5	7.7	145.0294 117.0338	1-Acetoxy-pinoresinol
21	36.30	$C_8H_8O_5$	184.03717	183.0299	0.4	2.2	124.0141 167.9081 135.9199 92.9978	Methylgallic acid
22	37.24	$C_{17}H_{26}O_4$	294.3910	293.22278	-1.4	7.4	220.1456 177.0906 193.1617	[6]-Gingerol

It was found that a gallotannin derivative, methyl gallate, showed anti-adipogenic activity by inhibiting pancreatic lipase activity (Kwon et al., 2013). The therapeutic potential of gingerol was reported against PL activity in the management of obesity (Saravanan et al., 2014). It has to be noted that all of these phenolic compounds have been identified and characteriaed in the ethyl acetate fraction, which may account for its inhibitory effect. In addition, the positive correlation value between TPC and inhibition rate further confirmed that higher pancreatic lipase inhibition properties may be contributed by the

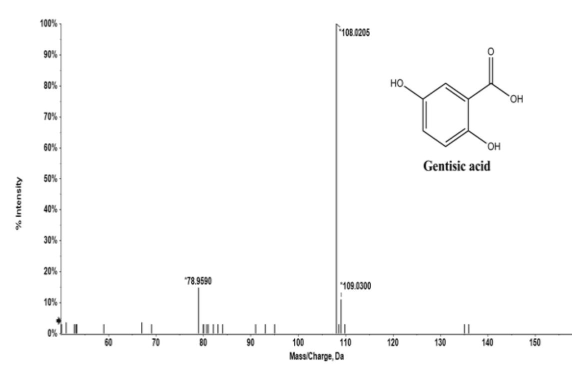
phenolic compounds present in ethyl acetate frcation of *M. charantia*. It can be postulated that the anti-lipase activity of the fraction of *M. charantia* extract seems to be related to the phenolic hydroxyl groups present in their active pharmacophore (Buchholz & Melzig, 2015).



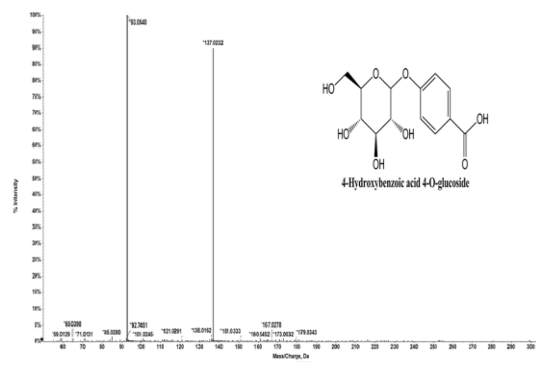
Feruloyl quinic acid



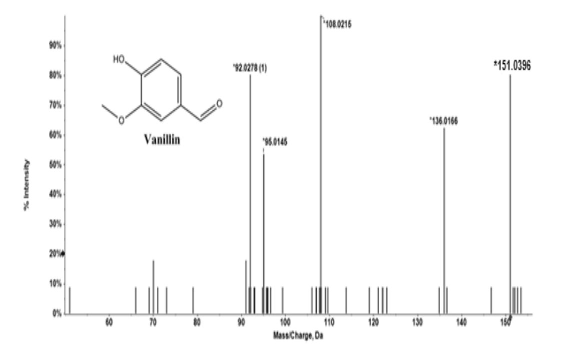
Vanillic acid



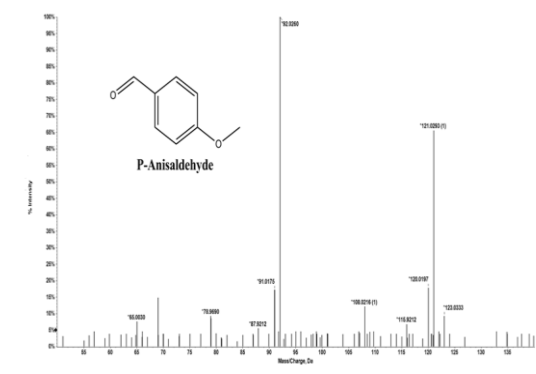
Gentisic acid



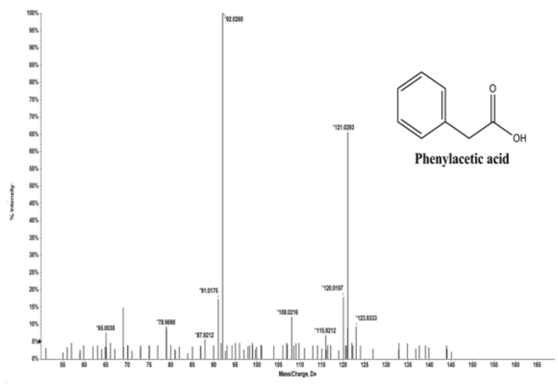
4-Hydroxybenzoic acid 4-O-glucoside



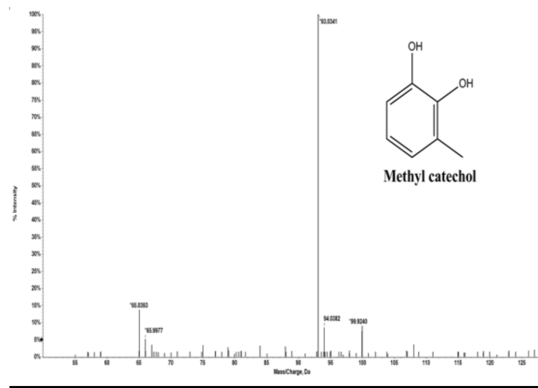
Vanillin



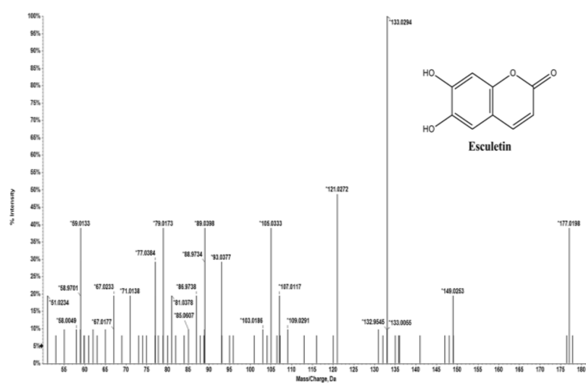
p-Anisaldehyde



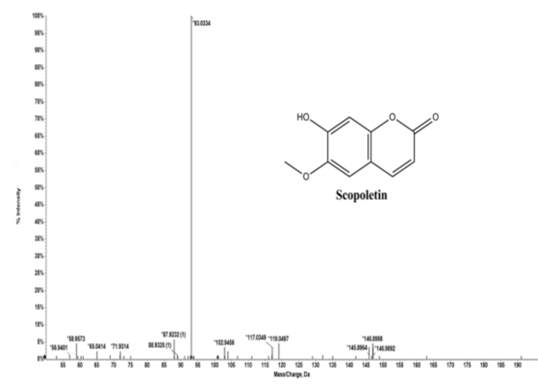
Phenyl acetic acid



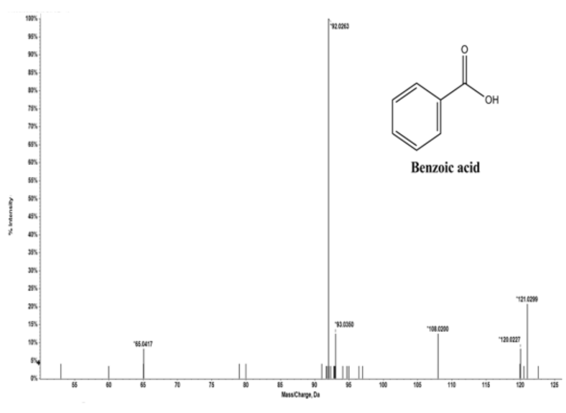
Methyl catechol



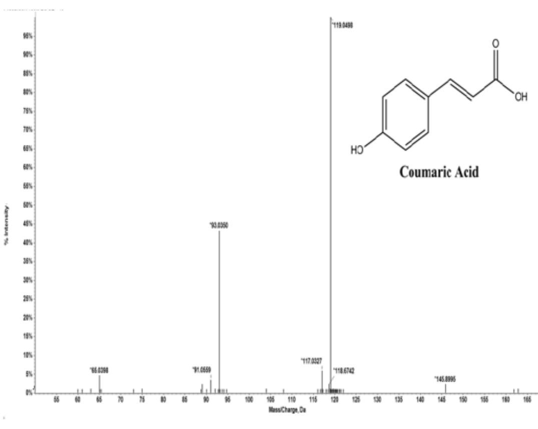
Esculetin



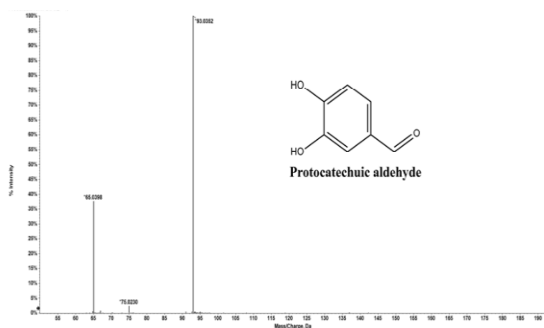
Scopoletin



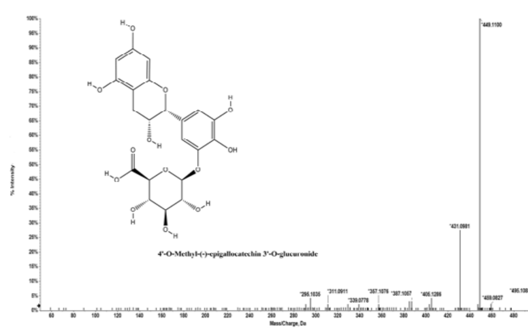
Benzoic acid



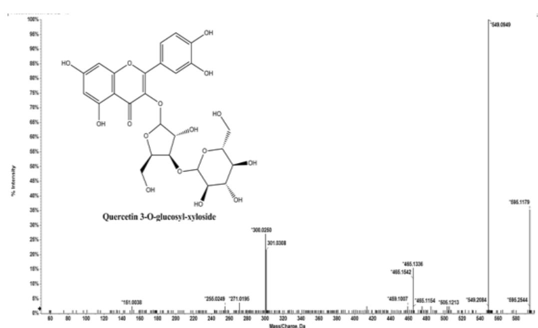
Coumaric Acid



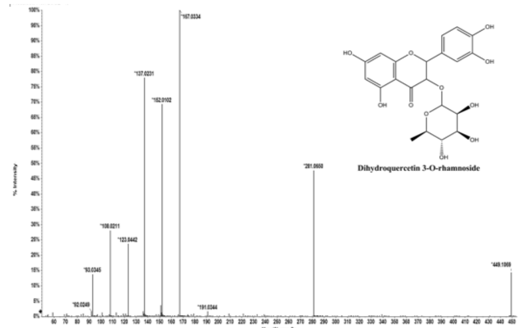
Protocatechuic aldehyde



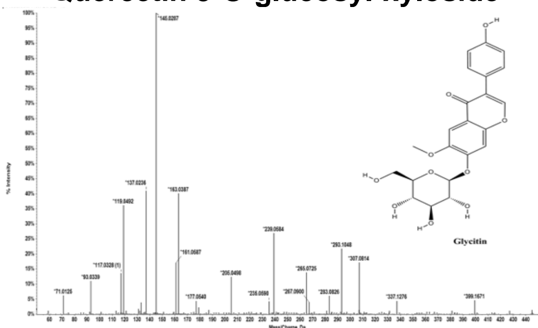
4’-O-Methyl-(-)-epigallocatechin 3’-O-glucuronide



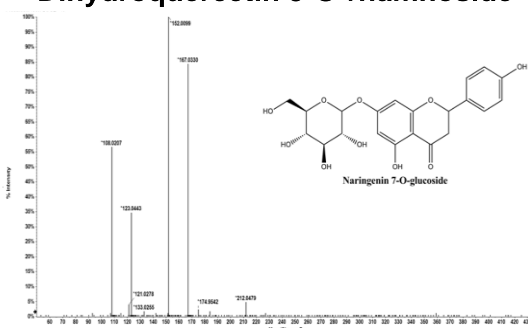
Quercetin 3-O-glucosyl-xyloside



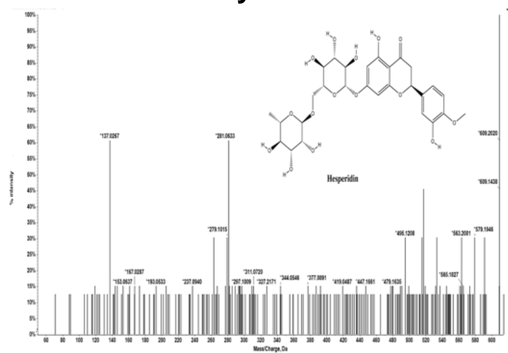
Dihydroquercetin 3-O-rhamnoside



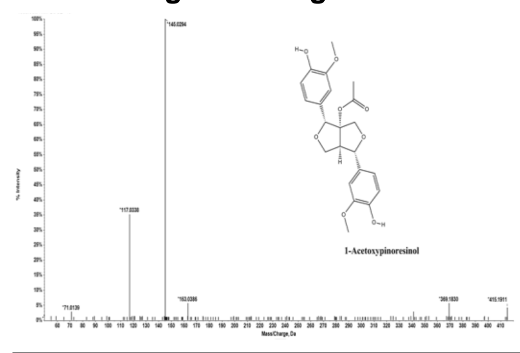
Glycitin



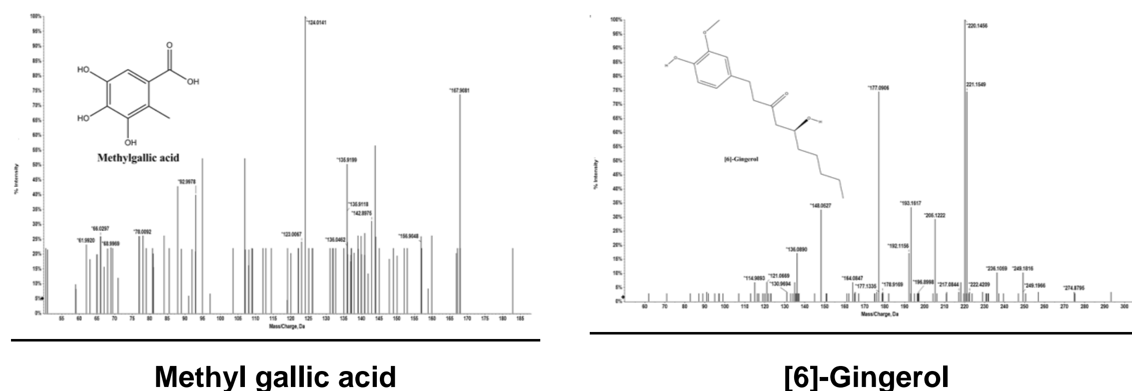
Naringenin 7-O-glucoside



Hesperidin



1-Acetoxypinoresinol



Methyl gallic acid **[6]-Gingerol**

Figure 9.8. LC-MS/MS Spectra of phenolic compounds of *M. charantia* ethyl acetate fraction

9.5. LC-QTOF-MS based metabolite profiling of *Coccinia grandis* fruit against α-glucosidase

The ethyl acetate fraction of *C. grandis* exhibited highest inhibition capacility with compared to others (Described in chapter 8). The chemical profiling of the bioactive fraction was done by using high-resolution liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–QTOF–MS).

9.5.1. LC-QTOF-MS methodology

UPLC-QTOF-MS analysis was carried out in Agilent 1290 Infinity LC System Coupled to Agilent 6545 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) with Agilent Jet Stream Thermal Gradient Technology to identify the major constituents present in ethyl acetate frcation of *C. grandis*. The chromatographic method was optimized based on the mobile phase composition, gradient, flow rate, and injection volume. The optimization was performed by changing those parameters in according to get better resolution, analysis time, and peak shape. The mobile phase consisted of 10 mM ammonium acetate in water (A) and acetonitrile 10 % B; flow 0.5 ml/min. The eluate was monitored at 210 nm and 254 nm. The sample was prepared by dissolving in acetonitrile and filtered through 0.45 μm filter before injection. The phenolic compounds were identified by their typical UV absorptions at 254, 280 and 320 nm, followed by their retention times and MS fragmentation pattern. Considering the MS conditions, negative ion mode was used to obtain better tandem mass spectra and high-resolution mass spectra. The operating parameters were optimized as follows: drying gas (N_2) flow, 8 l/mL; drying gas

temperature, 250ºC. Other parameters were set as nebulizer gas, 35 psig; capillary voltage, 3500 V; skimmer voltage, 60 V; nozzle voltage 1500 V. Mass spectra were recorded across the m/z range of 50–1500 with accurate mass measurement. The fragmentor voltage was set 150 V and sheath gas temperature was at 350°C, at a flow of 11 l/ml. The instrument was set to perform on TOF mode with an acquisition rate of 1 spectra/sec. The mass range of both scan types was 50–1000 m/z. The mass axis of the TOF was calibrated before analysis by infusing a sample that contains known masses into the source and measuring the actual flight times for ions of the known masses. The data acquisition on the LC-Q-TOF was performed using Agilent MassHunter Acquisition B.06.01 software (Agilent Technologies, Santa Clara, CA, USA). The data was deconvoluted into individual chemical peaks with Agilent MassHunter Qualitative Analysis B.07.00 (MassHunterQual, Agilent Technologies, Santa Clara, CA, USA), using Molecular Feature Extractor (MFE). A customized Personal Compound Database and Library (PCDL) was prepared from phenol database (Phenol-Explorer 3.0) (http://www.phenol-explorer.eu). This customized PCDL comprising of phenolic compounds was uploaded into MassHunterQual software's MFE algorithm for targeted data mining.

9.5.2. Results and discussion

LC-QTOF-MS analysis was carried out to identify the major constituents present in ethyl acetate frcation of *C. grandis* fruits. The fingerprinting of the active fraction was performed based on their respective retention times, experimental and calculated *m/z*, molecular formula, error (in ppm), and score value (on a scale of 0 to 100 of the measured mass (*m/z*) with respect to their theoretical formula. Table 9.6 summarizes 35 compounds identified in ethyl acetate fraction of *C. grandis*. The Total ion chromatogram has been represented in Figure 9.9. The analysis was carried out in the mass error window of 5 ppm for confirming their elemental composition. The Metlin database was used to identify the major phytoconstituents present in the bioactive fraction. The result of the study revealed the presence of hydroxybenzoic acids, hydroxycinnamic acid, flavonol, isoflavonoid, flavanone, hydroxycoumarin as major compounds. Interestingly, some of them are reported to have α-glucosidase inhibitory activity for example, some phenolic acids and their derivatives *viz.* caffeic acid, sinapic acid, ferulic acid, p-coumaroylquinic acid, caffeoylquinic acid, and feruloylquinic acid have been found to

possess significant α-glucosidase inhibitory activities (Chen et al., 2014; Tan et al., 2017). Likewise, some glyocosides and flavonoids (Isorhamnetin, isorhamnetin 3-glucoiside,esculetin, scopoletin) were reported to have significant *in-vitro* α-glucosidase inhibitory activity (Islam et al., 2013). Furthermore, luteoside (Luteolin 7-O-glucoside) and isorhoifolin were found effective in improving sucrose tolerance by inhibiting α-glucosidase (Veerapur et al., 2017).

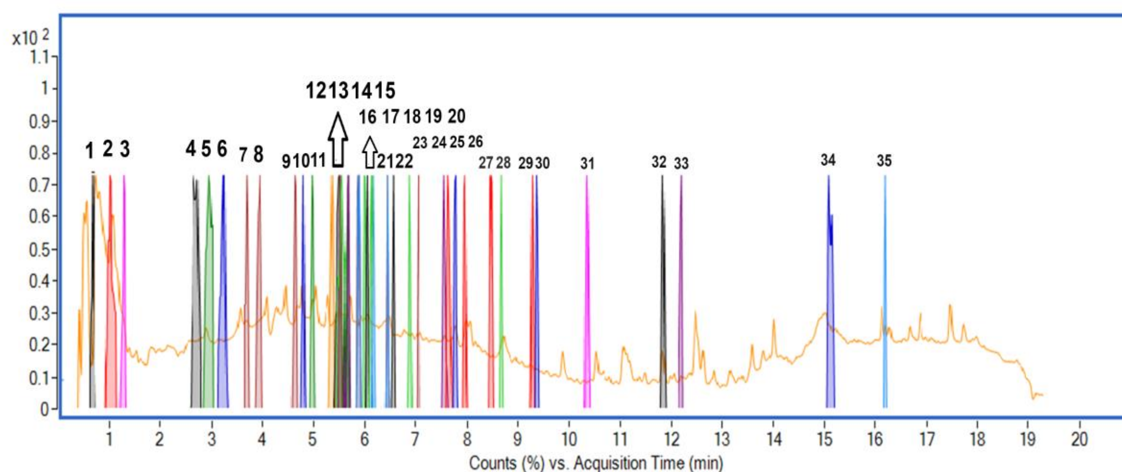


Figure 9.9. Total ion chromatogram of ethyl acetate fraction of *C. grandis*

Also, caffeic acid-3-O-glucoside and luteolin showed stronger α-glucosidase inhibition in comparison to acarbose (Ha et al., 2012). Moreover, some synergistic inhibition of α-glucosidase has been found between quercetin-3-rutinoside and acarbose in reduction of postprandial hyperglycemia (Adisakwattana et al., 2011). Enzyme kinetics study of quercetin 3-O-acetyl-rhamnoside and kaempferol 3-O-rhamnoside against α-glucosidase revealed the inhibition mechanism as mixed in nature whereas carnosic acid offers non-competitive type of inhibition (Chen et al., 2017; Sheng et al., 2018). Some anthocyanins, *viz.* cyanidin-3-galactoside, cyanidin-3-arabinoside and cyanidin-3-xyloside are also effective inhibitors of the α-glucosidase enzyme (Du et al., 2018). Formononetin (isoflavone), a compound isolated from the dried stem and bark of *Sophora flavescens* showed moderate α-glucosidase inhibitory effects (Yin et al., 2014). All of these reports led us to conclude that these phenolic compounds present in ethyl acetate fraction of *C. grandis* are presumably responsible for α-glucosidase inhibition as well as their antioxidant capacity. The results were further supported by the higher

correlation value between the total phenol content and the observed half maximal inhibitory concentration (IC_{50}) of ethyl acetate fraction of *C. grandis* fruit, both in terms of α-glucosidase inhibitory and antioxidant activity.

Table 9.6. Compounds identified in ethyl acetate fraction of *C. grandis*

S. No	Reten-tion time	m/z	Molecular formulae	Mass (Theo.)	Error	Scor e	Proposed compounds
1.	0.544	210.053	$C_{10}H_{10}O_5$	210.053	0.96	86.21	5 Hydroxyferulic acid
2.	0.89	149.0839	$C_9H_{11}N_O$	149.0839	-0.94	99.16	Cathinone
3.	1.142	743.2034	$C_{32}H_{39}O_{20}$	743.2034	-0.04	78.14	Cyanidin 3-O-sambubioside 5-O-glucoside
4.	2.58	180.0784	$C_{10}H_{12}O_3$	180.0784	-1.2	80.88	Coniferyl alcohol
5.	2.843	326.1003	$C_{15}H_{18}O_8$	326.1003	0.28	98.71	p-Coumaroyl glucose
6.	3.118	178.0265	$C_9H_6O_4$	178.0265	-0.66	87.16	Esculetin
7.	3.601	356.1108	$C_{16}H_{20}O_9$	356.1108	0.06	99.49	Feruloyl glucose
8.	3.832	192.0422	$C_{10}H_8O_4$	192.0422	-0.38	86.8	Scopoletin
9.	4.533	342.1317	$C_{16}H_{22}O_8$	342.1317	0.6	91.31	Coniferin
10.	4.696	224.0686	$C_{11}H_{12}O_5$	224.0686	0.72	80.61	Sinapic acid
11.	4.879	368.1102	C17H20O9	368.1102	-1.46	99.38	4-Feruloylquinic acid
12.	5.277	610.1526	$C_{27}H_{30}O_{16}$	610.1526	-1.26	98.79	Quercetin 3-O-rutinoside
13.	5.42	348.1111	$C_{20}H_{16}N_2O_4$	348.1111	0.35	75.62	Camptothecin
14.	5.47	288.1	$C_{16}H_{16}O_5$	288.1	0.66	82.55	7 2 -Dihydroxy-4 -methoxy-isoflavanol
15.	5.772	180.0422	$C_9H_8O_4$	180.0422	-0.25	87.34	Caffeic acid
16.	5.898	624.1691	$C_{28}H_{32}O_{16}$	624.1691	0.15	92.25	Isorhamnetin 3-O-glucoside 7-O-rhamnoside
17.	5.957	448.0989	$C_{21}H_{20}O_{11}$	448.0989	-3.61	99.41	Luteoside/Luteolin 7-O-glucoside
18.	6.079	478.111	$C_{22}H_{22}O_{12}$	478.111	-0.19	98.33	Isorhamnetin 3-O-galactoside
19.	6.364	578.164	$C_{27}H_{30}O_{14}$	578.164	0.8	78.51	Isorhoifolin
20.	6.49	300.0637	$C_{16}H_{12}O_6$	300.0637	0.92	81.46	(-)-Sophorol

21.	6.779	490.1117	$C_{23}H_{22}O_{12}$	490.1117	1.08	89.28	Quercetin 3-O-acetyl-rhamnoside
22.	6.959	419.0985	$C_{20}H_{19}O_{10}$	419.0985	1.72	76.36	Cyanidin 3-O-xyloside
23.	7.493	431.0989	$C_{21}H_{19}O_{10}$	431.0989	2.62	75.4	Kaempferol 3-O-rhamnoside
24.	7.581	286.0842	$C_{16}H_{14}O_5$	286.0842	0.38	98.3	2 7-Dihydroxy-4 -methoxyisoflavanone
25.	7.707	132.0575	C_9H_8O	132.0575	-0.31	97.84	Cinnamaldehyde
26.	7.898	268.0735	$C_{16}H_{12}O_4$	268.0735	-0.29	84.32	Isoformononetin
27.	8.411	287.1157	$C_{16}H_{17}NO_4$	287.1157	-0.25	86.04	(S)-Norlaudanosoline
28.	8.616	388.1524	$C_{21}H_{24}O_7$	388.1524	0.54	83.02	Trachelogenin
29.	9.218	416.1475	$C_{22}H_{24}O_8$	416.1475	0.84	79.59	1-Acetoxypinoresinol
30.	9.303	332.1987	$C_{20}H_{28}O_4$	332.1987	-0.21	81.65	Carnosic acid
31.	10.299	344.1627	$C_{20}H_{24}O_5$	344.1627	0.87	81.1	Rosmadial
32.	11.795	134.0732	$C_9H_{10}O$	134.0732	0.06	87.37	Cinnamyl alcohol
33.	12.159	335.1729	$C_{18}H_{25}NO_5$	335.1729	-1.07	81.03	Senecionine
34.	15.1	556.3032	$C_{32}H_{44}O_8$	556.3032	-0.76	98.9	Cucurbitacin E
35.	16.208	305.1622	$C_{17}H_{23}NO_4$	305.1622	-1.7	97.41	(6S)-Hydroxyhyoscyamine

The methodology of the work involves bioactivity guided fractionation followed by liquid chromatography coupled with high-resolution mass spectrometry for dereplication of major constituents present in *L. siceraria, L. acutangula, M. charantia* and *C. grandis* against some targeted enzyme activity. The results of the study indicated that phenolic compounds of *L. siceraria*, *L. acutangula* showed effective carbonic anhydrase inhibition property. Similarly, the tandem mass chromatography analysis also identified some phenolic and flavonoid compounds in the bioactive fraction of *M. charantia* against pancreatic lipase. The bioactivity guided study was also helped in profiling secondary metabolites present in α-glucosidase inhibitory fractions of *C. grandis*. The high-resolution QTOF-MS coupled with the compound database and Library searching provided a selective and highly sensitive procedure for rapid identification of bioactive constituents in the plant extract and fractions. This technique allows in dereplication in order to explore naturally occurring lead compounds which can be used as functional food components with several therapeutic benefits linked with the enzymes.

9.6. Publications

- UPLC-QTOF-MS analysis of a carbonic anhydrase inhibiting extract and fractions of *Luffa acutangula* (L.) Roxb (ridge gourd). Phytochemical Analysis. 2018; 30(2):148-155.
- Study of pancreatic lipase inhibition kinetics and LC-QTOF-MS based identification of bioactive constituents of *Momordica charantia* fruits, Biomedical Chromatography. 2019: 33(4):e4463.

Chapter 10

10. Summary & Conclusion

10.1. Summary
10.2. Conclusion
10.3. Future perspective
10.4. Publications & Reprints

10.1. Summary

The phytoconstituents present in the medicinal plants comprises of diverse biological activities, which have therapeutic efficacices in the management of metabolic diseases. The work presented in this thesis focuses on standardization, identification of bioactive compounds and elucidate probable enzymatic interaction mechanisms underlying the beneficial effects of some selected plants of cucurbitaceae family. The study was designed to screen four plants of cucurbitaceae family *viz. Lagenaria siceraia*, *Luffa acutangula, Momordica charantia* and *Coccinia grandis* based on their ethnopharmacological relevances found in the literature, against there enzymes *viz.* carbonic anhydrase, pancreatic lipase, α-glucosidase linked with hypertension, edema, obesity, cardiac hypertrophy etc. The results revealed that *L. siceraria* and *L. acutangula* possess significant carbonic anhydrase inhibitory activity whereas *M. charantia* and *C. grandis* showed potential pancreatic lipase and α-glucosidase inhibitory activity respectively. The study also focuses on the evaluation of enzyme kinetics behavior of all the related enzymes. Further, bioactivity guided fractionation coupled with chromatographic evaluation including was carried out for identification of active phytoconstituents present in the extract and fractions of the plants of cucurbitaceae family. The results of the study have been found useful for phytochemical and therapeutic validation of cucurbitaceae family plants against some metabolic disorders linked with the selected enzymes.

In Chapter 1, the detailed study on morphological, phytochemical and pharmacological aspects of plants of Cucurbitaceae family has been discussed extensively. This chapter highlights on complete literature survey on several aspects of cucurbitaceae family based on their phytochemical and pharmacological point of view. The literature review was carried out from several search engines, e.g. PubMed, SciFinder, Scopus, Science Direct and Google Scholar up to 2018. Starting from a different genus and geographical distribution, the morphological properties of the cucurbitaceae plants has been highlighted in this chapter. Concise information on phytochemical and pharmacological aspects of cucurbitaceae plants have been presented in the chapter. Several nutritional and economic importance and their toxicity profile have been reported in brief. Although the cucurbitaceae plants are widely used as a food crop and in culinary purposes in India, the uses of different parts of the plants are also reported in Indian traditional

system of medicine. Research on this context was carried out to validate the uses of the plants in order to explore their quality, safety, and efficacy.

Chapter 2, describes the scope, objective and plan of work in details. A framework of the study has been designed based on plant collection, authentication and extraction, HPLC and HPTLC based standardization, evaluation of the selected enzyme inhibition potential followed by their kinetics behavior as well as identification of bioactive constituents using high-resolution liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) present in the bioactive fraction of the plants

In Chapter 3, the rationale behind the selection of four species of cucurbitaceae family *viz. Lagenaria siceraia, Luffa acutangula, Momordica charantia*, and *Coccinia grandis* was established based on the reported pharmacological activities, and several ethnomedicinal practices in order to validate their quality and efficacy aspects. The morphological, phytochemical and pharmacological aspects of individual plants have been described in this chapter. In addition, the details methodology of plant collection, authentication, drying, extraction and phytochemical screening of the plants have been highlighted. In all cases, a significant amount of yield the aqueous ethanol extract was observed. The phytochemical screening of the plant extracts confirmed the presence of phenolics, flavonoids, glycosides, and tannins etc. in the four species of cucurbitaceae family.

In Chapter 4, the application of RP-HPLC in medicinal plant analysis and the development of a validated RP-HPLC method was described in order to standardize the aqueous extract of the selected plants by using cucurbitacin E as a marker compound.. The RP-HPLC method was developed with a reverse phase C_{18} column, using acetonitrile and water (1% glacial acetic acid) as mobile phase in the ratio of 70:30 v/v. The flow rate and λ_{max} were optimized at 1ml/min and 230 nm respectively. The HPLC method was validated in terms of accuracy, specificity, sensitivity, and repeatability as per ICH guidelines. The results of the study indicated that the calibration curve was found linear in the concentration range of 1-100 µg/ml. The % RSD of precision and recovery was found to be < 2%, which confirms high repeatability of the method. The limits of detection (LOD) and limit of quantification (LOQ) were estimated to be 3.45 and 8.82 µg/ml respectively, which reflect the high sensitivity of the method. The results

indicated that the content of cucurbitacin E was highest (0.0663 % w/w) in *Cucurbita pepo* whereas *Lagenaria siceraria* contains the lowest (0.0356 % w/w). The study was able to explore the variation of cucurbitacin E content in some selected food plants of the cucurbitaceae family. As the tolerable limit of cucurbitacin should be restricted for human consumption due to their high toxicity, the standardization of these fruits was necessary which may help to prevent the adverse events associated with the consumption of cucurbitaceae food plants. The applicability of the method was applicable in the nutraceutical industry for the effective quality control of cucurbitaceous fruits for safe human consumption.

In Chapter 5, the application of HPTLC in medicinal plant analysis and the development of a validated HPTLC method was reported for quantitive estimation of cucurbitacin E (as marker compound) in the aqueous extract of the selected plants. The densitometric analysis of cucurbitacin E was carried out in the absorbance mode at 254 nm. The R_f value of cucurbitacin E was found at 0.52 with the mobile phase consisted of petroleum ether and ethyl acetate in a ratio of 60:40 (v/v). The limit of quantification (LOQ) and limit of detection (LOD) was found at 10.53 and 3.47 ng per band, respectively, in the linearity range of 200 to 1000 ng per band. The method was validated for linearity, accuracy, precision, detection, and quantification limits, specificity, and robustness as per the ICH guidelines. The content of cucurbitacin E was determined using the calibration curve by plotting the mean peak area (y-axis) against the concentrations (x-axis). The % of cucurbitacin E in aqueous extract of *Lagenaria siceraria, Benincasa hispida, Momordica charantia, Coccinia grandis, Cucurbita pepo, Luffa acutangula* was found in the range of 0.024 to 0.057% w/w. This method may be useful for the development of quality control and marker analysis of the medicinal plants of cucurbitaceae family.

In Chapter 6, the carbonic anhydrase inhibitory activity of the selected cucurbitaceae plants was discussed. The role of carbonic anhydrase inhibition in disease pathophysiology was described in details with a schematic diagram. The results of the study indicated that *L. siceraria* offered highest inhibition capacity (IC_{50} value 356.03 ± 7.59 µg/ml) whereas the IC_{50} values of other extract were found at 431.9±3.28 (*L. acutangula*), 993.2±5.34 (*M. charantia*) and 1150±7.9 (*C. grandis*) respectively. Based on the above results *L. siceraria* and *L. acutangula* were furthered considered for dose response and enzyme kinetics study.

The carbonic anhydrase inhibition study of aqueous ethanol extract and fractions of *L. acutangula* were assessed along with their relationship with phenolic and flavonoid content. The study indicated that the ethyl acetate fraction of the aqueous ethanol extract of *L. acutangula* had the highest carbonic anhydrase inhibition activity. A positive correlation between total phenolics and flavonoids composition in the extract and fractions and their carbonic anhydrase inhibition activity was observed. The enzyme kinetics analysis indicated a mixed mode of inhibition. The correlation of total phenolic content with carbonic anhydrase inhibition suggested further research that might confirm that phenolic compounds of *L. acutangula* offer potential therapeutic benefits against carbonic anhydrase-related disorders.

The exploration of carbonic anhydrase inhibitory potential of the extract and fractions of *L. siceraria* fruits and the inhibitory mechanism of the bioactive constituents present in the most potent carbonic anhydrase-inhibiting fraction was carried out. The extraction and fractionation of the dried fruit of *L. siceraria* were carried out in different solvents based on polarity. The active fraction was purified by flash chromatography and screened for their *in vitro* carbonic anhydrase II (CA II) inhibitory activity. The method was based on the change in absorbance due to the liberation of 4-nitrophenol as the hydrolysis product of 4-nitrophenyl acetate as substrate. The underlying carbonic anhydrase inhibitory mechanism of the phenolic compounds was explained by enzyme kinetics and molecular docking study. The LC-Q-TOF-MS based identification of the most active fraction revealed the presence of phenolic compounds *viz.* ferulic acid (7.67 %), *p*-coumaric acid (8.24 %), and coniferyl alcohol (11.71% w/w) as major constituents. The results of the enzyme inhibition assay revealed that coniferyl alcohol, ferulic acid, and *p*-coumaric acid inhibited CA-II activity (IC_{50} value range of 80-250 μM) in a dose-dependent manner. The kinetics study of enzyme inhibition revealed that p-coumaric acid binds to the enzyme competitively whereas the non-competitive type of inhibition was observed for ferulic acid and coniferyl alcohol. The molecular docking study explored the interaction mechanism of phenolic compounds at the active site of CA II. The present research led us to conclude that, the phenolic compounds from *L. siceraria* serve as major contributors for carbonic anhydrase inhibition, which could play a useful role in the management of edema, hypertension, obesity and related metabolic disorders linked with carbonic anhydrase.

In Chapter 7, the pancreatic lipase inhibitory activity of the selected plants of cucurbitaceae family was performed. The inhibition of this enzyme prevents the absorption of dietary in the intestine, and thus exerts an anti-obesity effect. The *in vitro* pancreatic lipase inhibition assay was performed with the aqueous ethanol extract of *L. siceraria*, *L. acutangula, M. charantia, C. grandis*. The results of the study indicated that *M. charantia* possesses highest inhibitory activity whereas (655.24±21.88 µg/ml) wheres the IC_{50} values of other extract was found at 1183.19±77.04 µg/ml (*L. acutangula),* 256.03±32.81 µg/ml (*M. charantia*) and 893.19± 79.94 µg/ml (*C. grandis*) respectively. No significant difference (P value < 0.05) was observed between *M. charantia* extract and the positive control Orlistat (IC_{50} value 149.09±13.95 µg/ml).

The further investigation of pancreatic lipase (PL) inhibition and their kinetic behavior was studied with the extract and fractions of *M. charantia* fruits. The PL inhibitory activity was assayed spectrophotometrically by measuring the change of absorbance of the products at 405 nm, using p-nitrophenyl caprylate (pNPC) as a substrate. The total phenol and flavonoid content of the fractions was estimated by the Folin-Ciocalteu method. The results of the study indicated that the ethyl acetate fraction of *M. charantia* fruit offered significant, dose-dependent and mixed type inhibition against PL. A higher positive correlation between phenolic content of ethyl acetate fraction and its PL inhibitory activity was established statistically, which implied that higher inhibition potential was contributed by the phenolic compounds. The LC–QTOF–MS study helped in putative identification of phenolic constituents in the active fraction. This outcome of the study suggested that phenolic compounds of *M. charantia* fraction can serve as functional food components to address obesity-related disorders linked with pancreatic lipase.

In Chapter 8, the screening of α-glucosidase inhibitory activity of the selected plants of cucurbitaceae family was performed. The further study of antioxidant, α-glucosidase inhibitory properties and the inhibition kinetic parameters of different fractions of *C. grandis* was carried out through spectrophotometric assay, with p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate in varying concentration. The *in vitro* antioxidant potential was performed by DPPH (2, 2-diphenyl-1-picrylhydrazyl radicals) assay. The results of the study showed that the difference between the α-glucosidase inhibitory activity of ethyl acetate fraction of *C. grandis* (EFCG) (IC_{50} 2.43±0.27 mg/ml), and

standard inhibitor, acarbose (2.08±0.19 mg/ml) was not statistically significant at a p-value of 0.05. The results of the kinetics study calculated the apparent V_{max} and K_m value as 2.85 mM/min and 4.4 mM respectively. The inhibition constants, K_i and αK_i were estimated from the secondary plot as 2.4 mg/ml and 9.1 mg/ml respectively which confirmed the inhibition mode in a mixed manner. In addition, a significant correlation between antioxidant activity and α-glucosidase inhibitory activity of EFCG was observed. Additionally, Ultra-performance liquid chromatography-quadrupole/time-of-flight mass spectrometry (UPLC-QTOF-MS) analysis was carried out for metabolite profiling of bioactive fraction of *C. grandis* in the subsequent chapter.

In Chapter 9, the LC–QTOF-MS-guided dereplication methodology was employed for rapid identification of the bioactive constituents with major therapeutic activity linked with enzyme inhibition. The higher mass accuracy of LC-QTOF-MS based method was found very helpful to identify the bioactive constituents in order to explore the lead molecules with potential therapeutic activity from natural products.

The LC-QTOF-MS analysis of the bioactive subfraction of *L. siceraria* revealed the presence of six major polyphenolic compounds namely p-coumaric acid, ferulic acid (phenolic acids), coniferyl alcohol (monolignol), hesperidin and apigenin-7-glucoside (flavonoid glycosides). The compounds were identified with a retention time (RT) of 12.15, 15.14, 14.8, 8.63 and 10.86 min respectively. The *m/z* values were found at 163.0408, 193.0512, 179.0721, 609.1835 and 431.0979 respectively. The presence of these compounds was confirmed by their respective retention times, experimental and calculated m/z, molecular formula, error (in ppm), sigma value (an exact numerical comparison between the theoretical and measured isotope patterns), MS/MS fragmentation pattern. The HPLC-DAD analysis quantified CFA, 4-CA, and FA as 11.71, 8.24, and 7.67 % (w/w) respectively.

The LC-QTOF-MS based study of carbonic anhydrase inhibiting extract and fractions of *L. acutangula* fruit revealed in tentative identifications of various phenolic acids (ellagic acid, chlorogenic acid, gallic acid, hydroxycaffeic acid, gentisic acid, dihydroferulic acid, caffeic acid, coumaric acid etc.), flavonones (sakuranetin, gardenin B, chrysin, scutellarein, geraldone etc.), hydroxycoumarins (scopoletin, mellein etc.), guaiacol (methoxyphenol) in ethyl acetate fraction of aqueous ethanol extract of *L. acutangula* fruits. The high-resolution Q-TOF mass spectrometry coupled with custom Personal

Compound Database and Library searching was found as a highly sensitive procedure for rapid identification of phenolic compounds in ethyl acetate fraction.

The active phytoconstituents in *M. charantia* fruits against pancreatic lipase was putatively identified by LC–QTOF–MS analysis which identified phenolic compounds such as feruloyl quinic acid (RT 3.53 min), vanillic acid (RT 4.29 min), gentisic acid (RT 5.96 min), esculetin (RT 20.68 min), scopoletin (RT 21.17 min), coumaric acid (RT 24.30 min), epigallocatechin glucuronide (RT 28.01 min), glycitin (RT 30.69 min), naringenin-7-O-glucoside (RT 33.20 min), hersperidin (RT 34.34 min), 1-acetoxypinoresinol (liganan derivative) (RT 35.37 min) and methylgallic acid (RT 36.30 min). It has to be noted that, owing to its faster acquisition rate and accurate mass acquisition capacity, the high-resolution mass spectrometric analysis offered a reliable technique for putative identification of the polyphenolic compounds in *M. charantia* fraction.

The alpha-glucosidase inhibitory constituents of *C. grandis* fruits was also identified in LC-QTOF-MS platform based on respective retention times, measurement of accurate molecular mass and isotope peak pattern. Total 35 compounds were identified with their respective retention time, molecular formulae, error (in ppm) and score value (on a scale of 0 to 100) of the measured mass (*m/z*) with respect to their theoretical formula. The analysis was carried out in the mass error window of 5 ppm for confirming their elemental composition. The results revealed the presence of hydroxybenzoic acids, hydroxycinnamic acid, flavonol, isoflavonoid, flavanone, hydroxycoumarin, alkaloids, triterpenoids etc. as major compounds.

10.2. Conclusion

The development of functional foods generates newer insights into the discovery of molecular targets in the management of some chronic, lifestyle-associated disorders. It has been observed that a large number of bioactive metabolites present in the food plants can interact with some metabolizing enzymes in our body. The combination of diet and medicine also found a suitable treatment approach in hypertension, diabetes, obesity. The popularity of the functional food ingredients is increasing day by day which in term modulate the well-being of individuals either therapeutically or prophylactically. There are certain fruits and vegetables consists of potential phytochemicals having medicinal uses required for good health of humans. The Cucurbitaceae family introduces

a large number of plants which can be consumed as food as well as medicine. The reports of ethnopharmacological importance of several cucurbitaceae plants necessitate the validation of their traditional claim based on quality, safety, and efficacy.

In relation to the ethnomedicinal uses, the four plants of cucurbitaceae family were selected in order to perform standardization and therapeutic evaluation based on their enzyme inhibiting potential. The bioactive phytoconstituents and their inhibitory mechanisms were also studied in details. The standardization of the plants of cucurbitaceae family helps in the content estimation of cucurbitacin E through RP-HPLC method. The validated method can be beneficial for the nutraceutical industry in establishing effective quality control of these fruits. The results of the enzyme inhibition study indicated that the phenolic compounds of *Lagenaria siceraria* and *Luffa acutangula* serve as major contributors for carbonic anhydrase inhibition, which could play a useful role in the management of edema, hypertension, obesity and related metabolic disorders. In addition, the therapeutic potential *M. charantia* fruits against pancreatic lipase activity were reported to address obesity-related disorders associated with lipid digestion at large. Also, the therapeutic validation of *C. grandis* fruit in α-glucosidase inhibition was explored which can serve as functional food components be addressed postprandial hyperglycemia and related disorders.

10.3. Future perspective

Thus the present work addresses several quality, safety and efficacy related aspects of four plants of cucurbitaceae family widely used in diet and the ethnomedicinal system. The bioactivity guided separation, purification and dereplication strategy may be helpful in profiling the enriched fractions in order to identify their potential phytoconstituents, prior to isolation of any new compounds, if any. The application of metabolomics study will be useful in the characterization of the functional components in optimizing the therapeutic effect of the food plants of cucurbitaceae family. Also, the study will further insist the researchers to explore the synergism among the several bioactive constituents present in the plants. Thus the recent approaches in natural product-based research will validate the therapeutic claim of several ethnomedicinal uses of Cucurbitaceae plants. In further continuation, the isolation of the bioactive constituents may be beneficial for developing newer phytophramaceuticals. Moreover, in order to validate the therapeutic

efficacy of the plant extracts/isolated compounds in vitro in vivo corelationship may be established by performing animal studies. In the future, this work will help in developing the concept of food as medicine in order to develop “new generation therapeutics” to overcome the emerging threat of lifestyle-related disorder at large.

10.4. Publications & Reprints

1. UPLC-QTOF-MS analysis of a carbonic anhydrase inhibiting extract and fractions of *Luffa acutangula* (L.) Roxb (ridge gourd). Phytochemical Analysis. 2018; 30(2):148-155.
2. Study of pancreatic lipase inhibition kinetics and LC-QTOF-MS based identification of bioactive constituents of *Momordica charantia* fruits, Biomedical Chromatography. 2019, 33(4), e4463.
3. Determination of cucurbitacin E in some selected herbs of Ayurvedic importance through RP-HPLC. Journal of Ayurveda and Integrative Medicine (Elsevier Science), 2019, doi.org/10.1016/j.jaim.2019.01.002

Chapter 11

11. References

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RESEARCH ARTICLE

WILEY Phytochemical Analysis

UPLC-QTOF-MS analysis of a carbonic anhydrase-inhibiting extract and fractions of *Luffa acutangula* (L.) Roxb (ridge gourd)

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Abstract

Introduction: *Luffa acutangula* (L.) Roxb, commonly known as ridge gourd (cucurbitaceae), is a common vegetable cultivated in India. It is also a well-used medicinal plant in Indian traditional medicine.

Objectives: To analyse the phenolics content of the most potent carbonic anhydrase-inhibiting fraction from an extract of *L. acutangula*.

Materials and Methods: An aqueous ethanol extract of dried fruits of *L. acutangula* was successively fractionated into petroleum ether, dichloromethane and ethyl acetate. The extract and subsequent fractions were assessed for carbonic anhydrase-inhibitory activity and the enzyme inhibition kinetics were determined for the most active fraction. Total phenolic and flavonoid content of the extract and subsequent fractions were determined spectrophotometrically. Ultra-performance liquid chromatography-quadrupole/time-of-flight mass spectrometry (UPLC-QTOF-MS) analysis was used to tentatively identify the major phenolics in the most active fraction.

Results: The concentration of total phenolics and total flavonoids in the extract and each fraction thereof correlated with the level of carbonic anhydrase inhibition activity. The ethyl acetate fraction of the aqueous ethanol extract of *L. acutangula* had the highest carbonic anhydrase inhibition activity. The enzyme kinetics analysis indicated a mixed mode of inhibition. UPLC-QTOF-MS analysis of the ethyl acetate fraction indicated a number of phenolic acids, hydroxycoumarins, flavones, flavanones, and flavonoids.

Conclusion: The correlation of total phenolic content with carbonic anhydrase inhibition suggested further research that might confirm that phenolic compounds of *L. acutangula* offer potential therapeutic benefits against carbonic anhydrase-related disorders.

KEYWORDS

carbonic anhydrase, correlation analysis, *Luffa acutangula*, total phenol and flavonoid, UPLC-QTOF-MS

1 | INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc-containing metalloenzymes found in higher vertebrates including humans. They regulate several physiological and pathological processes, including transportation of CO_2 and bicarbonate ions between metabolising tissues and lungs, thus maintaining the pH of blood and homeostasis.[1,2] They also play a significant role in bone resorption, electrolyte

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secretion in various tissues and organs and some other biosynthetic reactions, namely gluconeogenesis, lipogenesis, and ureagenesis.[3,4] The inhibition of α-carbonic anhydrase (α-CA) isozymes may account for several significant physiological benefits against obesity,[5] hypertension,[6] cardiac hypertrophy,[7] oedema, glaucoma, epilepsy and osteoporosis.[8] In spite of sulfonamides like acetazolamide, dorzolamide, brinzolamide being the most widely used classical CA inhibitors (mainly CA II-mediated), they are associated with several adverse reactions. Hence, there is a need to develop potent and safe CA II inhibitors from natural resources in the management of CA mediated disorders. In this context, several phenolic compounds derived from medicinal plants have been reported to possess potential CA II inhibitory activity to date.[9]

Luffa acutangula (L.) Roxb (ridge gourd), belonging to the Cucurbitaceae family, is a widely found vegetative climber throughout south-eastern Asia. The fruits of this plant are also well known for their high nutritional and medicinal value.[10] In Ayurveda, the fruits of *L. acutangula* are utilised in the treatment of oedema due to its diuretic properties.[11,12] The plant also possesses hepatoprotective,[13] anti-diabetic, α-glucosidase inhibitory activity.[14] The plant contains a significant amount of polyphenols (mostly phenolic acids, namely gallic acid, *p*-coumaric acid, ferulic acid, protocatechuic acid), and its glycosides, flavonoids (catechin, quercetin, anthocyanins) especially in its fruits.[15] Various micronutrients, essential amino acids, triterpene saponins (acutosides), cucurbitacins and luffangulin have also been reported in the plant.[11,16]

The present study evaluated the extracts and partitioned fractions of *L. acutangula* for CA inhibition and total phenolic and flavonoid content; explored the mechanism of inhibition based on the reaction kinetics; tentatively identified phenolics in the most active fraction using ultra-performance liquid chromatography-quadrupole/time-of-flight mass spectrometry (UPLC-QTOF-MS).

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

CA II from bovine erythrocytes (bCA II) (lyophilised powder, 3848 Wilbur-Anderson (W-A) unit units/mg protein) (EC-232-576-6) and 4-nitrophenyl acetate (4-NPA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetazolamide IP (Batch No. AZM-V-P/131107) was procured as a gift sample (Mangalam Drugs and Organics Ltd, Mumbai, India). Tris buffer GR, sulphuric acid (purity 98%), acetone (HPLC grade), dimethyl sulfoxide, petroleum ether, dichloromethane, ethyl acetate, ethanol (synthesis grade) were bought from Merck (Mumbai, India). Acetonitrile, anhydrous acetonitrile (99.8%) and trifluoroacetic acid (HPLC grade ≥ 99.0%) were obtained from Sigma-Aldrich. All aqueous solutions were prepared using purified water (resistivity of 18.2 MΩ cm at 25°C) from a Mili-Q filtration system.

2.2 | Extraction and fractionation

Fresh fruits of *L. acutangula* were collected from the local market of Jadavpur, Kolkata, India in December 2014. The plant sample was authenticated by Dr S. Rajan, Field Botanist, Ministry of AYUSH, Government of India and the herbarium specimen was deposited at the School of Natural Product Studies (SNPS), Jadavpur University, Kolkata, India for future reference (Voucher specimen number SNPS-JU/2015/1096). The fruits were air-dried under shade at ambient temperature and pulverised by using a mechanical grinder to make a coarse powder. The powdered sample (1 kg) was steeped in 1.5 L aqueous ethanol (80% *v*/*v*) for three days at room temperature. The process was repeated three times. The aqueous ethanol extract was filtered and evaporated under vacuum using a rotary evaporator (EyelaN-1200, Tokyo Rikakikai Co. Ltd., Japan) at 45–50°C, to yield the dry extract (16% *w*/*w*). Afterwards, dried extract (120 g) was suspended in water and partitioned successively with petroleum ether, dichloromethane and ethyl acetate (100 mL each). The fractionation was carried out in a repetitive manner followed by vacuum evaporation at 40°C, to afford a yield of 2%, 4% and 8% (*w*/*w*), respectively. The fractions were stored at 4°C for 15 days prior to analysis.

2.3 | *In vitro* CA inhibition assay

Inhibition of CA inhibition assay by the *L. acutangula* aqueous ethanol extract and subsequent partitioned fractions was determined using a previously reported method with some modifications.[17] In brief, test sample (20 μL) was added to bCA II (115 U/mL in Tris-SO_4 buffer, 30 μL) in a 96-well plate. After incubation in BOD incubator at 25°C for 15 min, the substrate (10 mM 4-NPA in dry acetonitrile, 20 μL) was added to each well and further incubated at the same temperature. The test samples were prepared by dissolving aliquots of the dried aqueous ethanol and subsequent partitioned fractions in aqueous dimethyl sulfoxide (2% *v*/*v*) to obtain a concentration of 1 mg/mL. The stock solutions were serially diluted to different concentrations with dimethyl sulfoxide, in order to generate dose–response curve. The absorbance of each well was determined at 400 nm using a UV-visible spectrophotometer (SpectraMax Plus, Molecular Devices LLC, San Jose, CA, USA). The change of absorbance was due to the hydrolytic liberation of *p*-nitrophenol from the substrate, 4-NPA. The assay procedure was carried out in triplicate. Acetazolamide was used as a positive control. The CA inhibitory activity (%) was calculated based on the following equation:

$$\begin{aligned}\text{Enzyme activity} = {} & [\Delta A\ (400\ \text{nm}/\min_{\text{Test}}) - \Delta A\ (400\ \text{nm}/\min_{\text{Blank}})] \\ & [\text{Volume of assay}\ (\mu\text{L})]/ \\ & [\varepsilon_{\text{nitrophenolate}}\ (=18\,400\ \text{L/mol/cm})] \\ & [\text{Volume of enzyme used}\ (\mu\text{L})]\end{aligned}$$

where ε is the extinction coefficient of nitrophenolate.The half-maximal inhibitory concentration (IC_{50}) values of inhibitors were determined by plotting the percentage of relative enzyme activity (activity without inhibitor) against the inhibitor concentration. The enzymatic activity assay without inhibitor was defined as 100%.

2.4 | Estimation of total phenolic and flavonoid content

Total phenolic and flavonoid content of the aqueous ethanol extract of *L. acutangula* fruits and the subsequent three partitioned fractions was determined using a previously described method with some

modification.[18] The sample was prepared by weighing approximately 1 mg of each fraction, dissolved in 1 mL of methanol followed by filtration with 0.45 μm syringe filter to get a concentration of 1 mg/mL. The reaction mixture was prepared by mixing the sample (18 μL), 10% Folin–Ciocalteu's reagent (90 μL) and 7.5% sodium bicarbonate ($NaHCO_3$) (90 μL). The samples were thereafter incubated in a BOD incubator at 45°C for 45 min. Gallic acid was used to generate a five-point calibration curve at the concentration range of 5 to 30 μg/mL. The absorbance was measured at 725 nm using a spectrophotometer (SpectraMax Plus, Molecular Devices LLC). The total phenol content was calculated as milligrams of gallic acid equivalent (GAE) per gram of sample (mg GAE/g).

The total flavonoids content was also determined by spectrophotometric assay.[18] Quercetin was used as standard. The five-point calibration curve of quercetin was prepared with different concentration ranges (10–75 μg/mL) of methanol. Initially, 3% sodium nitrite ($NaNO_2$) (10 μL), 1 M sodium hydroxide (NaOH) (50 μL) and 15% aluminium chloride ($AlCl_3$)(10 μL) were added in the reaction mixture and total volume of 170 μL was made by adding methanol. The absorbance of the mixture was determined at 415 nm. The content of total flavonoids was calculated as milligrams of quercetin equivalents per gram of sample (mg QE/g). The results were represented as mean ± standard deviation (SD) (n = 3). In addition, to understand the interrelationship between CA inhibitory activity (IC_{50} value) and the total phenol and flavonoid content of *L. acutangula* aqueous ethanol extract and subsequent fractions, Pearson's correlation coefficient was calculated.

2.5 | CA inhibition kinetics study

To explore the inhibitory mechanism (reversible or irreversible) of ethyl acetate fraction, plots of reaction velocity (ΔOD/min) versus enzyme concentrations [E] at different sample concentrations were constructed.[19] The inhibition kinetics of ethyl acetate fraction was also performed using the *in vitro* CA inhibition assay method with different concentrations of substrate (0.5–4 mM) and the inhibitors (0–450 μg/mL). The amount of reaction product formed was monitored at a 20-s interval for 10 mins. Afterward, the V_{max} and K_{app} were calculated according to the Michaelis–Menten equation and Lineweaver′Burk plot based on the following equation:

For non-competitive, $1/V = K_m/V_{max} \times (1 + [I]/K_i) \times 1/[S] + 1/V_{max} \times (1 + [I]/\alpha K_i)$, where V is the reaction velocity; K_m is the Michaelis constant; V_{max} is the maximum velocity; [I] is the concentration of inhibitor; K_i is the inhibitor constant; [S] is the concentration of substrate; α is the apparent coefficient. The K_i and αK_i values were determined by the secondary plot of the slope (K_m/V_{max}) and Y-intercept ($1/V_{max}$) versus inhibitor concentration [I].

2.6 | UPLC-QTOF-MS conditions

UPLC-QTOF-MS analysis was carried out in Agilent 1290 Infinity LC system coupled to Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (QTOF) with Agilent Jet Stream Thermal Gradient Technology to identify the major constituents present in ethyl acetate fraction.

The chromatographic method was refined based on the mobile phase composition, gradient, flow rate, and injection volume. The separation was achieved on Agilent Zorbax Eclipse Plus C_{18} column (2.1 mm × 150 mm, 1.8 μm) as stationary phase. The mobile phase consisted of 10 mM ammonium acetate in water (A) and acetonitrile (B). The gradient profile was set to 20% B from 0 to 8 min, 35% B at 12 min, 50% B at 15 min, 75% B at 20 min followed by a post time run of 5 min to equilibrate the column. The flow rate of all separations was set at 0.5 mL/min. The sample (1 mg) was prepared by dissolving in methanol (1 mL) and filtered through 0.45 μm filter. The stock solution was diluted to 100 μg/mL before injection. The phenolic compounds were identified based on the high-resolution accurate mass analysis which corresponds to the score value (on a scale of 0 to 100) of the measured mass (m/z) with respect to their theoretical formula. Considering the MS conditions, negative ion mode was used to obtain high-resolution mass spectra. The operating parameters were optimised as follows: drying gas (N_2) flow, 8 L/min; drying gas temperature, 150°C. Other parameters were set as nebuliser gas, 35 psig; capillary voltage, 3500 V; skimmer voltage, 60 V; nozzle voltage 1500 V. Mass spectra were recorded across the range m/z 50–1500 with accurate mass measurement. The fragmentor voltage was set 150 V and sheath gas temperature was at 350°C, at a flow of 11 L/min. The data were acquired using the Extended Dynamic Range mode (2 GHz) and the mass range was set at 50–1000 Da. The data acquisition on the LC-QTOF was performed using Agilent MassHunter Acquisition B.06.01 software (Agilent Technologies, Santa Clara, CA, USA). The data were deconvoluted into individual chemical peaks with Agilent MassHunter Qualitative Analysis B.07.00 (MassHunterQual, Agilent Technologies), using Molecular Feature Extractor (MFE).

2.7 | Library searching

Agilent Personal Compound Database and Library (PCDL) was utilised to create the custom database with the phenolic compounds obtained from Phenol-Explorer database.[20] This customised database was uploaded into MassHunterQual software's algorithm for targeted data mining. The PCDL search process integrates the chromatogram within a specified mass window and generates match score, based on the combination of accurate mass, isotopic abundance and isotopic spacing of the identified compounds.[21]

2.8 | Statistical analysis

The statistical analysis of all the experimental data were expressed as mean value ± SD (n = 3). The significance of the difference between mean (control versus test) was determined by Student's t-test at the P value < 0.05. The Pearson correlation test was employed to analyse the correlation coefficients between the content of polyphenols and bioactivities. The statistical analysis was performed by Graph pad prism 6.0 software.

3 | RESULTS AND DISCUSSION

3.1 | Effect of *L. acutangula* fractions on CA activity

The CA inhibitory activity was represented as IC_{50}. The IC_{50} values of the aqueous ethanol extract and the three subsequent partitioned fractions and acetazolamide (positive control) were estimated to be 286.0 ± 2.41 (ethyl acetate fraction), 468.40 ± 4.32 (aqueous ethanol

extract), 609.60 ± 2.35 (dichloromethane fraction), 760.80 ± 3.85 (petroleum ether fraction) and 203.6 ± 2.08 (acetazolamide) μg/mL. The mean IC_{50} value of ethyl acetate fraction was found to be significantly similar (P-value < 0.05) to the standard inhibitor acetazolamide. The decrease in relative enzyme activity with increasing concentration of ethyl acetate fraction confirmed the dose-dependent inhibition against CA II (Figure 1). The plots of the reaction velocity versus enzyme concentration at different inhibitor concentrations gave a group of straight lines. It was noted that all the straight lines passed through the origin and the slope of the line decreased inversely with the increasing concentration of the inhibitors (Figure 2). This result suggested that ethyl acetate fraction inhibited CA, reversibly.[19]

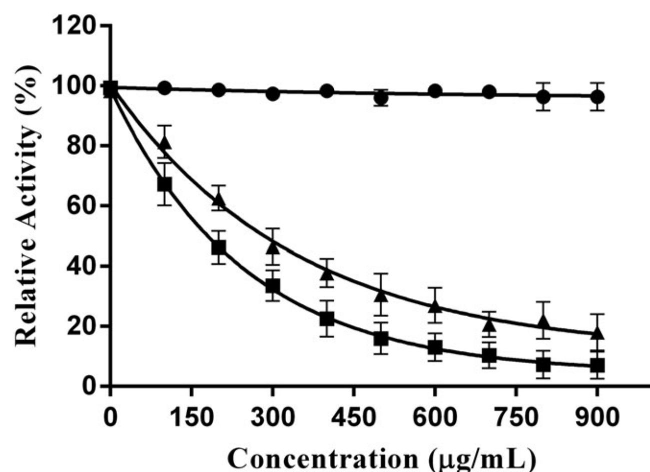

FIGURE 1 Dose dependent inhibition pattern of acetazolamide and ethyl acetate fraction on carbonic anhydrase inhibitory activity. [(●) without inhibitor; (▲) Ethyl acetate fraction; (■) acetazolamide; data are presented as mean ± standard deviation (n = 3)]

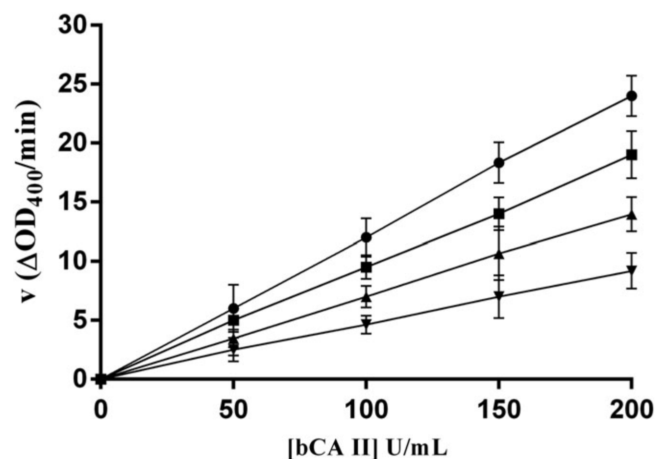

FIGURE 2 Reversible inhibition of ethyl acetate fraction against carbonic anhydrase activity (plots of velocity versus enzyme concentration). [The V value indicates the change in absorbance at 400 nm/min at ethyl acetate fraction concentrations of 0 (■), 250 (▼), 500 (▲) and 750 (●) μg/mL. Data presented as mean ± standard deviation (n = 3)]

3.2 | Kinetics analysis of CA inhibitory activity

The bCA II catalyzed 4-NPA esterase reaction rate was dependent on the concentration of ethyl acetate fraction as the initial reaction velocity decreased with its increasing concentration. Based on the best-fit values of the Michaelis–Menten parameters, it was observed that both the apparent values for V_{max} and the K_m change in presence of inhibitors. The V_{max} and K_{app} values were calculated as 0.70 nM/min and 2.66 mM, respectively (Table 1). In addition, Lineweaver–Burk plot was constructed by plotting 1/V (velocity) versus 1/[S] (substrate) which gave a family of straight lines with different slopes. The secondary re-plot of Y-intercept ($1/V_{max}$) versus [ethyl acetate fraction] gave αK_i value as 2.14 ± 0.22 μg/mL whereas the K_i value was calculated as 182.50 ± 0.32 μg/mL from the replot of the slope (K_m/V_{max}) versus [ethyl acetate fraction] (Figure 3B and 3C). These two different K_i values indicated that the inhibitor can bind to both enzyme (K_i) as well as Enzyme-substrate (ES) complex (αK_i), referring to mixed type of inhibition.[22]

3.3 | Correlation analysis between total phenol and flavonoid content and CA inhibitory activity

The total phenol and flavonoid content were found to be maximum in ethyl acetate fraction at concentration 102.80 ± 3.03 mg GAE/g and 47.80 ± 8.41 mg QE/g of sample, respectively followed by the aqueous ethanol and dichloromethane fractions. The petroleum ether fraction had a very low total phenol and flavonoid content. A statistically significant correlation (r = 0.9581) was obtained between the total phenol content and CA inhibition activity (IC_{50} value) of ethyl acetate fraction. However, no significant correlation was observed between total phenol and flavonoid content and CA inhibitory actions of other fractions, which may be due to the difference in the contents and varieties of phenolic compounds (Table 2). Several phenolic compounds extracted from plants, mushrooms and honey inhibited CA enzyme, which corresponds to their higher phenol and flavonoid content.[23] Some phenolic acids (namely benzoic acid, chlorogenic acid, *m*-hydroxybenzoic acid, *o*-coumaric acid, etc.) and flavonoids (namely genistein) have also been reported to be active against CA.[24] This study explored the abundant presence of phenolics as well as flavonoids in ethyl acetate fraction of *L. acutangula* fruit extract which may be effective to inhibit CA.

TABLE 1 Kinetics parameters of carbonic anhydrase inhibition of ethyl acetate fraction

Kinetics parameters	Value Mean ± SD (n = 3)
V_{max}[a]	0.70 nM/min
K_{app}[b]	2.66 mM
K_i[c]	182.50 ± 0.32 μg/mL
αK_i[d]	2.14 ± 0.22 μg/mL
IC_{50}	286.0 ± 2.40 μg/mL
Type of inhibition	Mixed

[a]maximum reaction velocity.

[b]apparent Michaelis–Menten constant.

[c]inhibition constant.

[d]inhibition constant.

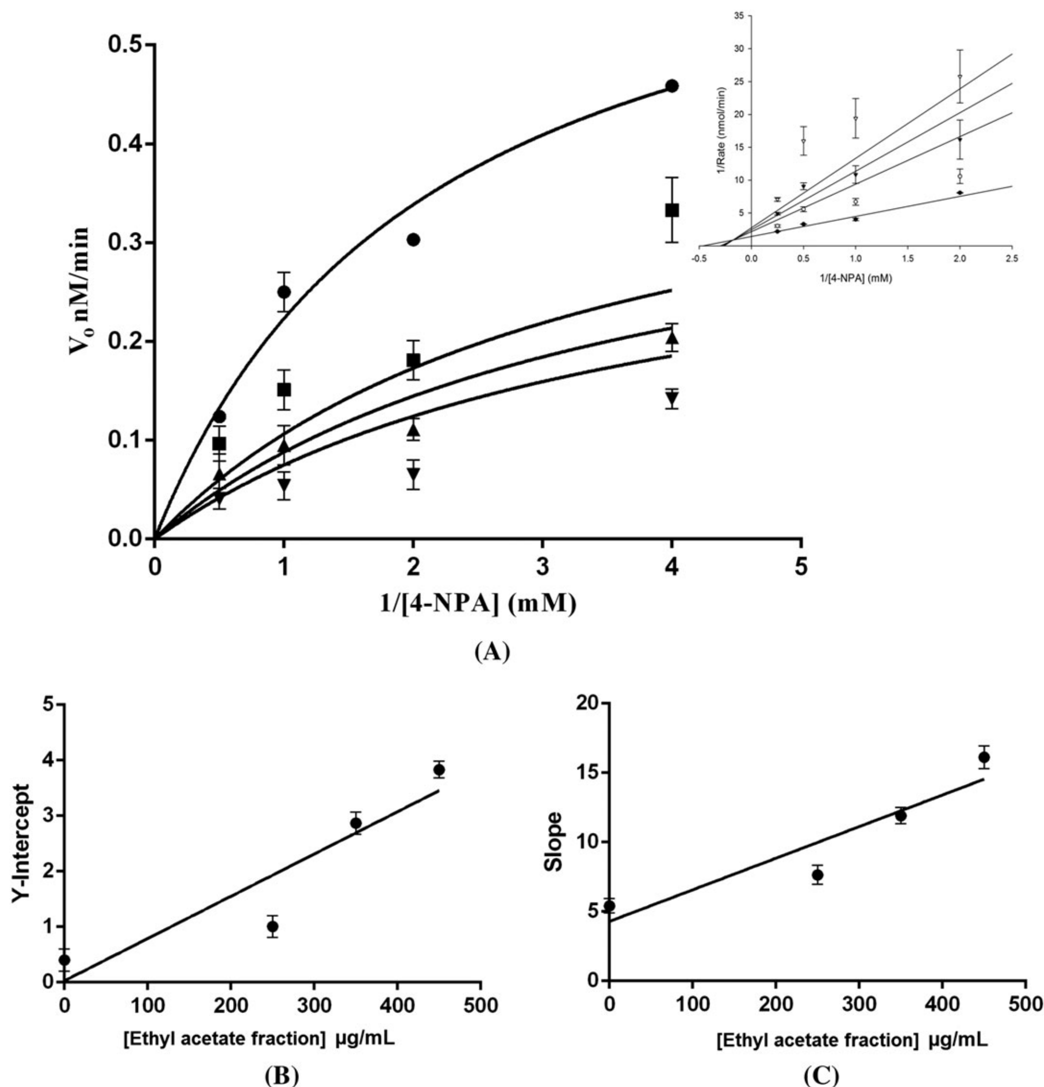

FIGURE 3 (A) Kinetics study of ethyl acetate fraction against carbonic anhydrase activity. Inset: Lineweaver–Burk plot of the Michaelis–Menten data, represent mixed inhibition behaviour of ethyl acetate fraction on bCA II. [The ethyl acetate fraction concentrations are (●) 0, (■), 250, (▲) 350 and (▼) 450 µg/mL. Data presented as mean ± standard deviation (SD) (n = 3). The data were fit to the Michaelis–Menten equation using non-linear regression analysis]. (B) Plot of Y-intercept ($1/V_{max}$) versus concentration of ethyl acetate fraction. (C) Plot of slope (K_m/V_{max}) versus concentration of ethyl acetate fraction. Secondary replots were constructed from the data, collected from Lineweaver–Burk plot. (Concentrations of ethyl acetate fraction were 0, 250, 350, 450 µg/mL)

TABLE 2 Correlations analysis between the total phenol and flavonoid content of *Luffa acutangula* fractions and their carbonic anhydrase inhibitory activity (IC_{50} value)

Correlations	Pearson correlation coefficient *(r value)*
Total phenol content and ethyl acetate fraction IC_{50}	0.9581**
Total flavonoid content and ethyl acetate fraction IC_{50}	0.5560^{ns}
Total phenol content and aqueous ethanol fraction IC_{50}	0.2393^{ns}
Total flavonoid content and aqueous ethanol fraction IC_{50}	0.5477^{ns}
Total phenol content and dichloromethane fraction IC_{50}	0.1017^{ns}
Total flavonoid content and dichloromethane fraction IC_{50}	0.6743^{ns}
Total phenol content and petroleum ether fraction IC_{50}	0.0296^{ns}
Total flavonoid content and petroleum ether fraction IC_{50}	0.0515^{ns}

Correlation is significant at: ns, not significant (two-tailed); **P-Value < 0.01.

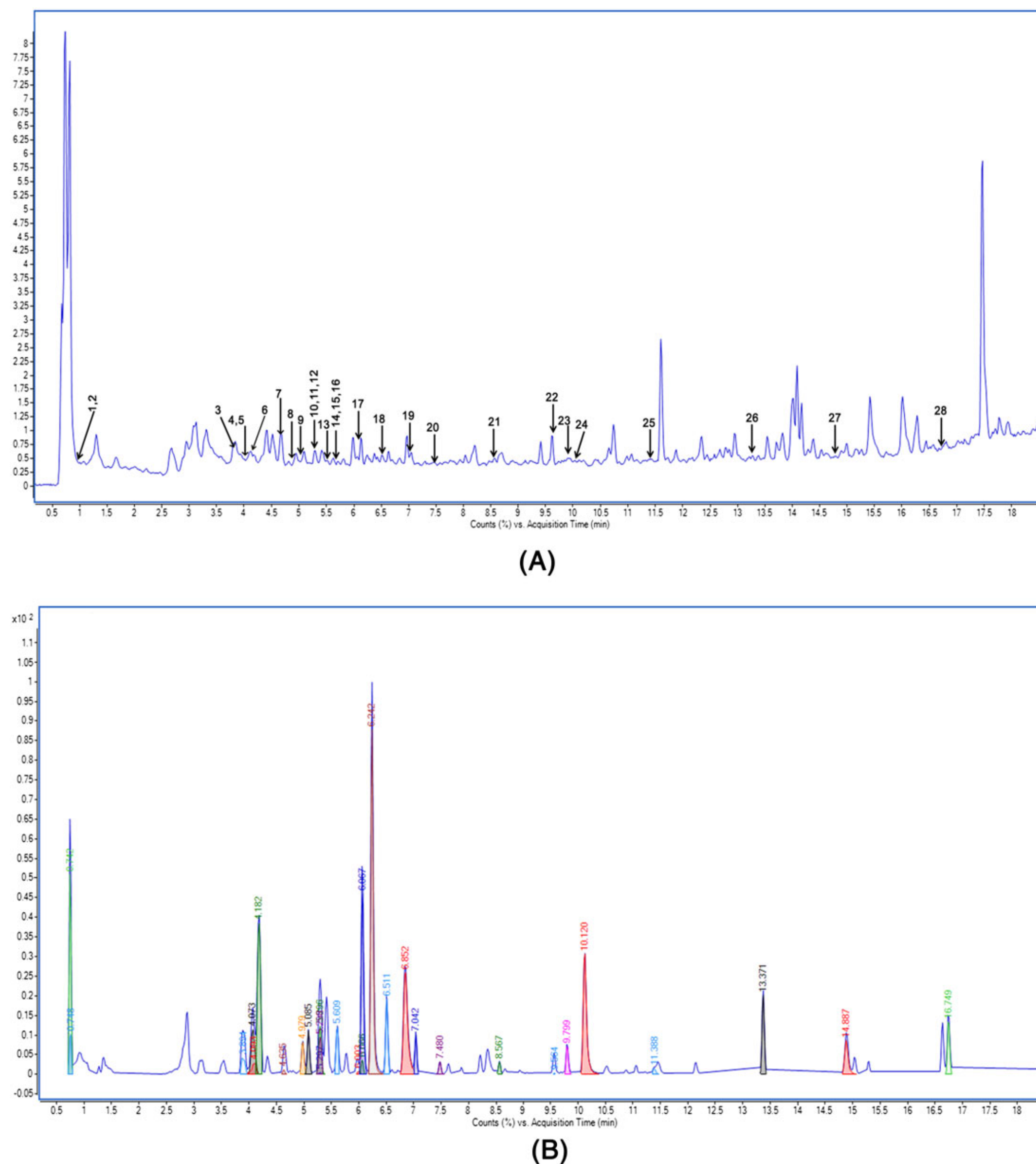

FIGURE 4 (A) Total ion chromatogram of ethyl acetate fraction in the negative ion mode. (B) Extracted ion chromatogram of ethyl acetate fraction in negative ion mode. Peak labels represent the identified compounds [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Phenolic compounds identified in ethyl acetate fraction by UPLC-QTOF-MS

Peak	Retention time	*m/z*	Molecular formulae	Mass (Experimental)	Mass (Theoretical)	Error (ppm)	Proposed compounds	Classification
1	0.742	302.194	$C_{14}H_6O_8$	302.0069	302.0063	2.01	Ellagic acid	Phenolic acids
2	0.748	353.0863	$C_{16}H_{18}O_9$	354.0948	354.0951	−1.92	Chlorogenic acid	Phenolic acids
3	3.894	207.0662	$C_{11}H_{12}O_4$	208.0732	208.0736	−1.82	Sinapaldehyde	Hydroxycinnamaldehydes
4	4.073	151.0398	$C_8H_8O_3$	152.0471	152.0473	−1.77	Hydroxyphenyl acetic acid	Phenolic acids
5	4.093	191.0353	$C_{10}H_8O_4$	192.0426	192.0423	1.89	Scopoletin	Hydroxycoumarins
6	4.182	137.0247	$C_7H_6O_3$	138.0323	138.0317	4.49	Salicylic acid	Phenolic acids
7	4.635	169.0146	$C_7H_6O_5$	170.0218	170.0215	1.92	Gallic acid	Phenolic acids
8	4.979	161.0248	$C_9H_6O_3$	162.0321	162.0317	2.73	4-Hydroxycoumarin	Hydroxycoumarins
9	5.296	135.0449	$C_8H_8O_2$	136.0521	136.0524	−2.25	Phenylacetic acid	Phenolic acids
10	5.297	123.0455	$C_7H_8O_2$	124.0528	124.0524	2.95	Guaiacol	Methoxyphenols
11	5.298	167.0347	$C_8H_8O_4$	168.042	168.0423	−1.41	Dihydroxyphenylacetic acid	Phenolic acids
12	5.609	195.0302	$C_9H_8O_5$	196.0375	196.0372	1.66	Hydroxycaffeic acid	Phenolic acids

(Continues)

TABLE 3 (Continued)

Peak	Retention time	m/z	Molecular formulae	Mass (Experimental)	Mass (Theoretical)	Error (ppm)	Proposed compounds	Classification
13	6.003	153.0199	$C_7 H_6 O_4$	154.0272	154.0266	3.84	Gentisic acid	Phenolic acids
14	6.066	151.0758	$C_9 H_{12} O_2$	152.0832	152.0837	−4.73	4-Ethylguaiacol	Alkylmethoxyphenols
15	6.067	195.0669	$C_{10} H_{12} O_4$	196.0742	196.0736	3.46	Dihydroferulic acid	Phenolic acids
16	6.242	179.0356	$C_9 H_8 O_4$	180.0428	180.0423	3.0	Caffeic acid	Phenolic acids
17	6.511	255.0671	$C_{15} H_{12} O_4$	256.0745	256.0736	3.68	Pinocembrin	Flavanones
18	6.852	121.0296	$C_7 H_6 O_2$	122.0368	122.0368	0.1	Benzoic acid	Phenolic acids
19	7.042	313.0731	$C_{17} H_{14} O_6$	314.0805	314.079	4.71	Cirsimaritin	Flavones
20	7.48	163.0769	$C_{10} H_{12} O_2$	164.0842	164.0837	2.95	Eugenol	Hydroxyphenylpropenes
21	8.567	163.0395	$C_9 H_8 O_3$	164.0467	164.0473	−3.67	Coumaric acid	Phenolic acids
22	9.564	285.0768	$C_{16} H_{14} O_5$	286.0841	286.0841	−0.03	Sakuranetin	Flavanones
23	9.799	357.0994	$C_{19} H_{18} O_7$	358.1067	358.1052	4.03	Gardenin B	Flavones
24	10.12	253.0513	$C_{15} H_{10} O_4$	254.0585	254.0579	2.35	Chrysin	Flavones
25	11.388	285.0407	$C_{15} H_{10} O_6$	286.048	286.0477	0.79	Scutellarein	Flavones
26	13.371	341.1041	$C_{19} H_{18} O_6$	342.1111	342.1103	2.27	Tetramethyl scutellarein	Flavones
27	14.887	177.0555	$C_{10} H_{10} O_3$	178.0628	178.063	−1.31	Mellein	Hydroxycoumarins
28	16.749	283.0621	$C_{16} H_{12} O_5$	284.0694	284.0685	3.35	Geraldone	Flavones

3.4 | UPLC-Q-TOF-MS analysis

The phenolic and flavonoid compounds of the ethyl acetate fraction of *L. acutangula* were tentatively identified based on their elution order, measurement of accurate molecular mass and isotope peak pattern. The total ion chromatogram and extracted ion chromatogram of ethyl acetate fraction were obtained by full scan mode under negative electrospray ionisation (ESI) condition as shown in Figure 4A and 4B, respectively. Table 3 summarises the mass spectrometric data of the identified compounds, including experimental and calculated *m/z*, molecular formula, error in parts per million (ppm), retention time as well as the classification of the proposed compounds. The high mass accuracy of the TOF-MS method was able to display a mass error of below 5 ppm, and thus support tentative identifications of various phenolic acids (ellagic acid, chlorogenic acid, gallic acid, hydroxycaffeic acid, gentisic acid, dihydroferulic acid, caffeic acid, coumaric acid, etc.), flavonones (sakuranetin, gardenin B, chrysin, scutellarein, geraldone, etc.), hydroxycoumarins (scopoletin, mullein, etc.), guaiacol (methoxyphenol) in ethyl acetate fraction of aqueous ethanol extract of *L. acutangula* fruits. The high-resolution QTOF-MS coupled with custom Personal Compound Database and Library searching provided a selective and highly sensitive procedure for rapid identification of phenolic compounds in ethyl acetate fraction. Thus, this study reports LC-QTOF-MS based identification of phenolic compounds in CA inhibiting extract and fractions of *L. acutangula* fruit.

DECLARATION OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

WILEY Biomedical Chromatography

Study of pancreatic lipase inhibition kinetics and LC–QTOF–MS-based identification of bioactive constituents of *Momordica charantia* fruits

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Abstract

The different parts of *Momordica charantia* have been reported to have several therapeutic applications against hyperglycemia and hypercholesterolemia associated with pancreatic lipase (PL). Inhibition of this enzyme prevents the absorption of dietary triglyceride in the intestine, and thus exerts an anti-obesity effect. This study aimed to investigate the bioactive constituents of the fruits of *M. charantia* (MCF) extract and fractions against pancreatic PL followed by study of their inhibition kinetics. The PL inhibitory assay was performed spectrophotometrically by measuring the change in absorbance of the products at 405 nm, using *p*-nitrophenylcaprylate as substrate. The results indicated that the ethyl acetate fraction of MCF (EFMC) offered significant, dose-dependent inhibition against PL, compared with the positive control, Orlistat. The enzyme kinetics study revealed the inhibition to be a mixed type in nature. Additionally, the total phenol and flavonoid content of the fractions was estimated. A positive correlation between phenolic content of EFMC and its PL inhibitory activity was established statistically, which implied that higher inhibition potential was contributed by the phenolic compounds. The identification of the bioactive constituents was further confirmed by LC–QTOF–MS study. This finding suggested that phenolic compounds of MCF can serve as functional food components to address obesity-related disorders linked with PL.

KEYWORDS

correlation analysis, enzyme kinetics, LC–QTOF–MS, *Momordica charantia*, pancreatic lipase, phenolics

1 | INTRODUCTION

Pancreatic lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) (PL) are carboxylesterases, ubiquitous enzymes required for fat metabolism in the human body. They are secreted from the pancreas into duodenum and catalyze the hydrolysis of triglycerides present in food into glycerol and fatty acids (Mendes, Oliveira, & de Castro, 2012). PL converts excess dietary fats and carbohydrates into fatty acids which are absorbed in the small intestine and promote the accumulation of body fat (de la Garza, Milagro, Boque, Campión, & Martínez, 2011). The elevation of the body fat may cause hyperlipidemia, obesity, cardiovascular disease, hypertension and diabetes marked by increased levels of total cholesterol, triglycerides and low-density lipoprotein cholesterol (Lean, Lara, & O'Hill, 2006). PL inhibition offers the most promising strategy to suppress triacylglycerol absorption/overproduction of free fatty acids. Thus the reduction of intestinal lipid digestion leads to a decrease in the intra-abdominal fat content, which offers a valuable therapeutic

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target to avert obesity (Yun, 2010). In the search for potential and safer therapeutic alternatives, there is a tremendous need to explore medicinal plants and their potential compounds as alternative lipase inhibitors.

There are several food plants of cucurbitaceae family reported to have anti-obesity properties by lowering lipid levels (de la Garza et al., 2011; Sharma, Sharma, & Seo, 2005). *Momordica charantia* (Family: Cucurbitaceae) is an annual fruity vegetable, traditionally used as both food and medicine. It consists of a wide variety of chemical constituents including triterpene (cucurbitane type), protein (polypeptide-P), steroid, alkaloid, inorganic and phenolic acids, phenolic glycosides and flavonoids (Joseph & Jini, 2013; Kenny, Smyth, Hewage, & Brunto, 2013). These different classes of secondary metabolites offer several therapeutic applications against hyperlipidemia, diabetes mellitus, obesity, cancer and various microbial infections (Grover & Yadav, 2004). In particular, *M. charantia* extract possesses potential hypocholesterolemic activity (Jayasooriya et al., 2000; Nerurkar et al., 2006). The whole fruit of bitter gourd has been reported to possess potential therapeutic activity against hyperglycemia and hyperlipidemia (Mahwish, Arshad, Nisa, Nadeem, & Arshad, 2017). The anti-obesity activity of *M. charantia* was reported in threeT3-L1 preadipocyte cell lines (Sahib et al., 2011). In addition, the fruit extract of *M. charantia* was also reported to have potential therapeutic effect on β-cells of isolated pancreatic islets of obese hyperglycemic rats (Singh, Gupta, Sirohi, & Varsha, 2008).

In the search for PL inhibitors from natural resources, the identification and characterization of the active phyto-constituents are necessary as therapeutic alternatives to treat obesity. Although the anti-pancreatic lipase activity of *M. charantia* fruit has been reported earlier (Sahib et al., 2012), the mode and type of inhibition are unknown to date. However, there is still an increasing interest in identifying bioactive constituents present in the fraction of *M. charantia*. With this background, the objective of the present study was to identify the phenolic constituents present in the most active fraction using high-resolution liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–QTOF–MS), evaluation of pancreatic lipase inhibition kinetics to explore the inhibition mechanism of the bioactive fraction of MCF against PL, correlation analysis between PL inhibitory activity and total phenolic and flavonoid content of the extract and fractions of MCF.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Lipase from porcine pancreas Type II (activity 59 units/mg protein) and *p*-nitrophenyl caprylate (*p*NPC) was purchased from Sigma Aldrich (St Louis, MO, USA). Orlistat was procured as a gift sample from Central Drugs Laboratory, Kolkata, India. Hexane, ethyl acetate and ethanol were purchased from Merk, Mumbai, India. Acetonitrile and formic acid (HPLC grade) were procured from Sigma Aldrich. All aqueous solutions were prepared using purified water (resistivity of 18.2 MΩ cm at 25°C) from Mili-Q filtration system.

2.2 | Extraction and fractionation

The fruits of *M. charantia* were collected from the South Bengal region in the month of December 2016. The plant sample was authenticated by Dr S. Rajan, Field Botanist, Ministry of AYUSH, Government of India and the herbarium specimen was deposited at the School of Natural Product Studies, Jadavpur University, Kolkata, India for future reference (voucher specimen number SNPS-JU/2015/1096). The fruits were dried under shade and pulverized using a mechanical grinder to make a coarse powder. A 500 g of dried powder was steeped in 1 L aqueous ethanol (80% *v*/v), for 3 days at room temperature. The process was repeated three times. The extract was filtered and evaporated under vacuum at 45–50°C using a Eyela rotary evaporator (Japan). The percentage yield of the aqueous ethanol extract of MCF (EEMC) was 16.68% (w/w). A 70 g aliquot of EEMC was suspended in water and partitioned successively with hexane and ethyl acetate (100 mL each). All of the fractions were collected, evaporated and weighed. The yields of the hexane (HFMC), ethyl acetate (EFMC) and aqueous (AFMC) fractions were calculated as 3.5, 12.7, and 14.2% (w/w), respectively. The samples were stored in borosilicate glass vials at 4°C prior to analysis.

2.3 | Estimation of total phenolic and flavonoid content

The estimation of total phenolic and flavonoid content of aqueous ethanol extract of MCF and partitioned fractions was performed based on the earlier method described from our laboratory with some modification (Biswas, Mukherjee, Kar, et al., 2016). The samples were prepared with methanol at the concentration 1 mg/mL. The reaction mixture was prepared by mixing 18 μL of sample, 90 μL of 10% Folin–Ciocalteu's reagent and 90 μL 7.5% $NaHCO_3$. The samples were thereafter incubated in a BOD incubator at 45°C for 45 min. Gallic acid was used as a standard at the concentration range of 5–30 μg/mL, to generate a five-point calibration curve. The absorbance was measured at 725 nm using a spectrophotometer (SpectraMax Plus, Molecular Devices LLC, USA). The total phenol content (TPC) was calculated as mg gallic acid equivalent (GAE)/g of sample.

The total flavonoids content (TFC) was also determined by spectrophotometric assay (Biswas, Mukherjee, Kar, et al., 2016). Quercetin was used as the standard. The five-point calibration curve of quercetin was prepared with a different concentration range (10–75 μg/mL) with methanol. Initially, 10 μL of 3% $NaNO_2$, 50 μL 1 M NaOH and 10 μL 15% $AlCl_3$ were added to the reaction mixture and a total volume of 170 μL was made by adding methanol. The absorbance of the mixture was determined at 415 nm. The content of total flavonoid was calculated as milligrams of quercetin equivalents (QE)/g of sample. The results were represented as means ± standard deviation (SD) ($n = 3$). In addition, to understand the interrelationship between pancreatic lipase inhibitory activity (IC_{50} value) and the total phenol and flavonoid contents of *M .charantia* fractions, Pearson's correlation coefficient was calculated.

2.4 | Pancreatic lipase inhibitory activity assay

The *in vitro* pancreatic lipase inhibition assay was performed with the aqueous ethanol extract and fractions of MCF, based on the method described by Gonçalves and his co-workers with minor modification (Gonçalves, Mateus, & de Freitas, 2010). The porcine pancreatic lipase was dissolved in 50 mM phosphate buffer at pH 7.0 and finally the enzyme concentration was made at 5.67 U/mL. *p*-Nitrophenyl caprylate was used as a substrate in a concentration of 200 μM (in phosphate buffer). The assay was initiated by adding *p*NPC in the enzyme solution, in the presence and absence of inhibitors. The rate of change of absorbance of the reaction mixture in each well was determined at 405 nm using a UV–visible spectrophotometer (SpectraMax Plus, Molecular Devices LLC, USA). The assay process was carried out in triplicate. Orlistat was used as a positive control. The PL inhibitory activity, assayed without inhibitor was calculated as 100%. Relative enzymatic activity (%) and IC_{50} value were determined based on the following equation. Relative enzymatic activity (%) = (catalytic rate of enzymatic reaction with inhibitor)/(catalytic rate of enzymatic reaction without inhibitor) × 100. IC_{50} values of inhibitors were determined by plotting percentage relative activity (*y* axis) against inhibitor concentration on a logarithmic scale (*x* axis).

2.5 | Inhibition kinetics study on pancreatic lipase

To explore the inhibitory mechanism, the reversibility of the enzyme-inhibitor reaction was studied. The plots of residual activity (expressed in ΔAbs/min) vs. PL concentrations (0–50 U/mL) (*E*) at different inhibitor concentrations (100–400 μg/mL) were constructed (Biswas, Chanda, Kar, & Mukherjee, 2017). Additionally, in order to determine kinetic parameters, an enzyme inhibition study was performed with the above spectroscopic method using different concentrations of substrate (50–200 μM) and the inhibitors (100–400 μg/mL). The catalytic effect of the active fraction was monitored as the function of initial reaction velocity and incubation time (from 0 to 30 min). The kinetic parameters, viz. K_m and V_{max} values, were estimated using the Lineweaver–Burk double reciprocal plot based on the equation: $1/V = K_m/V_{max} \times (1 + [I]/K_i) \times 1/[S] + 1/V_{max} \times (1 + [I]/\alpha K_i)$, where V is the reaction velocity, K_m is the Michaelis constant, V_{max} is the maximal velocity, $[I]$ is the concentration of inhibitor, K_i is the inhibitor constant, $[S]$ is the concentration of substrate and α is the apparent coefficient. The values were calculated using the software SigmaPlot 12.5. The K_i and αK_i values were determined by constructing the secondary plot of the slope (K_m/V_{max}) and *y*-intercept ($1/V_{max}$) vs. inhibitor concentration $[I]$.

2.6 | LC–QTOF–MS method

The LC–QTOF–MS analysis was performed to identify the phytoconstituents present in the bioactive fraction of MCF. Chromatographic separation was carried out using an LC800, GL Sciences equipped with a vacuum degasser, a binary pump, an autosampler and a diode array detector. The chromatographic separation was achieved on Agilent Zorbax Eclipse C_{18} column (50 × 2.1 mm, 1.7 μm). The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. The gradient profile was set to 10% B from 0 to 1 min, 30% B at 8 min, 40% B at 12 min, 80% B at 16 min, 95% B for 20–27 min, and finally 10% B at 28–45 min. The flow rate was set at 0.7 mL/min. The sample was prepared at a concentration of 100 μg/mL with acetonitrile and filtered through 0.45 μm filter before injection. The autosampler and column heater temperatures were maintained at 25°C, and the injection volume was 15 μL for all analyses. The HPLC system was hyphenated with a Triple TOF 5600 System (AB Sciex, Concord, Canada) supported with a DuoSpray ion source. The spectra were acquired in negative ionization modes over a mass-to-charge (*m/z*) range between 50 and 1100 Da. The source voltage was set to 4.0 kV for negative ionization mode. The declustering potential and source temperature were maintained at 80 V and 550°C respectively. The curtain gas flow, nebulizer

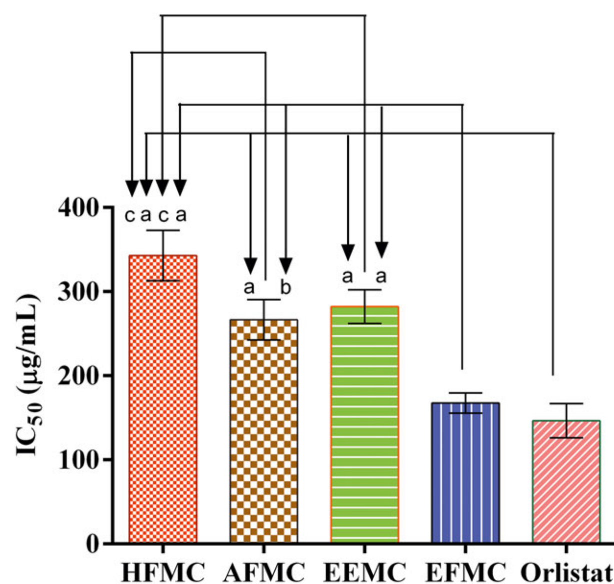

FIGURE 1 Half maximal inhibitory concentration (IC_{50} value) of fractions of *Momordica charantia* extract. Orlistat—positive control; results are represented as mean ± SD ($n = 3$); one-way ANOVA with Tukey's multiple comparison test; Significant value are represented as a = $p < 0.001$; b = $p < 0.01$; c = $p < 0.05$

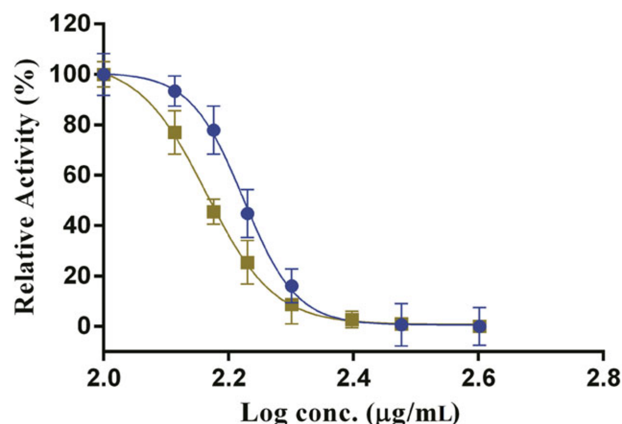

FIGURE 2 Log–dose response curve of EFMC and Orlistat on PL inhibitory activity. (●) EFMC, ethyl acetate fraction of MCF; (■) Orlistat; data are presented as mean ± SD ($n = 3$)

and heater gas were set to 35, 50 and 55 arbitrary units. The instrument was configured to perform one TOF–MS survey scan (150 ms) and 20 MS/MS scans (50 ms each) with a total duty cycle time of 1.2 s. The mass range of both scan types was 50–1000 *m/z*. The MS–MS spectra were acquired using the IDA function of the Analyst TF software (AB Sciex, Concord, Canada). The data files were subjected to isotope pattern matched peak mining using the extracted ion chromatogram manager add-on for PeakView (AB Sciex, Concord, Canada) and reported the most intense peak across the chromatogram. The metabolites were putatively identified by utilizing the custom database with the phenolic compounds obtained from Phenol-Explorer database (Neveu et al., 2010).

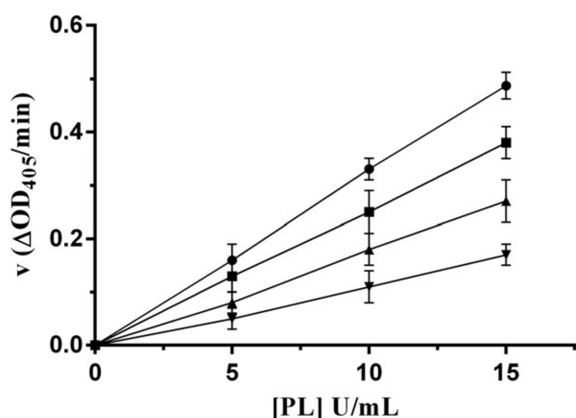

FIGURE 3 Reversible inhibition of EFMC against PL (plots of velocity vs. enzyme concentration). The *v* value indicates the change in absorbance at 405 nm/min at EFMC concentrations of 0 (●), 100 (■), 200 (▲), 400 (▼) µg/mL. Data presented as mean ± SD ($n = 3$)

2.7 | Statistical analysis

The statistical analyses of all of the experimental data were expressed as mean value ± standard deviation (SD) ($n = 3$). The one-way ANOVA test was performed to determine the significant mean difference ($n = 3$) between the samples followed by Tukey's multiple comparisons test ($p < 0.05$). The Pearson correlation test was employed to determine the correlation coefficients among means. The statistical analysis was performed by Graph pad prism 6.0 software.

3 | RESULTS

3.1 | Pancreatic lipase inhibitory activity

The extract and fractions of MCF were tested for their ability to inhibit pancreatic lipase activity *in vitro*, expressed as half-maximal inhibitory concentration (IC_{50}). The IC_{50} values of the aqueous ethanol extract, three subsequent partitioned fractions and Orlistat

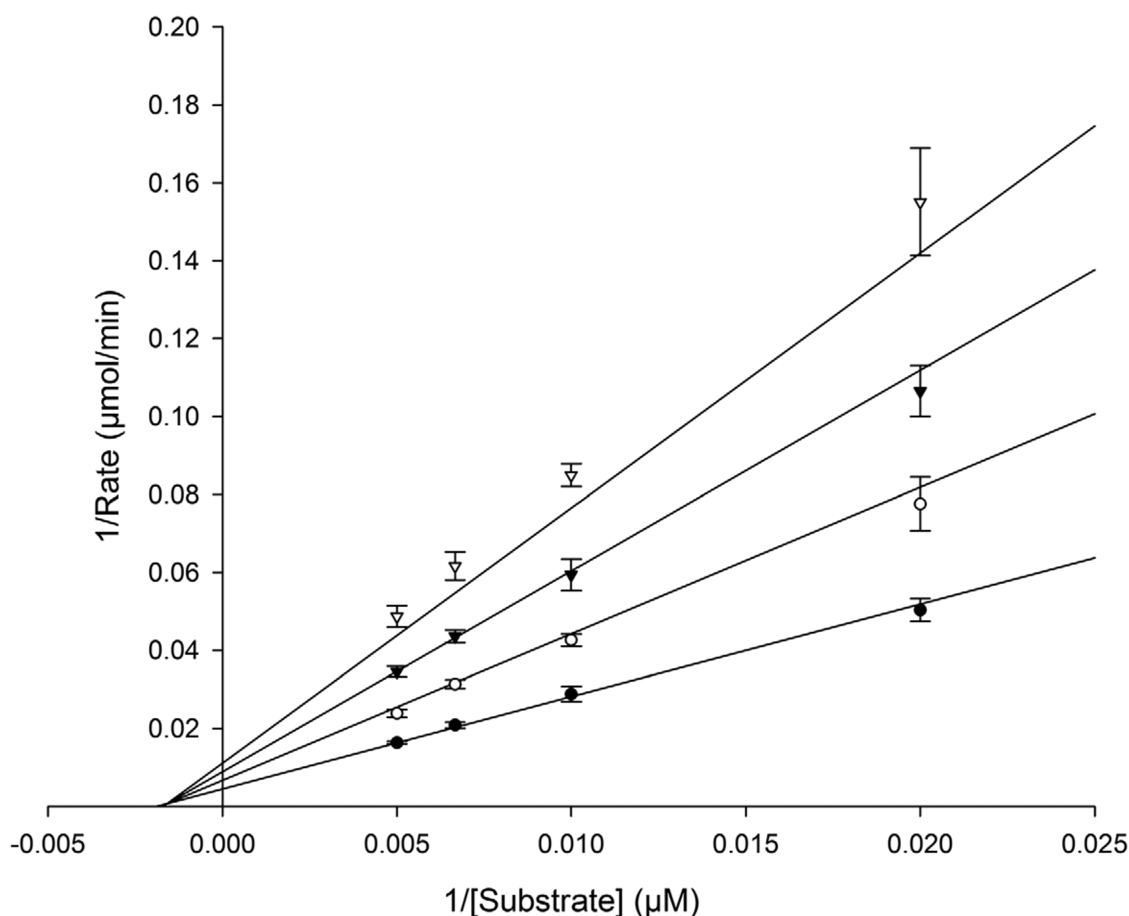

FIGURE 4 Enzyme kinetics study (LB plot) of EFMC against PL inhibitory activity. The *v* value indicates the change in absorbance at 405 nm/min at different concentrations of inhibitors: (●) 100, (■) 200, (▲) 300 and (▼) 400 µg/mL. Data presented as means ± SD ($n = 3$)

(positive control) were estimated to be 167.53 ± 12.45 (EFMC), 266.47 ± 24.58 (AFMC), 282.6 ± 20.44 (EEMC), 342.7 ± 37.12 (HFMC) and 146.33 ± 20.88 (Orlistat) μg/mL (Figure 1). No significant difference ($p < 0.05$) was observed in the case of EFMC compared with standard inhibitor Orlistat. The inhibitory action of EFMC was found to be dose dependent in a range of inhibitor concentrations of 100–400 μg/mL (Figure 2).

3.2 | Estimation of inhibition kinetics parameters

The plot of the reaction velocity (v) vs. enzyme concentration at different inhibitor concentrations gave a group of straight lines. It was observed that all of the straight lines passed through the origin and the slope of the line decreased inversely with the increasing concentration of the inhibitors (Figure 3). The data indicated that the enzymatic reaction followed first-order kinetics as the reaction rate (velocity) was dependent on the concentration of the reactant (enzyme). This result confirmed the reversible type of inhibition of EFMC against PL activity. The kinetics mode of pancreatic lipase inhibition was studied with respect to different substrate concentrations. It was observed that both of the values of apparent V_{max} and K_m were changed with an increasing concentration of the inhibitor. This indicated a probability of a mixed type of inhibition on lipase with the test sample. From the Lineweaver–Burk plot, the K_m and V_{max} values were 2.0 mM and 271.9 μM/min (Figure 4). The inhibition constants (K_i) and αK_i were also calculated from the secondary plot as 123.60 ± 0.09 and 4.24 ± 1.12 μg/mL, respectively (Figure 5.a and b).

3.3 | Correlation between PL inhibitory activity and total phenolic and flavonoid content

The TPC and TFC were the highest in EFMC at 3.79 ± 0.28 mg GAE/g and 2.44 ± 0.48 mg QE/g of sample, respectively. The TPC and TFC of other fractions were in the range of EEMC > AFMC > HFMC. A correlation between PL inhibitory activity and TPC and TFC of all of the fractions was studied through Pearson's correlation coefficient (R^2 value). A statistically significant correlation (R^2 value 0.9261) between the TPC of ethyl acetate fraction and its PL inhibition potential (IC_{50} value) was observed (Table 1). A lower correlation value was observed with other fractions and their phenolic and flavonoid content.

3.4 | LC–QTOF–MS identification of bioactive constituents

LC–QTOF–MS-based fingerprinting of the ethyl acetate fraction led to the identification of phenolic compounds based on their accurate mass, experimental and calculated m/z, molecular formula, error (in ppm), isotopic ratio and MS fragmentation pattern (matched within 25 ppm error tolerance). Table 2 summarizes 22 phenolic compounds with their respective retention time, molecular formulae, exact and deprotonated mass, isotopic difference and MS/MS fragment ions. The total ion chromatogram and the representative MS/MS spectra of the identified compounds have been represented in Figure 6 and Figure S1–S22 (Supplementary Materials). The results demonstrated that EFMC contains a large number of phenolic compounds, including hydroxybenzoic acids, hydroxycinnamic acid, flavonol, isoflavonoid, flavanone and hydroxycoumarin. The high mass accuracy of the TOF–MS method was able to display a mass error of <5 ppm, and thus confirmed the authenticity of the chemical composition.

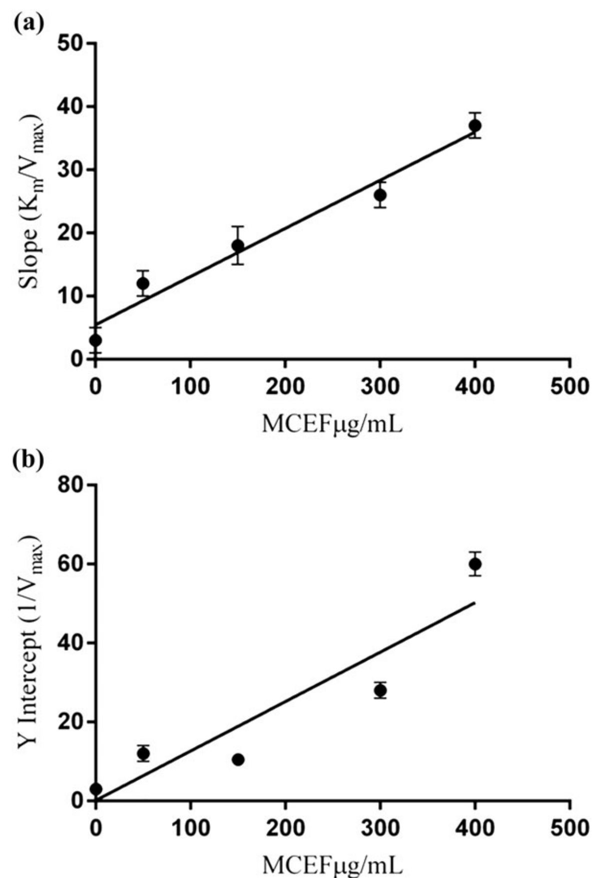

FIGURE 5 (a) Plot of slope (K_m/V_{max}) vs. EFMC concentration. (b) Plot of y-intercept ($1/V_{max}$) vs. EFMC concentration; Secondary replots were constructed from the data, collected from the Lineweaver–Burk plot

TABLE 1 Correlation analysis (R^2) between lipase inhibitory action (IC_{50} value) of *Momordica charantia* fractions and total phenol and flavonoid content

R^2	EFMC	EEMC	AFMC	HFMC
Total phenol content	0.9261**	0.2361^{ns}	0.3214^{ns}	0.1017^{ns}
Total flavonoid content	0.4560^{ns}	0.4417^{ns}	0.4894^{ns}	0.3243^{ns}

Correlation is significant at: ^{ns}not significant (two-tailed); **p-value <0.01.
EFMC, ethyl acetate extract of MCF; EEMC, ethanol extract of MCF; AFMC, aqueous extract of MCF; HFMC, hexane extract of MCF.

4 | DISCUSSION

Pancreatic lipase plays a major role in digestion of dietary triglycerides into monoacylglycerides and free fatty acids. Inhibition of PL leads to a

TABLE 2 Phenolic compounds identified in ethyl acetate fraction of *M. charantia* by LC–QTOF–MS

Peak no.	Retention time	Molecular formulae	Exact mass	Deprotonated mass	Error (ppm)	Isotopic difference	Major MS/MS fragments	Proposed compounds
1	3.53	$C_{17}H_{20}O_9$	368.11073	367.10346	1.3	7.5	115.0201, 187.0396, 157.0303, 127.0203	Feruloyl quinic acid
2	4.29	$C_8H_8O_4$	168.04226	167.03498	1.3	7.5	108.0221, 152.0096, 80.0265	Vanillic Acid
3	5.96	$C_7H_6O_4$	154.02661	153.01933	0	3.1	108.0205, 78.9590	Gentisic acid
4	8.31	$C_{13}H_{16}O_8$	300.08452	299.07724	-1	3.1	93.0340, 137.0232	4-Hydroxybenzoic acid 4-*O*-glucoside
5	12.07	$C_8H_8O_3$	152.04734	151.04007	0.2	9.1	108.0215, 92.0278, 136.0166, 95.0145	Vanillin
6	16.07	$C_8H_8O_2$	136.05243	135.04515	−0.4	9	92.0260, 121.0293	*p*-Anisaldehyde
7	18.84	$C_8H_8O_2$	136.05243	135.04515	−0.4	9	92.0260, 121.0293	Phenylacetic acid
8	19.76	$C_7H_8O_2$	124.05243	123.04515	−5	7.9	93.0341, 65.0393	Methyl catechol
9	20.68	$C_9H_6O_4$	178.02661	177.01933	0.7	1.7	133.0294, 121.0272, 93.0334, 71.0138	Esculetin
10	21.17	$C_{10}H_8O_4$	192.04226	191.03498	0	6.8	93.0334	Scopoletin
11	23.93	$C_7H_6O_2$	122.03678	121.0295	0.6		92.0263	Benzoic acid
12	24.30	$C_9H_8O_3$	164.04734	163.04007	−1	2.6	119.0498, 93.0350	Coumaric Acid
13	26.00	$C_7H_6O_3$	138.03169	137.02442	0.7	1.1	93.0352, 65.0398	Protocatechuic aldehyde
14	28.01	$C_{22}H_{24}O_{13}$	496.12169	495.11441	2.5	6.9	449.1100, 431.0981	4′-*O*-Methyl-(−)-epigallocatechin 3′-*O*-glucuronide
15	28.50	$C_{26}H_{28}O_{16}$	596.13774	595.13046	−2.2	9.6	549.0949, 300.0250	Quercetin 3-*O*-glucosyl-xyloside
16	30.22	$C_{21}H_{22}O_{11}$	450.11621	449.10894	−2.2	2.6	167.0334, 137.0231, 152.0102, 281.0650	Dihydroquercetin 3-*O*-rhamnoside
17	30.69	$C_{22}H_{22}O_{10}$	446.1213	445.11402	−2.8	1.9	145.0287, 137.0236, 163.0387, 119.0492	Glycitin
18	33.20	$C_{21}H_{22}O_{10}$	434.1213	433.11402	−3	8.6	152.0099, 167.0330, 108.0207, 123.0443	Naringenin 7-*O*-glucoside
19	34.34	$C_{28}H_{34}O_{15}$	610.18977	609.18249	−0.5	7.7	281.0633, 137.0267, 579.1946, 495.1208	Hesperidin
20	35.37	$C_{22}H_{24}O_8$	416.14712	415.13984	−1.5	7.7	145.0294, 117.0338	1-Acetoxypinoresinol
21	36.30	$C_8H_8O_5$	184.03717	183.0299	0.4	2.2	124.0141, 167.9081, 135.9199, 92.9978	Methylgallic acid
22	37.24	$C_{17}H_{26}O_4$	294.3910	293.22278	−1.4	7.4	220.1456, 177.0906, 193.1617	[6]-Gingerol

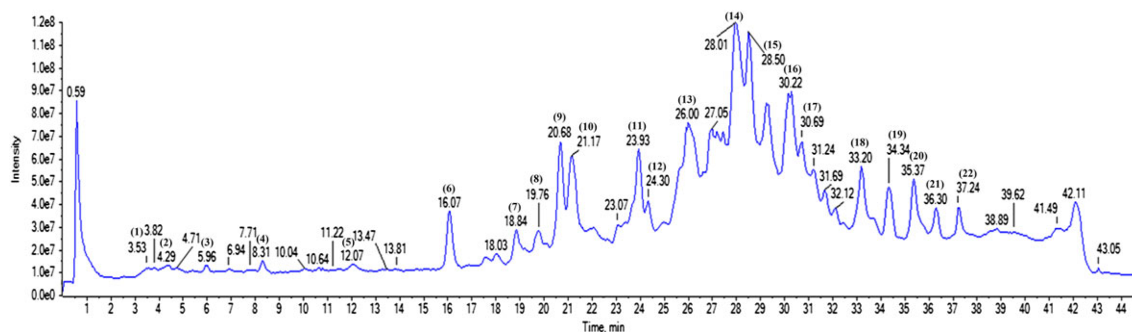

FIGURE 6 LC–ESI–MS total ion chromatogram of bioactive fraction of MCF (EFMC) in negative ionization mode

reduction in fat absorption, and thereby energy uptake, which is considered to be the most effective way to treat obesity (Birari & Bhutani, 2007). In spite of a wide therapeutic application of specific PL inhibitors, Orlistat, their use should be restricted owing to certain unpleasant gastrointestinal side effects. So, in the search for potential and safer therapeutic alternatives, there is a need to explore medicinal plants and their potential compounds as alternative lipase inhibitors. In the current context, there are several food plants containing a large number of polyphenolic compounds that are reported to have potential pancreatic lipase inhibitory activity and thus avert obesity and related disorders (Sergent, Vanderstraeten, Winand, Beguin, & Schneider, 2012).

This study highlights the pancreatic lipase inhibitory kinetics of the fraction of MCF. The results indicated that the ethyl acetate fraction exhibited better PL inhibitory activity among the other fractions. The increase in EFMC concentration resulted in the decrease in relative enzyme activity, which confirmed that EFMC inhibits PL in a dose-dependent manner. From the Lineweaver–Burk plot, two different K_i values were observed which indicates that inhibitor can bind to both enzyme (K_i and αK_i values) as well as ES complex (αK_i), hence it implied a mixed type of inhibition (Biswas, Mukherjee, & Chaudhary, 2016). Moreover, a higher value of K_i implies that the inhibitor binds to the enzyme alone, rather than the ES complex. In order to identify the chemical constituents present in the EFMC, LC–QTOF–MS analysis was performed which identified phenolic compounds such as feruloylquinic acid (RT 3.53 min), vanillic acid (RT 4.29 min), gentisic acid (RT 5.96 min), esculetin (RT 20.68 min), scopoletin (RT 21.17 min), coumaric acid (RT 24.30 min), epigallocatechin glucuronide (RT 28.01 min), glycitin (RT 30.69 min), naringenin-7-*O*-glucoside (RT 33.20 min), hersperidin (RT 34.34 min), 1-acetoxypinoresinol (liganan derivative) (RT 35.37 min) and methylgallic acid (RT 36.30 min). It has to be noted that, owing to its faster acquisition rate and accurate mass acquisition capacity, high-resolution mass spectrometric analysis offered a reliable technique for putative identification of the polyphenolic compounds in *M. charantia* fraction.

A large number of studies have suggested that phytochemicals present in vegetables offer some prophylactic benefits against several lifestyle-related disorders, including obesity, owing to their synergistic interaction (Williams et al., 2013). Many polyphenolic compounds such as phenolic acids, flavones, flavonols, tannins and chalcones are found to be active against PL (Birari & Bhutani, 2007). A variety of hydroxycinnamic acids (*viz*. vanillic acid, gentisic acid, gallic acid, feruloylquinic acid, coumaric acid and benzoic acid derivatives) found in fruits and different parts of plants have shown promising lipase inhibitory activity (Buchholz & Melzig, 2015; Tan, Chang, & Zhang, 2017). In 2011, Kim and co-workers reported moderate pancreatic lipase inhibition activity of vanillin, obtained from radiolytic transformation of curcumin (Kim, Kim, Ito, & Jo, 2011). The inhibitory effect of catechol and related hydroxyl phenol compounds on PL was exhibited earlier (Weinstein & Wynne, 1936). Two coumarin derivatives, esculetin and scopoletin, were reported to have potential anti-obesity activity by inhibiting adipocyte differentiation in three T3-L1 cells (Shin et al., 2010). Another phenolic metabolite, protocatechuic aldehyde (isolated from *Taraxacum ohwianum*), was reported to exhibit PL inhibitory activity (Kim & Kim, 2011). Some catechins and their metabolites/derivative (viz. epigallocatechin) lower cholesterol level by inhibiting pancreatic lipase (Juhel et al., 2000). The efficacy of quercetin-3-xyloside and quercetin-3-glucoside in inhibiting the catalytic activity of PL was reported in the literature (Martinez-Gonzalez et al., 2017). The dihydroflavonol derivatives, *viz.*quercetin-3-O-rhamnoside, glycitin (isoflavone glycoside) were reported to inhibit pancreatic lipase in a dose-dependent manner (Choi, Kim, Park, Seog, & Choi, 2007; Zhang et al., 2018). Another study showed that flavonoids (*viz.* naringenin, hesperidin) and lignans (*viz.* pinoresinol derivative) offer promising lipid-lowering activity by inhibiting pancreatic lipase (Ahn et al., 2012; Assini, Mulvihill, & Huff, 2013; Kawaguchi, Mizuno, Aida, & Uchino, 1997). It was found that a gallotannin derivative, methyl gallate, showed anti-adipogenic activity by inhibiting PL activity (Kwon et al., 2013). The therapeutic potential of gingerol was reported against PL activity in the management of obesity (Saravanan, Ponmurugan, Deepac, & Senthilkumard, 2014). It has to be noted that all of these phenolic compounds have been identified and characteriaed in the ethyl acetate fraction, which may account for its inhibitory effect. In addition, the positive correlation value between TPC and inhibition rate further confirmed that higher PL inhibition properties may be contributed by the phenolic compounds present in EFMC. It can be postulated that the anti-lipase activity of the fraction of *M. charantia* extract seems to be related to the phenolic hydroxyl groups present in their active pharmacophore (Buchholz & Melzig, 2015).

5 | CONCLUSION

This study explored the inhibitory potential of MCF against pancreatic lipase and its mode of inhibition. The results indicated that the ethyl acetate fraction of MCF offered reversible, dose-dependent inhibition among the other fractions. The enzyme kinetics study revealed that the inhibition of PL was mixed type in nature. A significant correlation was observed between total phenol content of ethyl acetate fraction of *M. charantia* and PL inhibitory activity. The LC–QTOF–MS study identified the bioactive phenolic constituents which were probably accountable for the PL inhibition. Based on the findings, it can be concluded that *M. charantia* fruits can serve as functional food components to address obesity-related disorders associated with lipid digestion at large.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

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Original Research Article

Determination of cucurbitacin E in some selected herbs of ayurvedic importance through RP-HPLC

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ABSTRACT

Background: The consumption of the fruits of cucurbitaceae plants is widely popular among Indians due to their various nutritional and medicinal purposes. Some of these plants are well reported in Ayurveda due to their potential therapeutic importance. In particular, the plants of this family are well-characterized by the presence of its bitter principle, Cucurbitacin E which differs within the species due to its genetic variations.

Objectives: The objective of the study was to develop a validated RP-HPLC method for standardization in some widely consumed cucurbits with cucurbitacin E as a marker compound.

Materials and methods: The RP-HPLC method was developed with a reverse phase C_{18} column, using acetonitrile and water (1% glacial acetic acid) as mobile phase (70:30 v/v). The flow rate and λ_{max} were optimized at 1 mL/min and 230 nm respectively. The HPLC method was validated in terms of accuracy, specificity, sensitivity, and repeatability as per ICH guideline.

Results: The calibration curve was found linear in the concentration range of 1–100 μg/mL. The % RSD of precision and recovery was found to be <2%, which confirms high repeatability of the method. The results indicated that the content of cucurbitacin E was highest (0.0663% w/w) in *Cucurbita pepo* whereas *Lagenaria siceraria* contains the lowest (0.0356% w/w).

Conclusion: The study was able to explore the variation of cucurbitacin E content in some selected food plants of Cucurbitaceae family. The applicability of the method can be established in nutraceutical industry for the effective quality control of cucurbits for safe human consumption.

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1. Introduction

Cucurbitaceae is a large plant family, consisting of about 125 genera and 960 species. The various parts (fruit, seeds, stems, leaves) of the plants belonging to the cucurbitaceae family are very popular for their uses in culinary purposes from the ancient time. It is also used in Ayurvedic and folk medicine for their several therapeutic values due to the presence of a large number of metabolites (both primary and secondary). The importance of cucurbitaceae species has been highly recognized for effective control of lifestyle diseases such as diabetes, obesity and related disorders [1]. The cucurbits are a good source of glucose, fructose, essential amino acids, vitamins, water-soluble polysaccharides, dietary fibers, phenolic glycosides, flavonoids, terpenoids, and minerals etc. Apart from the diverse chemical constituents, this family is very well characterized by their presence of cucurbitacin. Cucurbitacin consists of tetracyclic cucurbitane nucleus skeleton with a variety of oxygenation functionalities at different positions with diverse chemical categories. The cucurbitacins are present as non-glycosylated or glycosylated triterpenoids and divided into twelve categories, incorporating cucurbitacins A-T [2]. Various biochemical studies suggested that cucurbitacins have a potential cytotoxic property which is responsible for making it a prominent lead for anti-cancer drug development [3]. The hydrophobic property of the cucurbitacin nucleus is a major regulating factor for their cytotoxic effects and it increases linearly with their hydrophobicity. In particular, cucurbitacin E (Fig. 1) and their glycosides are the most widely distributed chemical constituents in food plants of Cucurbitaceae family. Cucurbitacin E has been reported to possess anti-

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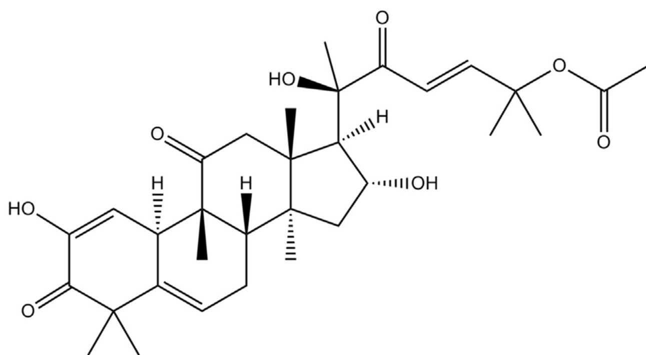

Fig. 1. General structure of Cucurbitacin E.

inflammatory [4], anti-angiogenic, immunomodulatory, cytotoxic [5], cytostatic and hepatoprotective [6] properties in both *in vitro* and *in vivo* model. It has been observed that the combination of cucurbitacin E with other synthetic anti-cancer drugs results in synergistic action in terms of cytotoxicity with greater efficacy in tumor growth inhibition [7]. Despite the potential therapeutic activity of Cucurbitacin E and cucurbitacin E glycoside, their chronic exposure is undesirable due to their extremely bitter and disagreeable taste as well as their toxicological effects found in experimental animals [8]. It has been presumed that back mutated fruits produce more toxicity and bitterness whereas the suppressor gene is responsible for the absence of cucurbitacins [9].

Although a large number of gourd family plants are grown and consumed, six species namely *Lagenaria siceraria, Benincasa hispida, Momordica charantia, Coccinia grandis, Cucurbita pepo,* and *Luffa acutangula* have potential nutraceutical benefits. The therapeutic benefits of these plants are also well documented in Ayurveda. *Lagenaria siceraia* (Bottle gourd) is known as Tumbini or Alabu in Ayurveda which is indicated in *Jwara* (fever), *Kasa* (cough), Svasa (respiratory distress), Visa Roga (poisoning), Sopha (inflammation/swelling), Vrana (Ulcers) *Sula* (colic pain) [10]. It is also reported as a diuretic, cardioprotective, antihyperlipidemic, anti-hyperglycemic, and antioxidant. The major bioactive constituents in the fruit consist of cucurbitacin B, D, E, phenolic compounds viz. phenolic glycosides, phenolic acid, flavonoids, flavon-C-glycoside such as isovitexin, isoorientin, saponarin sterols like fucosterol, campesterol etc [11,12]. In Ayurveda *B. hispida* (Wax gourd) is known as kusmanda, indicated in *Mutraghata* (Urethritis), *Prameha* (*Diabetes mellitus*), *Ashmari* (kidney stone), *Manasa Vikara* (psychological problems) [13]. It possesses several pharmacological properties including antioxidant, ACE inhibitory, anti-ulcer, anti-inflammatory, anti-obesity, anti-diarrheal activity. The presence of a large number of chemical constituents have been reported in this plant viz. lupeol, sitosterol, pentacyclic triterpenes, cucurbitacin B, E, triterpenoid (isomultiflorenol), trigonelline, β-sitosterol, alkaloids such as 5-methylcytosine, triterpenoids such as cucurbitacin B, sterols, glycosides [14]. *M. charantia* (Bitter gourd) is known as karabellak in Ayurveda indicated in *Kasa* (cough), *Svasa* (Asthma), Jvara (fever), *Raktavikara* (blood disorder), *Kamala* (jaundice), *Krmiroga* (helminthiasis), *Kustha* (skin disorder) [15] (Anonymous 1999). It consists of a wide variety of chemical constituents including triterpene (cucurbitane type), protein (Polypeptide P), steroid (diosgenin), alkaloid (vicine), inorganic and phenolic acids, phenolic glycosides, flavonoids etc. [16]. In particular, *M. charantia* extract possesses potential hypocholesterolemic, antidiabetic, antiobesity, antimicrobial, lipid-lowering properties [1,17]. Another food plant, *Coccinia indica* (Ivy gourd) is also known as Bimbi in Ayurveda, indicated in *Kasa* (cough), *Svasa* (Asthma), *Jwara* (fever), *Raktavikara* (blood disorder), *Daha* (burning sensation) [18]. *C. grandis* is used in folklore medicine as antibacterial, hepatoprotective, hypoglycemic, hypolipidemic, antioxidant properties. The fruits of this plant contain Cucurbitacin B, E, taraxerone, taraxerol, β-carotene, carotenoids, β-sitosterol, Stigma-7-en-3-one etc. as active constituents [19]. *C. pepo* is also mentioned as a variety of Kushmandu in Ayurveda and widely used in the treatment of mental disorder, epilepsy, urinary disorders, diabetes etc. [20]. It contains a large number of chemical constituents including cucurbitacin B, cucurbitacin E, dihydrocucurbitacin, acylated phenolic glycosides (cucurbitosides), spinasterol, β-sitosterol, palmitic, palmitoleic, stearic, oleic, linoleic acids etc. [21]. In Ayurveda, *Luffa acutangula* is known as Kosataki, indicated in *Kustha* (skin disorder), *Pandu* (jaundice), *Pliharoga* (Splenic disease), *Sopha* (inflammation) [22]. It has also been reported to possess several pharmacological properties like diuretic, hepatoprotective, anti-diabetic etc. The fruits of *L.acutangula* contain cucurbitacin B, E as bitter principles. The plant contains a significant amount of polyphenols (mostly phenolic acids *viz.* gallic acid, p-coumaric acid, ferulic acid, protocatechuic acid, and its glycosides, flavonoids (catechin, quercetin) [23,24].

With this background, the present study was aimed to develop a validated RP-HPLC method for standardization of the selected fruits of cucurbitaceae family by using cucurbitacin E as a marker compound. The validation of RP-HPLC method was further carried out based on the ICH guidelines. This validated method can be applied for quantitative estimation of cucurbitacin E in the cucurbitaceae food plants and their related preparations.

2. Experimental

2.1. Instrumentation and reagents

The RP-HPLC system (Waters, Milford, MA, USA) consisted of a 600 controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) detector equipped with an in-line degasser AF 2489 and a rheodyne 7725i injector having 20 μL loop volume. Membrane filters (0.45 μm pore size) (Millipore) were used for filtration of the mobile phase. Quantitative estimation was performed with Empower 2 software programs using the external standard calibration method. Acetonitrile (HPLC grade) and glacial acetic acid (HPLC grade) were procured from Merck (Mumbai, India). All the other solvents (AR grade) procured from Merck. Cucurbitacin E (purity $\geq$ 95% HPLC) was purchased from Chromadex Inc. USA. All aqueous solutions were prepared using purified water (resistivity of 18.2 MΩ cm at 25 °C) from a Mili-Q filtration system.

2.2. Extraction of plant material

The mature fruits of *L. siceraria, B. hispida, M. charantia, C. grandis, C. pepo*, and *L. acutangula* were collected from local market of West Bengal, India. They were authenticated and the voucher specimen of all of them has been retained in the School of Natural Product Studies, Jadavpur University, Kolkata, India vide voucher specimen numbers SNPS-1462/2016- SNPS-1467/2016 for future references. The juice was squeezed from the fruits and then filtered through Whatman no. 1 filter paper. The aqueous extract was lyophilized and stored at −20 °C for further use. The % yield of the extracts was calculated.

2.3. RP-HPLC conditions

The chromatographic method was developed based on the previous method with some modification [25]. The RP-HPLC method was refined by changing the mobile phase composition

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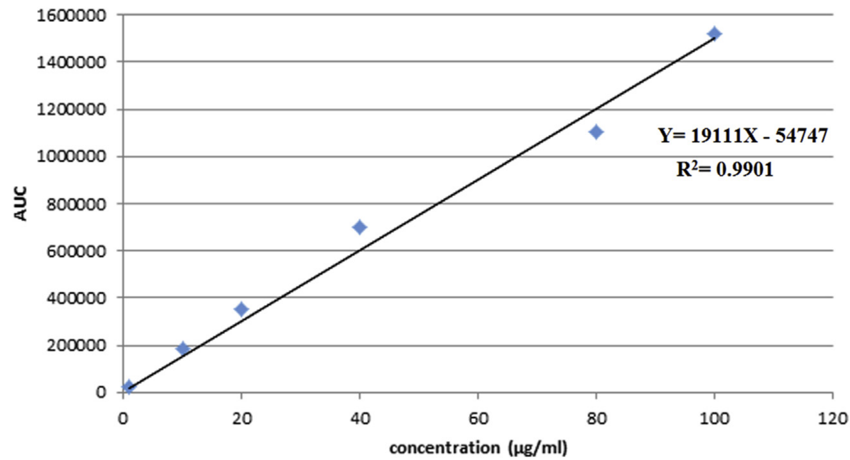

Fig. 2. Calibration curve of Cucurbitacin E.

Table 1
Cucurbitacin E content in cucurbits by RP-HPLC.

Plant name	Voucher specimen no.	Common name	Cucurbitacin E content (%w/w)
Lagenaria siceraria	SNPS-1462/2016	Bottle gourd	0.0356
Benincasa hispida	SNPS-1463/2016	Wax gourd	0.0446
Momordica charantia	SNPS-1464/2016	Bitter gourd	0.0523
Coccinia grandis	SNPS-1465/2016	Ivy gourd	0.0511
Cucurbita pepo	SNPS-1466/2016	Pumpkin	0.0663
Luffa acutangula	SNPS-1467/2016	Ridge gourd	0.0556

Table 2
Accuracy study.

Excess CuE added (ng)	Expected CuE in extract (ng)	Average CuE found (ng)	Average Recovery (%)	RSD (%)
0	66.3	63.21	95.35	1.25
10	77.3	74.20	95.99	0.98
40	107.3	103.8	96.82	1.41
80	147.3	143.2	97.23	1.05

in a gradient manner and finally, isocratic method was optimized with the mobile phase of acetonitrile (solvent A) and water (solvent B) in the ratio of 70: 30 (v/v). The pH of the solvent B was adjusted at 3.8 by using 1% (v/v) glacial acetic acid. The mobile phase was filtered through a 0.45 µm pore size (Millipore) membrane filter followed by sonication to degas the solvent. The separation was carried out on a Waters Spherisorb 5 mm ODS2 column (C_{18}, 250" × 4.6", 5 µm particle size). The temperature of the column was kept at 25 °C and the injection volume was 20 µL. The total run time was set at 10 min. The flow rate was set at 1.0 mL/min and the λ_{max} was set at 230 nm for maximum absorption of the compound. A baseline was recorded with the optimized chromatographic method for about 15 min prior to standard and sample injection. Each chromatographic analysis was followed by a blank run to wash out any carryover from the previous analysis.

2.4. Preparation of standard and sample solutions

A standard stock solution of Cucurbitacin E was prepared by dissolving approximately 1 mg of cucurbitacin E in 1 mL methanol. Further dilution was carried out to prepare calibration samples in the concentration range of 1–100 µg/mL. The sample solutions were prepared by taking 10 mg of extract in 1 mL methanol. The solution was filtered through 0.45 µL syringe filter prior to injection.

2.5. Method validation

The RP-HPLC method validation was carried out by determining linearity, specificity, accuracy and precision, limit of quantification and limit of detection on the basis of International Conference on Harmonization guidelines [26]. Method specificity was determined by comparing the retention time of both standard and test samples. Sensitivity was evaluated by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) and calculated based on the equation: $LOD = 3.3\ \sigma/S$ and $LOQ = 10\ \sigma/S$, where σ is the standard deviation and S is the slope of the calibration curve. The standard deviation (σ) was calculated by measuring the deviations of the background response of an appropriate number of blank samples ($n = 6$). The accuracy of the method was determined by the standard addition technique and expressed in terms of % RSD for the mean recovery of the theoretical concentration. The samples were spiked with three different amounts of standard compounds in triplicate. For estimation of spike recovery, *C. pepo* extract was

Table 3
Intra-day and inter-day precision study.

Intra-day (n = 6)				Inter-day (n = 6)			
RT (min)		Response (AU)		RT (min)		Response (AU)	
Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD
4.70	0.87	4,753,208	1.20	4.68	1.50	4,593,228	1.28
4.65	1.47	7,612,069	1.30	4.55	1.17	7,292,664	1.81
4.69	1.46	16,198,361	1.25	4.70	1.10	18,105,372	1.50

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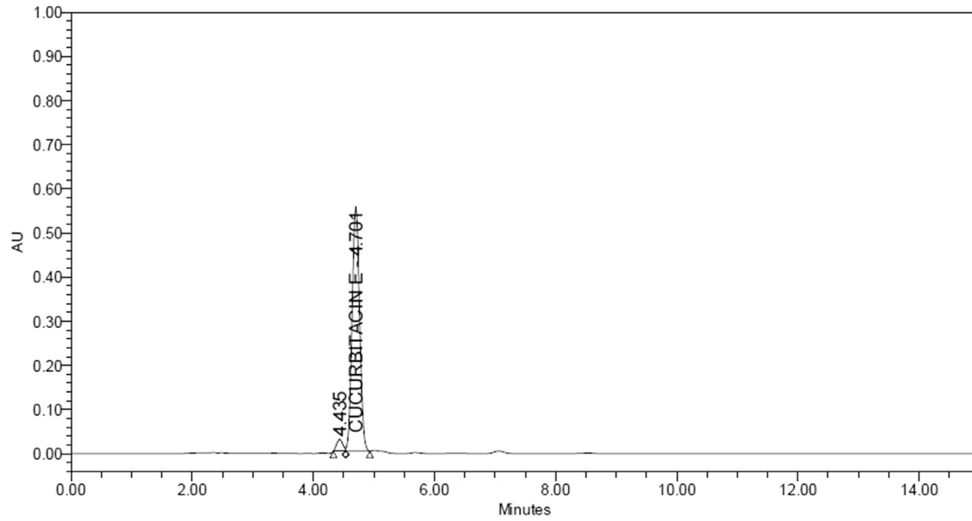

Fig. 3. RP-HPLC/UV chromatogram of Cucurbitacin E standard.

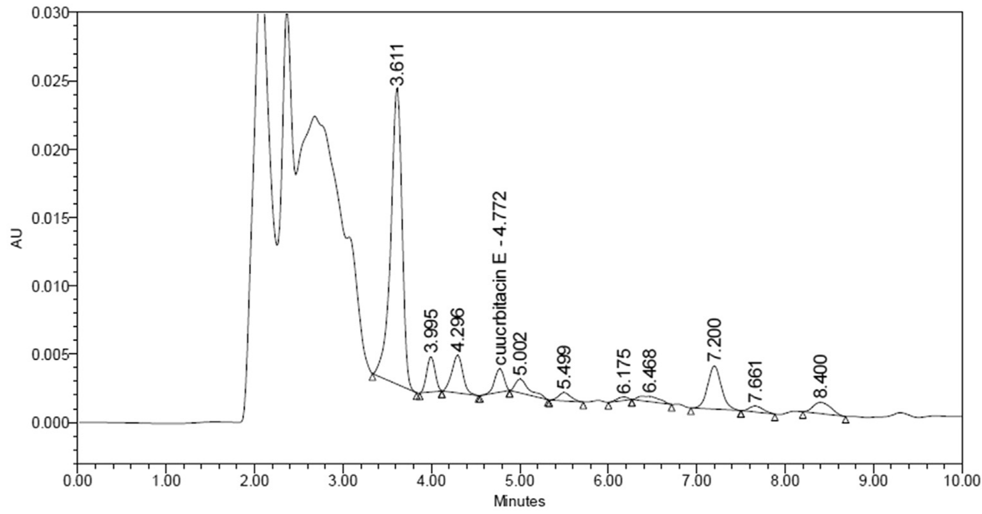

Fig. 4. RP-HPLC/UV chromatogram of *Lagenaria Siceraria* lyophilized extract.

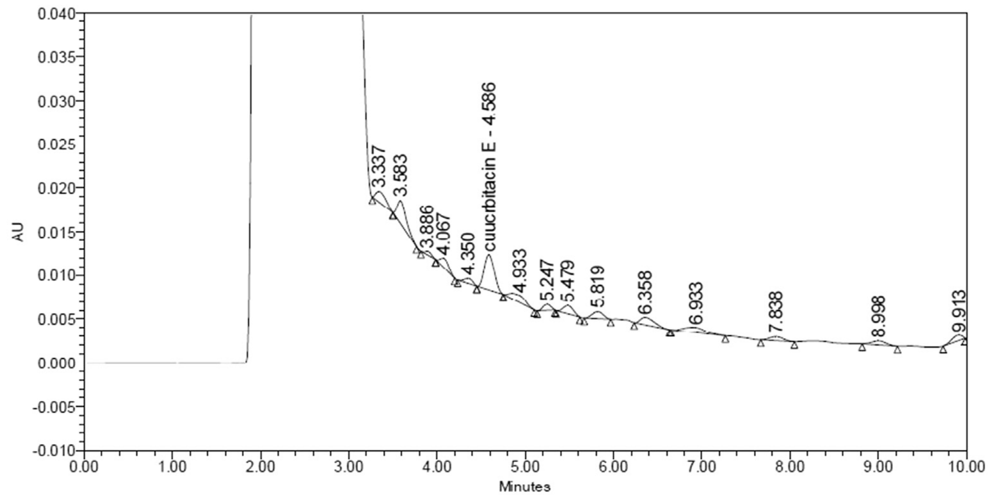

Fig. 5. RP-HPLC/UV chromatogram of *Benincasa hispida* lyophilized extract.

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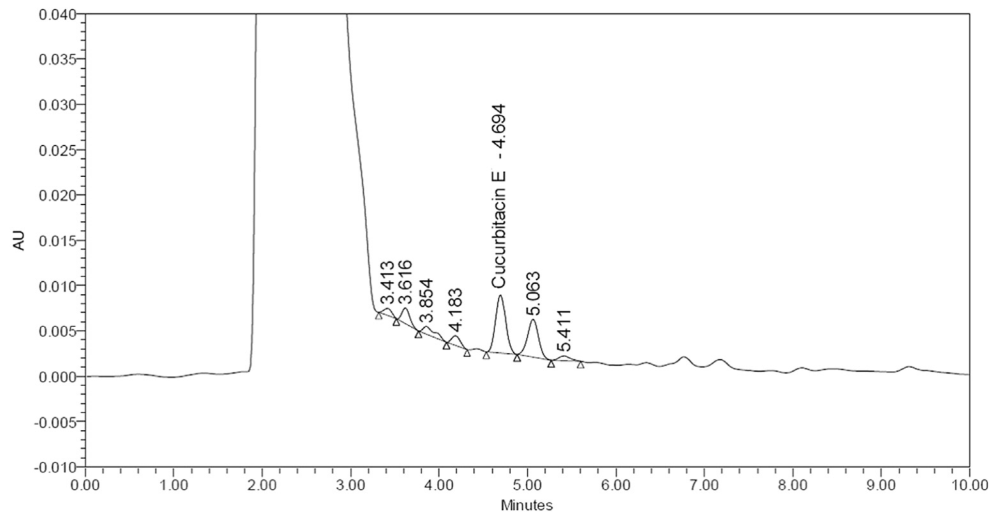

Fig. 6. RP-HPLC/UV chromatogram of *Momordica charantia* lyophilized extract.

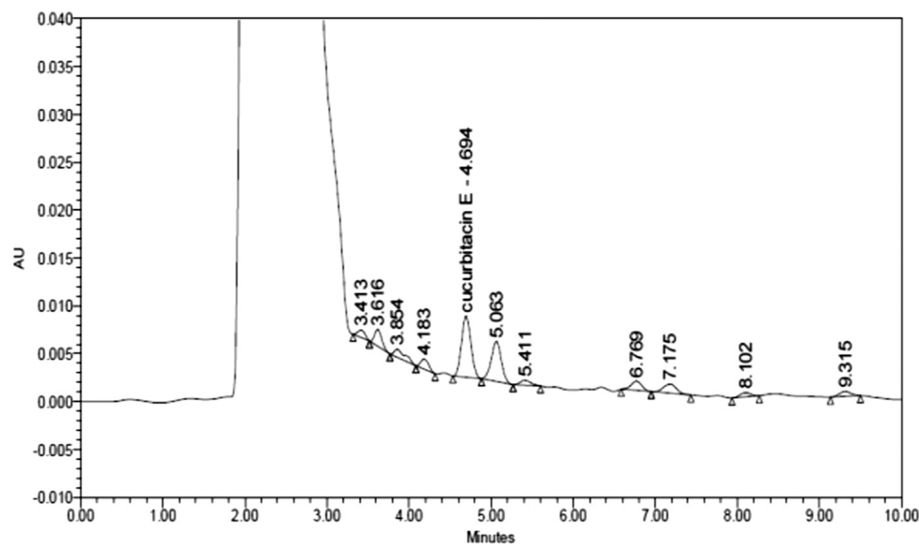

Fig. 7. RP-HPLC/UV chromatogram of *Coccinia grandis* lyophilized extract.

considered as it contains highest amount of cucurbitacin E. The precision of the method was assessed by injecting six replicates at three different concentrations, LQC (low-quality control), MQC (medium quality control) and HQC (high-quality control) for both standard and extract solutions to determine the repeatability of the method. The intra-day precision of the assay was determined by analyzing three concentrations in a day whereas the inter-day precision was carried over three successive days by analyzing the same concentrations. The robustness of the proposed method was carried out by varying different experimental conditions *viz.* flow rate, mobile phase composition, detection wavelength, column temperature and columns of the same configuration to check their influences on the retention time. Values were represented as % RSD in both cases. System suitability test was performed by using six replicates of test concentrations. A variation in the number of theoretical plates, capacity factor, and tailing factor was also calculated. Statistical analysis was performed using the Graph Pad Prism Version 5.0. The data has been represented as the mean ± % RSD.

3. Results

3.1. Extraction yield

The extracts were weighed and the percentage yields were calculated. The percentage yield (%) the aqueous extracts were found to be 5.21, 4.08, 7.25, 5.88, 3.83, 4.2% (w/w) for *L. siceraria, B. hispida, M. charantia, C. grandis, C. pepo* and *L. acutangula* respectively. The % yield was found the maximum for *M. charantia* whereas *C. pepo* was found to be lowest.

3.2. Method validation results

In RP-HPLC, the linearity range of the response was found to be 1–100 μg/mL. The correlation coefficient was found from the calibration curve as > 0.99, which confirms that the data is closer to the line of best fit. The regression equation was found to be Y = 19111X-54747 (Fig. 2). The specificity of the proposed method confirmed no interference among the peak of standard and test samples. The

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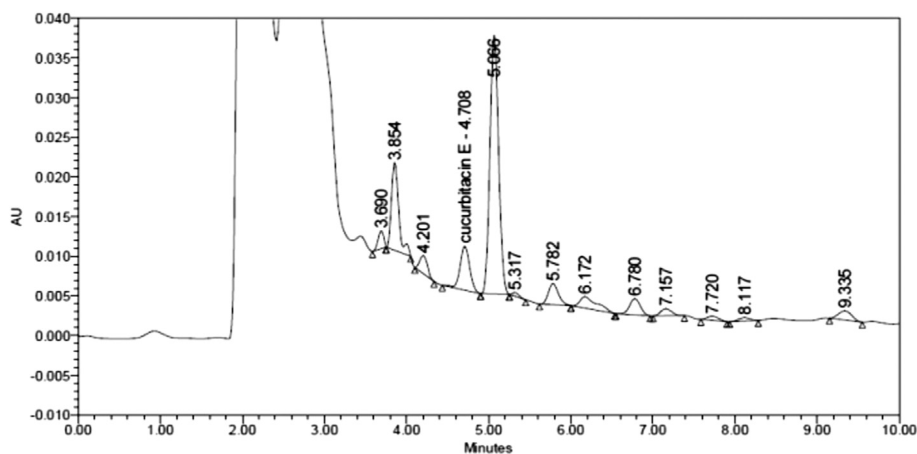

Fig. 8. RP-HPLC/UV chromatogram of *Cucurbita pepo* lyophilized extract.

limits of detection (LOD) and limit of quantification (LOQ) were estimated to be 3.45 and 8.82 μg/mL respectively, which reflect the high sensitivity of the method. The % recovery value (95.35–97.23%) indicated the good accuracy of the method (Table 2). The % RSD of intra-day and inter-day precision was reported to be <2% for in cases of both peak area (response) and retention time, which confirms high repeatability of the method (Table 3). The robustness of the experimental method was found to be in the range <2%. The number of theoretical plates, capacity factor and tailing factor were found to be 4092 (desirable > 2000), 6.72 (desirable 2–10), 1.35 (desirable < 1.5), respectively, from the mean of six determinations of test concentration.

3.3. Estimation of cucurbitacin E by RP-HPLC

The content of cucurbitacin E in the lyophilized extract was determined using the calibration curve by plotting the mean peak area (y-axis) against the concentrations (x-axis). The study confirmed that *C. pepo* contains the highest amount of cucurbitacin E (0.0663% w/w) whereas the lowest amount of was reported in *L. siceraria* as 0.0356% (w/w). The content of cucurbitacin E in the other species varied within this range. The content of cucurbitacin E was presented in Table 1. The chromatogram of standard cucurbitacin E has been shown in Fig. 3. RP-HPLC chromatograms of the six species have been shown as *L. siceraria* (Fig. 4), *B. hispida* (Fig. 5), *M. charantia* (Fig. 6), *C. grandis* (Fig. 7), *C. pepo* (Fig. 8) and *L. acutangula* (Fig. 9).

4. Discussion

The aqueous extract of Cucurbitaceae fruits is widely used by practitioners of Ayurveda in India and also in other systems of Indian medicine. The juice and powder of the fruits are widely marketed as a dietary supplement. In India, the fresh juice of *L. siceraria* and *M. charantia* are consumed for their anti-obesity and antidiabetic properties [27,28]. Although cucurbitacin class of compounds (specifically Cucurbitacin D & E) possesses immense pharmacological potential *viz.* antitumor, hepatoprotective, anti-inflammatory etc. [29] (Miro, 2015), their unpredictable occurrence may lead to colitis with bloody diarrhea, severe abdominal cramps, vomiting, and hypotension [30]. In October 2010, Indian Council of Medical Research (ICMR), Ministry of Health & Family Welfare, Government of India conducted a pilot study on the adverse effects of *L. siceraria* after consumption of its juice. The patients were reported to have suffered from diarrhea, vomiting, elevated levels of liver enzymes and excessive ulceration in distal oesophagus [31]. There were several other cases of cucurbit toxicity which have been reported in India as well as in other countries like

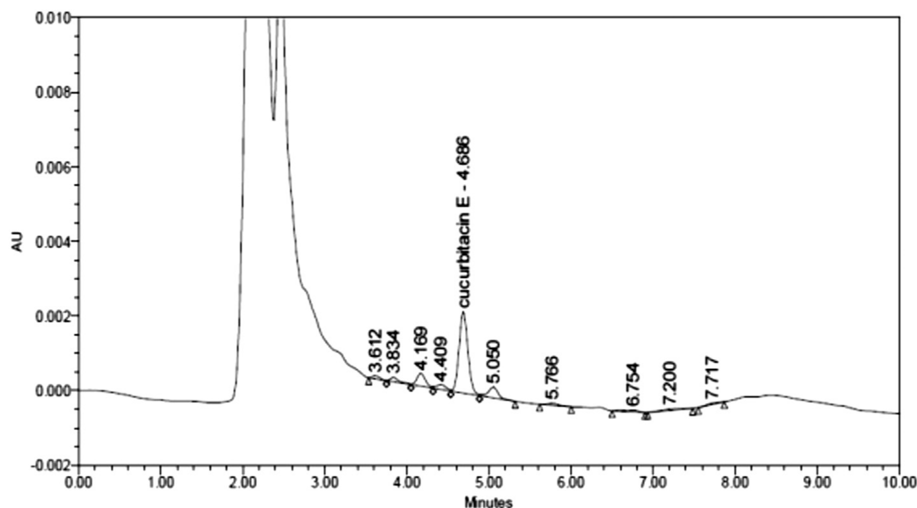

Fig. 9. RP-HPLC/UV chromatogram of *Luffa acutangula* lyophilized extract.

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Australia, Alabama and California [32]. The probable cause of the toxicity lies in is the presence of the active principle, cucurbitacin. It was further observed that the toxicity of cucurbitacin was closely related to their chemical structure, specifically due to the presence of a double bond at C-23 and acetyl group at C-25 in their structure [33]. Reports have been found that cucurbitacin and their glycoside exerts potential cytotoxicity in several cell lines. In specific, cytotoxic behavior of cucurbitacin E was reported at lower IC_{50} value, when studied in human hepatocellular carcinoma HepG2 cell line [34]. The *in-vivo* toxicity study reported the LD_{50} values of cucurbitacin E at a dose of 2–12.5 mg/kg body weight in mice after oral administration of cucurbitacin derivatives [33]. The toxic effects of cucurbitacin are rendered by increasing the blood pressure and subsequently accumulates fluid in thoracic and abdominal cavities by enhancing capillary permeability in human volunteers [34]. It has been reported that maximum, tolerable limit of cucurbitacin should be restricted for human consumption, although the content of cucurbitacin may vary due to mutations, lack of irrigation and environmental factors [30]. As a large population of India consumes fruit juices of Cucurbitaceae family regularly, the standardization of these fruits with cucurbitacin E as phytomarker is very necessary. This may help in preventing toxicity associated with the Cucurbitaceae food plants at a large.

5. Conclusion

The RP-HPLC study confirmed the highest cucurbitacin E content in *C. pepo* whereas the lowest amount of was reported in *L. siceraria* fruit. The developed RP-HPLC method is robust, accurate, precise and reproducible for quantification of cucurbitacin E with a narrow linear range. This validated method can be beneficial for the nutraceutical industry in establishing effective quality control of these fruits for safe human consumption.

Declarations of interest

None.

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