CHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF *DREGEA VOLUBILIS* FLOWER AND ITS APPLICATION IN FORMULATION DESIGNING

Thesis submitted by

Bhaskar Das

Doctor of Philosophy (Pharmacy)

Department of Pharmaceutical Technology

Faculty Council of Engineering and Technology

Jadavpur University

Kolkata, India

2019

JADAVPUR UNIVERSITY

KOLKATA-700032, INDIA

INDEX NO. 242/16/Ph

1. Title of the thesis:

CHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF *DREGEA VOLUBILIS* FLOWER AND ITS APPLICATION IN FORMULATION DESIGNING.

2. Name, Designation & Institution of the Supervisor:

Dr. Amalesh Samanta, Professor, Division of Microbiology & Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India.

3. List of Publication:

- Mitra D, Jena AK, De A, Das M, **Das B**, Samanta A. Prebiotic potential of gum odina and its impact on gut ecology: *in vitro* and *in vivo* assessments. ***Food & Function.*** 2016; 7(7): 3064-72.

- Mitra D, Basu A, **Das B**, Jena AK, De A, Das M, Bhattacharya S, Samanta A. Gum odina: an emerging gut modulating approach in colorectal cancer prevention. ***RSC Advances***. 2017; 7(46): 29129-42.

- **Das B**, De A, Das M, Das S, Samanta A. A new exploration of *Dregea volubilis* flowers: Focusing on antioxidant and antidiabetic properties. ***South African Journal of Botany***. 2017; 109: 16-24.

- Bera S, **Das B**, De A, Samanta A. Preventive effect of *Cycus revoluta* in 1, 2-dimethylhydrazine-induced colon cancer in wistar rat model. ***Asian Journal of Pharmaceutical and Clinical Research***. 2018; 11(5): 120-3.

- Podder S, Chanda D, Mukhopadhyay AK, De A, **Das B**, Samanta A, Hardy JG, Ghosh CK. Effect of Morphology and Concentration on Crossover between Antioxidant and Pro-oxidant Activity of MgO Nanostructures. ***Inorganic chemistry***. 2018; 57(20): 12727-39.

- Bera S, **Das B**, De A, Barua A, Das S, De B, Samanta A. Metabolite profiling and in-vitro colon cancer protective activity of *Cycas revoluta* cone extract. ***Natural Product Research***. 2018; 1-5.

- Maitra S, De A, **Das B**, Roy SN, Chakraborty R, Samanta A, Bhattacharya S. Seasonal Variation of Phyto-Constituents of Tea Leaves Affects Antiproliferative Potential. ***Journal of the American College of Nutrition***. 2019; 1-9.

- Das M, **Das B**, De A, Banerjee S, Samanta A. Antimicrobial investigation and binding mode analysis of some newly synthesized 4-amino-5((aryl substituted)-4H-1,2,4-triazole-3-yl)-thio linked hydroxamic acid derivatives. ***Asian Journal of Pharmaceutical and Clinical Research***. 2019; 12(3): 469-79.

- **Das B**, De A, Das P, Nanda A, Samanta A. Pharmacognostic studies on flowers of *Dregea volubilis*: Evaluation for authentication and standardization. ***Asian Journal of Pharmaceutical and Clinical Research***. 2019; 12(5): 79-89.

- Das M, **Das B**, Samanta A. Antioxidant and anticancer activity of synthesized 4-amino-5-((aryl substituted)-4H-1,2,4-triazole-3-yl) thio-linked hydroxamic acid derivatives. ***Journal of Pharmacy and Pharmacology***. 2019; 71: 1400-11.

4. List of Patents:

Nil.

5. List of Presentations in National/International:

- Bera S, **Das B**, De A, Samanta A. Antioxidant Activities of *Cycas revoluta* (Cycadaceae) Extract: Modulatory Effect on Colon Cancer. Poster presentation in the **International Conference on Current Scenario in Pharmaceutical Technology & Healthcare: A Move Towards Patient-Centric Approach** from 9^{th} -10^{th} March, 2018 at Science City Auditorium, Kolkata & NSHM Knowledge Campus, Kolkata.

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"CHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF *DREGEA VOLUBILIS* FLOWER AND ITS APPLICATION IN FORMULATION DESIGNING"** submitted by **Shri Bhaskar Das**, who got his name registered on **29.03.2016** for the award of Ph. D. (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of **Prof. (Dr.) Amalesh Samanta** and that neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

Signature of the Supervisor
and date with Office Seal

Prof. (Dr.) Amalesh Samanta
Division of Microbiology & Biotechnology
Department of Pharmaceutical Technology
Jadavpur University
Kolkata-700032

DECLARATION

This research work entitled **"CHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF *DREGEA VOLUBILIS* FLOWER AND ITS APPLICATION IN FORMULATION DESIGNING"** has been carried out by me under the supervision of **Prof. (Dr.) Amalesh Samanta** in the Department of Pharmaceutical Technology, Faculty Council of Engineering and Technology, Jadavpur University, Kolkata-700032, India. I hereby declare that the work is original and has not been submitted so far, in part or full for any degree/diploma courses of any University.

Date: Full signature of the candidate

Place: (BHASKAR DAS)

যাদবপুর বিশ্ববিদ্যালয়

DR. B.KARMAKAR
PRINCIPAL SECRETARY
FACULTY OF ENGINEERING & TECHNOLOGY

*JADAVPUR UNIVERSITY
KOLKATA-700 032, INDIA

Ref. No. D-7/E/202/16
Date: March 16, 2016
21

To
Bhaskar Das,
C/o Bimal Kumar Ghosh,
Jaynagar Banerjee Para,
305, N. S. Road,
P.O. – Jaynagar Mazilpur,
P.S. – Jaynagar, Dist. – South 24 Parganas,
PIN – 743 337

Sub : Ph.D. (Pharmacy) Registration.

With reference to your application for registration to Ph.D. (**Pharmacy**) degree, you are hereby informed that you have been permitted to register your name on payment of requisite Ph.D.Programme fee of **Rs.22000/-** (Rupees twenty two thousand only) payable in three semi annual installments (8,000/- + 8,000/- + 6,000/-). You may be noted that the date of payment of registration fee will be considered as date of registration.

Registration shall remain valid for five years from the date of registration. The period of validity may be extended on sufficient grounds, if the Doctorate Committee satisfied. But such extension shall not exceed two years. Such application for extension duly forwarded by the supervisor/s shall have to be submitted before expiry of the validity of registration.

The approved title of your thesis is: **"CHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF *DREGEA VOLUBILIS* FLOWER AND ITS APPLICATION IN FORMULATION DESIGNING".**

If the registration fee is not paid within a month from date of issue of this letter, your application may be treated as cancelled. Your research will be governed by this New Ph.D.Rules and Regulation **Please visit our University website www.jadavpur.edu for New Ph.D. Rules and Regulations**. For any further clarification you may contact the undersigned.

You are directed to complete **2 (Two)** course work within a maximum period of two years from the date of registration as per Revised New Ph. D. rules & regulation.

You are informing to bring the following documents at the time of deposition of Ph.D. programme fee.

1. Original Migration Certificate.

(Dr. B. Karmakar)
Principal Secretary, FET

Copy : Secretary, FET (vide Res. No. B (11) of D.C meeting. Dt. 17.12.15
Records – 2
Supervisor/s – Prof. Amalesh Samanta,
Pharm. Tech. Department, J.U.

Sm

Phone No: +91(033) 2668 3235
GOVERNMENT OF INDIA
MINISTRY OF ENVIRONMENT & FORESTS
BOTANICAL SURVEY OF INDIA
OFFICE OF THE SCIENTIST -' D'
CENTRAL NATIONAL HERBARIUM
P O - BOTANIC GARDEN
HOWRAH- 711 103

Fax: +91(033) 2668 6226
भारत सरकार
पर्यावरण एवं वन मंत्रालय
भारतीय वनस्पति सर्वेक्षण
वैज्ञानिक 'डी' का कार्यालय
केन्द्रीय राष्ट्रीय पादपालय
पत्रालय-बोटेनिक गार्डेन
हावड़ा -711103

No.: CNH/Tech.II/2014/63/154

Dated: 1-08-2014

To,
Mr. Bhaskar Das
Division of Microbiology
Department of Pharmaceutical Technology
Jadavpur University
Kolkata 700032
West Bengal

Sub.: Identification of 1 plant specimen – reg.

Dear Mr. Das,

Please refer to your letter dated 5th July 2014 along with a plant specimen for identification. The specimen has been identified by the concerned expert as:

Sl. No.	Specimen No.	Scientific Name	Family
1.	BD-1	**Dregea volubilis** (L.f.) Benth. ex Hook.f.	Apocynaceae*

You are hereby requested to remit a sum **of** Rs. **50/-** (Rupees Fifty only) by cash or Demand Draft drawn in favour of **'Accounts Officer, P.A.O. (BSI & ZSI), Kolkata'** towards identification charges.

Your specimen is returned herewith.

With regards,

Yours sincerely

1-8-2014

(V.P. PRASAD)
Scientist –'D'

N.B. '*' Accorging to APG-III system of classification.

Acknowledgements

No research work is ever the outcome of individual efforts and this work is also no exception. I wish to acknowledge the assistance I have received from various people and organizations during the course of the research work.

To begin with, I must express my deepest gratitude and indebtedness to Prof. (Dr.) Amalesh Samanta, Professor, Division of Microbiology & Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, for tremendous inspiration, encouragement, valuable guidance throughout the research work and shaping me up to this point. His unremitting advice, affection, help and vision are the guiding factors responsible for successful completion of the thesis.

I would like to thank to The Head of the Department, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, for his cooperation in completion of this research work.

I am also grateful to Mr. Swapan Kumar Mondal, Director; Mr. Amal Kumar Dhar, Ex-Director; and Dr. Tapan Kumar Das, Ex-Assistant Director of State Drugs Control & Research Laboratory, 2 Convent Lane, Kolkata, for their valuable support and cooperation in completion of the research work.

I am grateful to Dr. Chandan Kumar Ghosh, Assistant Professor, School of Materials Science and Nanotechnology, Jadavpur University, Kolkata, for his invaluable support, encouragement and suggestion.

I am also thankful to Dr. Bratati De, Professor, Phytochemistry and Pharmacognosy Laboratory, Department of Botany, University of Calcutta, Kolkata, India, for providing suggestion and encouragement.

I would like to express my special thanks to my all lab mates: Dr. Anurup Mandal, Dr. Partha Sarathi Roy, Dr. Durbadal Ojha, Dr. Sayantan Maitra, Dr. Aditya Kumar Jena, Dr. Debmalya Mitra, Dr. Samit Bera, Mr. Arnab De, Mrs. Mousumi Das, Mrs. Piu Das, Miss Shilpa Das, Miss Piu Das, Miss Ahana Hazra, Miss Paloma Patra, Mr. Pranab Naskar, Mr. Partho Pratim Biswas, Miss Mousumi Tudu and all others who have extended their cooperation to carry out this work successfully.

I wish to express my thanks to my friends Mr. Saptarshi Bose, Mr. Soumik Podder, Mr. Amalesh Nanda, Mr. Pradipta Sarkar, Mr. Sandip Dolui, Mr. Avijit Das, Mr. Debashis Das, and Dr. Subrata Pandit for their encouragement and kind support.

I owe my deepest sense of heartfelt gratitude beyond any limit to my parents & wife for their blessing, moral support, constant encouragement and giving me a chance to fulfill my ambition.

Jadavpur University, 2019 Bhaskar Das

Preface

Medicinal plant materials play a vital role in basic healthcare needs in many developing countries wherein 80 % of people depend on traditional medicine due to its affordability and cultural acceptability. The ethnic communities and tribes in various parts of the world acquired and preserved different versions of Indigenous or Traditional Knowledge regarding natural resources for healing and treating various ailments. Herbal medicines have been widely used as effective remedies in the management of multiple health problems for centuries and there is still large number of plants or plant parts which are scientifically unexplored. Proper identification and authentication of medicinal plant or crude drug is the prerequisite step before conducting investigation and research on traditional medicines to guarantee its quality, safety and effectiveness. Nanobiotechnology is the intersection of nanotechnology and biotechnology wherein the use of plant materials has been considered as a green route and a reliable method for the biofabrication of nanoparticles due to its environmental friendly nature.

The subject matter of the thesis has been divided into several chapters covering pharmacognostic studies on *Dregea volubilis* flowers for its standardization, and authentication; antioxidant, and antidiabetic studies on *Dregea volubilis* flower in order to explore the medicinal importance of the plant which is consumed as a seasonal vegetable; and application of *Dregea volubilis* flower in the biofabrication of silver nanoparticles.

Dedicated to my family

Contents

List of Abbreviations

AA	:	Ascorbic acid
AgNPs	:	Silver nanoparticles
DNS	:	3, 5-dinitrosalicylic acid
DPPH	:	1,1-diphenyl-2-picryl-hydrazyl
DVHA	:	Hydroalcoholic flower extract of *D. volubilis*
EDTA	:	Ethylenediaminetetraacetic acid
EDX	:	Energy-dispersive X-ray
$FeCl_3.6H_2O$	:	Ferric chloride
FESEM	:	Field emission scanning electron microscopy
$FeSO_4.7H_2O$	:	Ferrous sulphate
FRAP	:	Ferric reducing antioxidant power
FTIR	:	Fourier transform infrared spectroscopy
FWHM	:	Full width at half maxima
GAE	:	Gallic acid equivalent
HCl	:	Hydrochloric acid
H_2O_2	:	Hydrogen peroxide
HRTEM	:	High resolution transmission electron microscopy
IC_{50}	:	Half maximal inhibitory concentration
LOD	:	Loss on drying
MHB	:	Mueller-Hinton broth
MIC	:	Minimum inhibitory concentration
NBT	:	Nitro blue tetrazolium
NED	:	N-(1-naphthyl)-ethylenediamine dihydrochloride

PBS	:	Phosphate buffer saline
PNPG	:	*p*-nitrophenyl-α-D-glucopyranoside
QE	:	Quercetin equivalent
ROS	:	Reactive oxygen species
SAED	:	Selected area electron diffraction
SEM	:	Standard error of mean
TAC	:	Total antioxidant capacity
TBA	:	Thiobarbituric acid
TCA	:	Trichloroacetic acid
TFC	:	Total flavonoid content
TPC	:	Total phenolic content
TPTZ	:	2,4,6 tripyridyl-S-triazine
T. S	:	Transverse sections
UFLC	:	Ultra fast liquid chromatography
UHPLC	:	Ultra high performance liquid chromatography
UV	:	Ultraviolet
XRD	:	X-ray diffraction
ZOI	:	Zone of inhibition

List of Tables

List of Figures

Synopsis

Chemical characterization and biological evaluation of *Dregea volubilis* flower and its application in formulation designing.

1. Objective: Plants have diverse structures with valuable medicinal properties that inspired scientists for millennia. Herbs used as therapeutic agent is as old as human civilization and has developed gradually along with it. Majority of people in the world still depend on their indigenous system of medicine and use herbal remedies for maintenance of health, prevention of various types of ailments, and amelioration of physical and mental illness. Medicinal plants contain complex mixtures of bioactive secondary metabolites which are responsible for imparting the biological activities of the plant material. There is a growing demand of medicinal plant materials in herbal industry and standardization of raw materials is very important to confirm its quality and to prevent deliberate adulteration and substitution. India is one of the richest nations in the world in terms of exhibiting a wide range of plant diversity and many plants are consumed as food or in the form of traditional medicine. Many of the plants are scientifically unexplored. There has been a correlation between lower risk of diseases like cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases with consumptions of natural products like vegetables, fruits and herbs. Thus, scientific studies on unexplored edible plants or plant parts give us valuable information for the development of nutraceuticals. Metallic nanoparticles have multifunctional applications particularly in biomedical science owing to smaller particle size, various shapes and increased surface area. The nanoparticles are prepared by employing physical, chemical, and biological methods. Biofabrication of nanoparticles using plant materials have enhanced stability, biocompatibility, and reduced toxicity. In Ayurveda, *Dregea volubilis* is used to treat various ailments like inflammation, piles, leucoderma,

asthma, tumours, application to boils, rat bite, urinary discharge etc. *D. volubilis* flowers are consumed as a seasonal vegetable in West Bengal in the month of April to June.

The present study is aimed to lay down Pharmacopoeial standards for *D. volubilis* flowers which will serve as a reference standard for the quality control of the plant material and to investigate the total phenolic content, total flavonoid content, antioxidant effects, α-amylase and α-glucosidase inhibitory activities of flowers of *D. volubilis* to establish its therapeutic and nutritional potentialities. The present work is also aimed to biosynthesize and characterizes silver nanoparticles using aqueous extract of *D. volubilis* flowers along with investigation of its antioxidant, antidiabetic, and antibacterial properties.

2. Experimental design and results

Chapter 1: *D. volubilis* flower was investigated in detail for organoleptic, histological, quantitative standards, physicochemical, spectroscopic and chromatographic characteristics. The total ash, acid insoluble ash, water soluble ash, loss on drying, water and alcohol soluble extractive values were found to be 11.767 ± 0.130 % (w/w), 1.287 ± 0.106 % (w/w), 9.140 ± 0.344 % (w/w), 14.110 ± 0.061 % (w/w), 21.600 ± 0.133 % (w/v) and 9.603 ± 0.104 % (w/v), respectively. Different extracts showed the presence of carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, flavonoids, tannins, and phenolics. Chromatographic study showed that the flowers of *D. volubilis* contained rhamnose (103.229 ± 4.994 µg/gm), fructose (738.670 ± 25.714 µg/gm), glucose (285.532 ± 24.465 µg/gm) and maltose (49.082 ± 5.206 µg/gm).

Chapter 2: The hydroalcoholic flower extract of *D. volubilis* was investigated for antioxidant and antidiabetic activities *in vitro*. The total phenolic content (TPC) and total flavonoid content (TFC) of the extract were found to be 39.82 ± 1.22 mg gallic

acid equivalent (GAE)/g and 27.50 ± 0.87 mg quercetin equivalent (QE)/g, respectively. The extract showed notable antioxidant activity for scavenging DPPH radical (IC_{50}, 237.86 ± 1.05 µg/ml), hydroxyl radical (IC_{50}, 170.67 ± 0.98 µg/ml), superoxide radical (IC_{50}, 219.07 ± 1.25 µg/ml), nitric oxide radical (IC_{50}, 196.38 ± 1.49 µg/ml), and ferric reducing antioxidant power (176.47 ± 3.18 µmol Fe^{2+}/g), total antioxidant capacity (39.68 ± 1.62 mg GAE/g). The extract showed remarkable inhibitory effects on α-glucosidase (IC_{50}, 3780.09 ± 21.19 µg/ml) and α-amylase (IC_{50}, 360.68 ± 1.26 µg/ml). The characterization of the extract was studied by Fourier transform infrared spectroscopy (FTIR) and Ultra High Performance Liquid Chromatography (UHPLC) analysis. The liquid chromatography study revealed that the extract contained gallic acid (412.36 ± 2.29 µg/g), ferulic acid (162.72 ± 0.89 µg/g), rutin (386.25 ± 2.00 µg/g), ellagic acid (208.8 ± 2.00 µg/g), quercetin (306.85 ± 2.24 µg/g) and cinnamic acid (213.71 ± 2.14 µg/g), respectively.

Chapter 3: New and innovative experimental processes are of potential interest for the synthesis of silver nanoparticles (AgNPs) which are used in the field of science, technology and medicine. Bio-inspired methodologies for synthesis of nanoparticles are receiving significant attention in nanobiotechnology. Plants are an attractive resource for the biosynthesis of the AgNPs due to the presence of a wide verity of secondary metabolites which have the potentials to act as reducing and capping agents. The present work was conducted to biofabricate the AgNPs in single step employing aqueous extract of *Dregea volubilis* flowers and to investigate its antioxidant, antidiabetic and antibacterial activities. The biosynthesized AgNPs were purified and characterized by UV-Visible spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), field emission scanning electron microscopy (FESEM), energy-dispersive X-ray (EDX) spectrometry, high resolution transmission electron microscopy

(HRTEM) and zeta potential study which revealed their purity , small size range and stability. The surface plasmon resonance was found at~ 420 nm which confirmed the formation of AgNPs. The FTIR study confirmed that the extract contained polyphenols, protein and saccharides which might play a key role in reducing, capping and subsequent formation of the AgNPs. The XRD and the selected area electron diffraction (SAED) patterns proved the crystalline nature of the biogenic AgNPs with face centered cubic (fcc) geometry. FESEM and HRTEM images revealed that the AgNPs were spherical in nature and uniformly distributed. The total phenolic content (TPC) and total flavonoids content (TFC) of the green synthesized AgNPs were found to be 42.33 ± 1.00 mg gallic acid equivalent (GAE)/g and 117.59 ± 1.96 mg quercetin equivalent (QE)/g, respectively whereas the Ultra High Performance Liquid Chromatography (UHPLC) study confirmed the role of gallic acid, catechin, protocatechuic acid, para hydroxybenzoic acid, para coumaric acid, ferulic acid, rutin and cinnamic acid present in the aqueous extract of *D. volubilis* flowers as reducing and capping agents. The biofabricated AgNPs showed potential antioxidant activity for scavenging DPPH radical (IC_{50}, 40.45 ± 5.06 µg/ml), ABTS radical (IC_{50}, 78.49 ± 1.41 µg/ml), total antioxidant capacity (148.83 ± 2.99 mg GAE/g) along with remarkable inhibitory effects on α-amylase (IC_{50}, 10.62 ± 0.22 µg/ml) and α-glucosidase (IC_{50}, 6.49 ± 0.03 µg/ml). The biogenic AgNPs showed notable antibacterial activity against four different pathogenic bacterial strains of both Gram-negative like *Pseudomonas aeruginosa, Escherichia coli* and Gram-positive like *Bacillus subtilis, Staphylococcus aureus*. The present work illustrated the potential use of *D. volubilis* flowers as a novel source for biosynthesis of AgNPs and the biological activity of the AgNPs could play an important application in the field of nanomedicine.

3. Conclusion:

Standardization of herbal drugs is an important topic of great concern. The present study of pharmacognostical evaluation on the flowers of *D. volubilis* has laid down standard parameters for proper identification, authentication and for distinguishing the material from its adulterants and substituents. The detailed study also set the parameters which can be utilized as a Pharmacopoeial reference for recognition of its distinctiveness, genuineness and quality. The study also contributes to the documentation of the nutritional composition on the flowers of *D. volubilis* which are consumed as vegetable.

The hydroalcoholic extract of *D. volubilis* flowers shows promising antioxidant potential towards different systems *in vitro* and α-glucosidase and α-amylase inhibiting activities *in vitro*. The phytochemical profiling of the edible vegetable provides data relating to phenolic characterization of the extract from the view of being a source of bioactive compounds with health beneficiary effects. The flower of the plant is therefore recognized as powerful antioxidant as well as carbohydrate hydrolyzing enzymes inhibitor which is helpful in the field of nutrition and medicine. The biological activities might be due to the presence of the phenolic compounds in the flower of the plant which were identified via UHPLC. *D. volubilis* flower can be considered as a nutraceutical because of their health beneficial properties which deserve to be further researched and developed in food and pharmaceutical industries.

A facile, quick, economically viable, readily scalable and non-pathogenic production of the AgNPs was depicted in our study, thus this method will be an alternative to whole cell, physical and/or chemical methods of AgNPs synthesis. We have demonstrated a green chemistry approach for the synthesis of AgNPs using *D. volubilis* flowers as a source of plant material. The present study demonstrated the synthesis, characterization and evaluation of antioxidant, antidiabetic and antibacterial activity of the biogenic Ag

NPs prepared using *D. volubilis* flowers extract. The biofabricated AgNPs possessed the added advantage of incorporation of active biomolecules as capping agent which made it as a potent free radical scavengers, antidiabetic and antibacterial agent. The phytochemical synthesis of the AgNPs using aqueous extract of *D. volubilis* flowers can play an important role in the field of nanotechnology and nanomedicine as it can offer alternative therapeutic options which are safe, free of side effects and effective. The green synthesized AgNPs are more acceptable for medicinal applications due to the toxic chemical-free nature. The study highlights the fact that the AgNPs can be formed and stabilized without the addition of synthetic stabilizing agents from the outside. The surface reactivity of the functionalized AgNPs by the capping agents facilitates it as potential candidate for various pharmaceutical, biomedical and environmental applications. Further future avenues can be directed towards formulating new capping agents for the synthesis of AgNPs with better biological activities.

Introduction

The use of plants, various parts of plants, plant extracts, and isolated bioactive phytomolecules has been in practice from time immemorial in healthcare system for the prevention and treatment of various ailments. It is calculated that about 25% of the drugs prescribed worldwide are obtained from plants and the drugs derived exclusively from plant sources contribute to about 11 % of WHO's essential medicines list of 252 drugs (Rates, 2001). Nearly 80% of rural population uses medicinal herbs or indigenous systems of medicine in India. The Indian herbal industry utilizes about 960 plant species of which 178 plant species are of great demand exceeding100 metric tonnes a year (Sahoo et al., 2010). Herbal marketing sector is growing significantly of which about 70% of export consists largely of raw materials and 30% of export consists of finished products including herbal extracts. Herbal drugs are of three types based on the nature of active ingredients (Sahoo and Manchikanti, 2013). The first category comprises of drugs which are used in crude form and the second category includes the active constituents isolated from the plant extracts whereas the third category of herbal drugs is those for which data on acute and chronic toxicity studies in animals are available (Iwu et al., 1999). Herbal medicines are gaining importance for the management of various diseases which require lifelong treatment due to their safer pharmacological profile and lesser side effects.

Herbal medicines contain more than one bioactive molecules and the active principle is frequently unknown which make them different compared to the synthetic drugs. The phytochemical profiles of medicinal plants depend upon the conditions of cultivation, manufacturing, marketing and distribution apart from the physiological, genetic and environmental variables (photoperiod, conditions of soil, climate, moisture and availability of nutrients). The content of secondary metabolites in plant depends upon

time of harvesting, storage, drying, extraction and processing for final packaging. A comprehensive understanding of biological, chemical, genetic, and agronomic aspects of plants are required for the development of herbal medicines and the chemical consistency of the herbal medicines at all stages of manufacturing processes such as extraction, stability, shelf-life and purity is of paramount importance to ensure medicinal efficacy and consumer safety (Sahoo et al., 2010). Various types of markers such as taxonomic, chemical, genomic and proteomics are helpful in the identification of components of herbal drugs. Such methods include morphological identification, anatomical identification, chemical analysis such as TLC, HPLC, protein analysis and the use of molecular markers. The chemical markers are chemically defined molecules or groups of molecules of herbal product which are of great interest for quality control purposes regardless whether they possess any health beneficiary activity. Chemical markers are grouped into analytical markers which deal with the phytomolecule or groups of phytomolecules that serve solely for analytical purposes, and active markers which are the bioactive molecule or groups of bioactive molecules that contribute to therapeutic activities. Secondary metabolites have been extensively used as markers in quality control and standardization of herbal drugs. The percentage of extractable matter with a solvent may be used as a quality control parameter where no active constituent or marker can be defined. Conventional quality control and botanical techniques are insufficient as the sole means for identifying, authenticating, evaluation for safety and efficacy due to the variations and inherent complexities of herbal drugs. A multi-technique approach is essential in proper identification and authentication of herbal drugs. The concept of 'Phytoequivalence' was developed in Germany wherein the chemical profile, such as chromatographic fingerprint, of the herbal drug is compared with the profile of a clinically proven reference product (Liang et al., 2004).

Dregea volubilis, commonly known as ‘‘Jukti’’ in Bengali, is a large twining shrub growing in India and Southeast Asia (Nandi et al., 2012). The plant bears green bisexual sweet scented flowers in drooping umbel with long glabrous branches (Jadhav et al., 2012) which are consumed as a seasonal vegetable in early summer in India (Hossain et al., 2013). *D. volubilis* is traditionally used to treat inflammation, boil, abscesses, dyspepsia, piles, asthma, tumors, leucoderma, anthelmintic, paralysis, rheumatism, tonsils, neck pain etc (Sreeramulu et al., 2013) and the flowers of the plant is known to contain volubiloside A, volubiloside B, volubiloside C, dregealol, volubilogenone, volubilol, drevogenin D, iso-drevogenin P, 17α-marsdenin, dregeanin, vicenin-2, vitexin, isovitexin, isoorientin, rutin, quercetin, luteolin, apigenin have been isolated from the flower of the plant (Sahu et al., 2002; Panda et al., 2003; Panda et al., 2006).

Oxidative stress has been defined as an imbalance between the generation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), hypochlorous acid (HOCl) and hydroperoxyl radical ($HO_2^{\bullet}$), or reactive nitrogen species (RNS), such as nitric oxide synthase (NOS)-catalyzed nitric oxide (NO) and peroxynitrite, and the cellular antioxidant capacity (Heshmati et al., 2018). RNS are of relatively low reactivity compared to the ROS. Free radicals are chemical species containing one or more unpaired electrons which are produced by enzymatic reactions (respiratory chain, phagocytosis, prostaglandin synthesis, and cytochrome P450 system) and non-enzymatic reactions (involving oxygen reaction with organic compounds or the exposition to ionizing radiations) taking place in peroxisomes and endoplasmic reticulum but mostly in the mitochondria (Pisoschi and Pop, 2015). Oxidative stress has been associated with various non-communicable diseases such as Alzheimer, Parkinson, Wilson's disease, stroke, diabetes, rheumatoid arthritis, multiple sclerosis, lateral amyotrophic sclerosis, thalassemia, hypertension, infertility, melanin

abnormalities, cataracts, asthma, allergies, heart failure, dyslipidaemia, angina and many more (Carocho et al., 2018). Human body has a set of endogenous antioxidants that act as the first line of defense to counter these diseases and this battery of enzymes together with other molecules are not enough to protect the health from the range of diseases, and hence, human body depends upon dietary antioxidants.

An antioxidant is a substance that significantly delays or prevents oxidation of oxidizable substrate when present at low concentrations compared to that substrate (Halliwell, 1990). Antioxidants play an important role in our internal defence system protecting against harmful metabolites and other substances. The antioxidants show their activity by following one of the seven mechanisms of action depending upon the type of oxidants: a) sequestration of free radicals from the medium; b) chelation of metallic ions; c) inhibition of free radical producing enzymes; d) activation of endogenous antioxidant enzymes; e) prevention of lipid peroxidation; f) prevention of DNA damage; g) prevention of protein modification and sugar destruction. Antioxidants present in diet have remarkable benefits and valuable properties that play an irreplaceable role in the maintenance of human health. Dietary antioxidants are found extensively in fruits and vegetables particularly brightly coloured varieties such as oranges, apricots, mangos, carrots, peppers/capsicum, and tomatoes and in green leafy vegetables which have been claimed to be magic bullets for the maintenance of healthy living and even for prolonging life span. The antioxidants fight free radicals within human body and the dietary polyphenols are one of the important groups of natural antioxidants present in human diets including fruits, vegetables, grains, tea and essential oils. Phenolic compounds can be classified into three main categories depending upon their structural characteristics such as phenolic acids, flavonoids and non-flavonoids (Zhang and Tsao, 2016).

Diabetes mellitus is a common and very prevalent chronic disease characterized by high blood glucose levels (hyperglycaemia) affecting the citizens of both developed and developing countries (Choudhury et al., 2018). The World Health Organization (WHO) describes diabetes mellitus as having a fasting plasma glucose value ≥ 7.0 mM/L (126 mg/dl) or being on medication for raised blood glucose. Diabetes is broadly categorized into two types: Type I diabetes mellitus, previously known as insulin dependent, or juvenile onset diabetes, wherein there is a deficiency of insulin production due to damage of β-cells of the pancreas leading to a decreased insulin supply to the blood circulation; and Type II diabetes mellitus, previously known as non-insulin dependent or adult-onset diabetes, which accounts for 90% of all diabetes cases, and results from the body's ineffective use of insulin. There are other groups of diabetes which can be categorized into gestational diabetes and rarer forms of genetic or acquired diabetes (Giovannini et al., 2016). It is predicted that diabetes mellitus will be the seventh leading cause of death worldwide by 2030 whereas the number of diabetes cases is supposed to increase to 592 million worldwide by 2035. Diabetes mellitus can cause acute fatal complications including diabetic ketoacidosis, coma and vascular complications, if the disease left untreated. The other complications of diabetes include retinopathy, neuropathy, dementia, sexual dysfunction, depression, and lower-limb amputations (Forbes and Cooper, 2013). There are different categories of antidiabetic medications available in the market which comprises insulin analogues, sulphonylureas, biguanides, dipeptidyl peptidase-4 inhibitors, thiazolidiones, α-amylase inhibitors, α-glucosidase inhibitors, etc., with different mechanism of actions of counteracting the increased glucose level. The long term treatment and side effect of the hypoglycaemic medications necessitates the discovery for more efficacious, decreased side effects and affordable agents for the treatment of diabetic complications. The use of medicinal

plants in the management of various ailments is one of the ancient traditions and the use of herbal medicines for the prevention of diabetes is still continued in the modern society. There are large numbers of drugs in the market which are produced commercially from medicinal plants. Many medicinal plants have exhibited antidiabetic activity by different mechanism of actions like regulating insulin secretion, insulin sensitivity to the cells, glucose abruption, etc. in order to improve the glycaemic control of the patients. The vast interest in the ethnopharmaceutical field to control diabetes mellitus compel establishment of validated testing protocols for the evaluation of the quantity and quality of active pharmaceuticals present in the final products, which will be tested in human subjects via proper clinical trials, confirmed and certified by the concerned regulatory authorities of the country to raise confidence of the consumers for the safety and efficacy of the herbal formulations.

Nanotechnology has been emerged as an important interdisciplinary approach in biochemical applications with a focus on the synthesis of nanoparticles with the improved biomedical applications. The metal nanoparticles have gained considerable scientific interest compared to all other nanoparticles owing to their exceptional magnetic, optical and electronic properties. Silver nanoparticles (AgNPs) has gained more scientific applications in the various fields of science and technology such as catalysis, optoelectronics, photonics, pharmaceuticals, antimicrobial products, electronics, sensing, and therapeutics (Maddinedi et al., 2017). The AgNPs are also being used in the consumer products such as food packaging, coatings, clothes, and bandages due to their antibacterial, antifungal activity, and low cost of production compared to other metal nanoparticles (Benn and Westerhoff, 2008; Gensel et al., 2012). The synthesis of nanoparticles involves either a “top down” approach or a “bottom up” approach (Meyers et al., 2005). Top down production method for the

synthesis of nanoparticles involves size reduction of a suitable starting material through various physical and chemical treatments leading to the production of nanoparticles with imperfections in the surface structure which is a major limitation because the physicochemical properties of the nanoparticles are highly dependent on the surface structure (Thakkar et al., 2010). Bottom up synthesis method involves chemical and biological methods wherein nanoparticles are produced from smaller entities by joining of atoms, molecules, and smaller particles (Mukherjee et al., 2001). The AgNPs can be produced through various physicochemical methods and the common synthetic approaches for the synthesis of AgNPs use hazardous chemicals which are harmful to the ecosystem. The eco-friendly methods which utilize several green reagents for the biosynthesis of AgNPs are of major interest to the researchers for the large scale production.

The present work have been designed to evaluate the *Dregea volubilis* flower pharmacognostically, chemically, biologically, and its ability to biosynthesize AgNPs. The whole study is divided into following chapters:

Chapter I:

Pharmacognostic studies on *Dregea volubilis* flower.

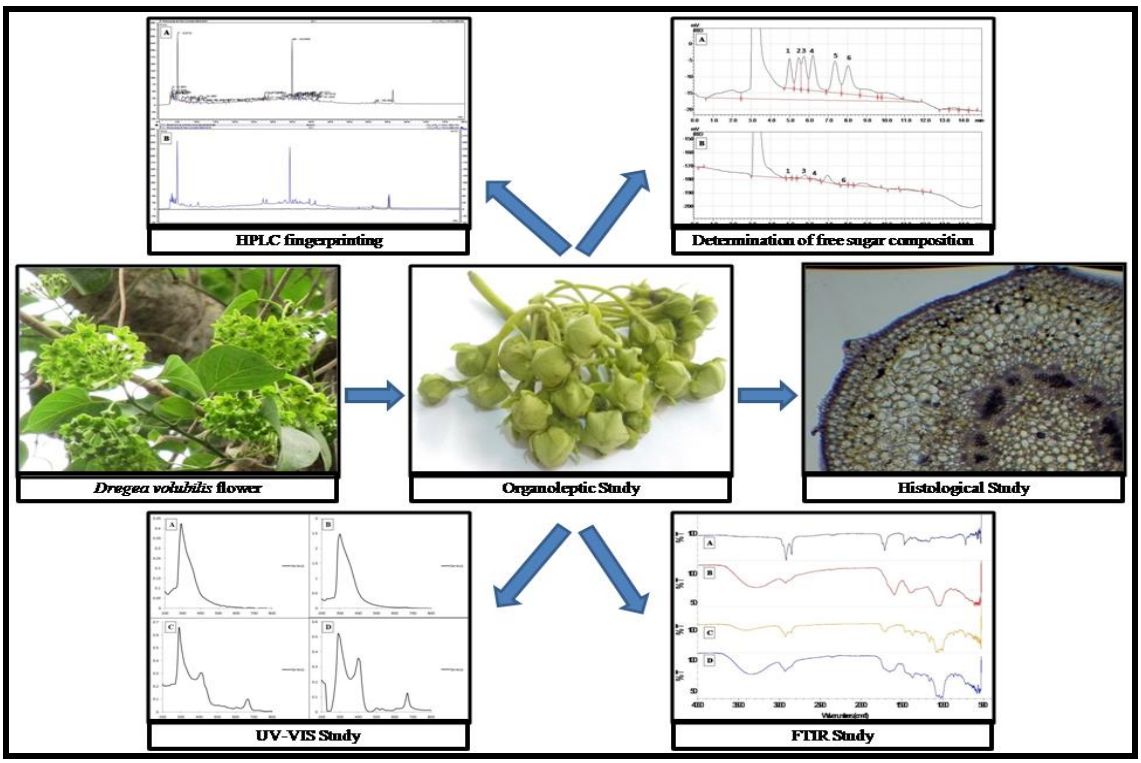
Chapter II:

Antioxidant and antidiabetic studies on *Dregea volubilis* flower.

Chapter III:

Application of *Dregea volubilis* flower in the biofabrication of silver nanoparticles.

Chapter 1



Pharmacognostic studies on *Dregea volubilis* flower

1. Introduction

Herbal medicines play an important role in the healthcare system to alleviate and treat various types of diseases due to the presence of bioactive phytoconstituents present in it. There is a growing demand of medicinally important plants in the herbal industry due to its health beneficiary properties with diverse chemical structures of the biomolecules. Adulteration and substitution of crude drugs partially or fully with other substances is undesirable and a major problem. Standardization of the medicinal plants is essential to confirm the authenticity and quality to avoid deliberate adulteration and substitution (Sharma et al., 2017).

Dregea, a genus of vines, is a rich source of steroidal pregnanes with potential biological activities (Sanyacharernkul et al., 2009). *Dregea volubilis* (L.f.) Benth. ex Hook.f. (Synonym: *Wattakaka volubilis* (L.f.) Stapf., *Marsdenia volubilis* (L.f.) Cooke) belongs to the kingdom of Plantae, subfamily of Apocynoideae, family of Apocynaceae, order of Gentianales, series of Bicarpellatae, subclass of Gamopetalae, class of Dicotyledons, and is distributed widely in the tropical zone and South East Asia (Karthika et al., 2012). *D. volubilis,* a large twining perennial shrub, grows as a woody climber having woody vines and is scattered throughout the India and Car-Nicobar ascending to an altitude of 1500 m (Biswas et al., 2010). The plant blooms during March and April. The young branches of the plant are green, slender, and smooth; the older branches are gray, very long, and glabrous, often with lenticels or small black dots. Leaves are broadly ovate or somewhat rounded, sub-orbicular, acuminate, 7.5-15 cm long, 5-10 cm wide. Flowers are green or pale green, about 1 cm in a radius, bisexual and sweet scented in drooping umbel. Follicles are usually two, slightly tapering to a very blunt point, glabrous and striated. The seeds are elliptic,

concave, flattened, smooth and shining. Different parts of the plant has been traditionally used in Ayurveda in India for the treatment of various ailments such as asthma, inflammation, tumours, piles, leucoderma, application to boils, rat bite, urinary discharge etc. (Nandi et al., 2012). The vernacular names of the medicinally important plant are Jukti (Bengali), Akadbel (Hindi), Harandodi (Marathi), Velipparuthi (Malayalam), Dudhipaala (Telugu), Koti-p-palai (Tamil), Dugdhive (Kannada), Dudghika (Oriya), Khamallata (Assamese), Kadvokharkhodo (Gujarati) and Hemajivanti (Sanskrit). The flowers of *D. volubilis* are eaten as a seasonal vegetable in early summer with bitter aesthetic principles. Flowers of the plant is a rich source of biologically active phytochemicals and was reported to contain volubiloside A, volubiloside B, volubiloside C, dregealol, volubilogenone, volubilol, drevogenin D, iso-drevogenin P, 17α-marsdenin, dregeanin, vicenin-2, vitexin, isovitexin, isoorientin, rutin, quercetin, luteolin and apigenin (Sahu et al., 2002; Panda et al., 2003; Panda et al., 2006).

In spite of a lot attention to health beneficiary effects, flowers of *D. volubilis* have been less explored pharmacognostically. A detailed study on the structural morphology and other physicochemical parameters of the flowers of *D. volubilis* are required. The present study is aimed to evaluate the flowers to fix the pharmacognostical parameters for proper identification, authentication and quality standardization of the plant.

2. Materials and methods

2.1. Plant material

The fresh flowers of *D. volubilis* were collected in the month of April, 2017 from Jaynagar Mazilpur, South 24 Parganas, West Bengal, India. It was taxonomically identified and

authenticated by Dr. V.P. Prasad, Central National Herbarium, Botanical Survey of India, Botanical Garden, Howrah, West Bengal, India. A voucher specimen (voucher no DV/H/141) of the plant was kept at Division of Microbiology & Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India. The flowers were dried under shade, powdered using a mechanical grinder and preserved at 25 ± 2 °C in air tight container at dry place.

2.2. Chemicals and instruments

Rhamnose, xylose, fructose, glucose, trehalose, and maltose were purchased from Sigma-Aldrich, St Louis, MO, USA. Ethanol was purchased from EMD Millipore, Bedford, MA, USA. Chloroform, acetone, 2-propanol, ethyl acetate, diethyl ether, petroleum benzine (40-60 °C), methanol for liquid chromatography, acetonitrile for liquid chromatography, sodium carbonate, anthrone, glacial acetic acid, hydrochloric acid, nitric acid, acetic acid, sulphuric acid, phosphoric acid, ammonia solution 25%, coomassie brilliant blue G250, sodium hydroxide, potassium hydroxide, chloral hydrate, glycerin were procured from Merck Life Sciences Private Limited, Mumbai, India. The water was purified by a Milli-Q water purification system (EMD Millipore, Bedford, MA, USA) and used for all experiments. All other reagents used were of analytical grade.

2.3. Macroscopic evaluation

The macroscopic study of crude drug includes evaluation of its morphological characteristics which are examined by naked eye and magnifying lens. The method is the simplest and quickest mean to check the authenticity of a crude drug (Chanda, 2014).

2.4. Microscopic evaluation

Fresh flowers were collected and washed with water for carrying out microscopical study. Different parts of the flower were cut into very thin transverse sections (T. S) and boiled in 10 % potassium hydroxide solution to remove fatty materials and colouring substances. The sections were stained and observed under Magnus microscope (Olympus (India) Pvt. Ltd., Noida, India). Photomicrographs were captured with Magnus photomicrography units (MIPS USB 2.0. Capture and Display Software) at 40 X magnification (Borah et al., 2014).

2.5. Powder characteristics

The mechanically grinded dried powdered material was sieved through mesh number 60 to get uniform powder. It was cleared with chloral hydrate, stained and mounted in glycerin to observe under Magnus microscope (Olympus (India) Pvt. Ltd., Noida, India) (Gogoi et al., 2016).

2.6. Quantitative Standards

The shade dried powdered material of flowers of *D. volubilis* were evaluated for the determination of ash values, extractive values and loss on drying.

2.6.1. Ash values

The total ash, water soluble ash and acid insoluble ash of the plant material were performed (Upreti et al., 2013).

2.6.1.1. Total ash

1 gm of material was taken in a previously ignited and tarred silica crucible. The material was spread in even layer and ignited at 450 °C by gradually increasing the temperature until it was white indicating the absence of carbon. It was then allowed to cool in a desiccator. The total ash content (% w/w) of the material was calculated according to the following equation:

Total ash (% w/w) = (weight of ash/weight of sample) × 100.

2.6.1.2. Acid insoluble ash

The acid insoluble ash was determined by boiling the total ash with 25 ml of 2 (N) hydrochloric acid (HCl) into a china dish. It was covered with a watch glass and gently boiled for 5 minutes. The watch glass was rinsed with 5 ml of boiled water and the rinsed contents were transferred to the contents of china dish. The insoluble matter of the contents of the china dish was collected on tarred gooch crucible, washed with boiled acidulated water, ignited, cooled in a desiccator and weighed. Acid insoluble ash content (% w/w) of the material was calculated with reference to the crude drug according to the following equation:

Acid insoluble ash (% w/w) = (weight of ash/weight of sample) × 100.

2.6.1.3. Water soluble ash

25 ml of water was added to the total ash in a china dish and was gently boiled for 5 minutes. The water insoluble ash was collected on tarred gooch crucible, washed with

boiled acidulated water, ignited, cooled in a desiccator and weighed. The water soluble ash was calculated by subtracting the weight of insoluble matter from the weight of total ash. The water soluble ash content (% w/w) was determined with respect to the air dried material using the following equation:

Water soluble ash (% w/w) = (weight of water soluble ash/weight of sample) × 100.

2.6.2. Extractive values

The extractive values are indicative weights of the extractable chemical constituents of crude drugs in different solvents. The extractive values of the crude drugs were determined in water and alcohol (Arambewela and Arawwawala, 2010). 5 gm each of the crude drugs was taken in a 250 ml stoppered conical flask. 100 ml of the respective solvent was added to the 250 ml stoppered conical flask and was allowed to macerate for 24 hours with the aid of mechanical shaker for 6 hours. It was then filtered and 25 ml of the filtrate was taken in a tarred petridish. It was evaporated to dryness in an oven at 105 °C and weighed it again. The extractive value (% w/w) was calculated with respect to the air dried material using the following equation:

Extractive value (% w/w) = (weight of extracted residue/weight of sample) × 100.

2.6.3. Loss on drying

The loss on drying (LOD) was performed (Aslam and Afridi, 2018) by taking 1 gm of the crude drug in previously weighed LOD weighing bottle. It was dried in an oven for 1 hour,

cooled in a desiccator and weighed. The LOD (% w/w) was calculated with respect to the crude drug using the following equation:

LOD (% w/w) = (weight loss/weight of sample) × 100.

2.7. Fluorescence analysis

The fluorescence analysis was performed by treating the dried powdered material with different chemicals and was observed in day light and ultraviolet (UV) light (Akbar et al., 2014). Some of the phytochemicals present in plant material show fluorescence in the visible range in daylight. The ultraviolet ray produces fluorescence in many crude drugs which do not fluorescence in day light. A more powerful source of ultra violet ray is often needed to produce fluorescence in crude drugs. Different types of reagents are often applied to the crude drugs which do not fluoresce to convert them into fluorescent derivatives. The fluorescence analysis is an important parameter for pharmacognostic evaluation for assessing crude drugs qualitatively. The behaviour of powdered drugs after treatment with different chemical reagents and their fluorescent characteristics were observed under UV (254 and 366 nm) and visible light using CAMAG UV CABINET 4.

2.8. Preliminary phytochemical studies

The shade dried powdered material of flowers of *D. volubilis* weighing about 200 gm was soaked with sufficient amount of light petroleum benzine (40-60 °C) in a glass Beaker for 24 hours and then the flowers were extracted with petroleum benzine (40-60 °C), chloroform, methanol successively using soxhlet apparatus and the exhausted material was boiled with water. The petroleum benzine (40-60 °C) fraction obtained after extraction

using soxhlet apparatus was combined with the initial fraction of petroleum benzine (40-60 °C) obtained after soaking. The extracts of organic solvents were concentrated using rotary evaporator under reduced pressure and evaporated to dryness. Aqueous extract was concentrated using water bath and lyophilized. The extracts were preserved in well closed container and kept in the dark at a temperature of 10 °C for future use. The different extracts obtained were tested individually for the presence of different phytoconstituents (Rabinarayan et al., 2018). TLC fingerprinting of the extracts were performed using Silica Gel G as an adsorbent. TLC plates (Millipore Corporation, USA) were activated in hot air oven at 110 °C for 30 minute. The plates were kept in a desiccator for future use. Different combinations of solvents were used for the preparation of the mobile phases.

2.9. HPLC fingerprinting

The chromatographic fingerprinting was performed by ultra high performance liquid chromatography using a UHPLC+ focused system consisting of a Dionex Ultimate 3000 Pump, a Dionex Ultimate 3000 auto sampler column compartment and a Dionex Ultimate 3000 variable wavelength detector (Adams et al., 2013). 1 gm of dried and finely powdered (mesh size 85) sample was taken in 10 ml volumetric flask and sufficient Milli-Q water was added and heated in boiling water bath for 20 minute and cooled and made up to the volume with Milli-Q water. The solution was filtered through a 0.45 µm syringe filter (Millex, Merck, Germany) which was injected as a test solution and Milli-Q water was injected as blank. Chromatographic separations of phytochemicals of the extract was performed using a C18 column (250 mm × 4.6 mm i.d.) with a particle size of 5 µm, Hypersil GOLD (Thermo Fisher Scientific, U.S.A.) and column oven temperature was maintained at 25 °C. The chromatographic separation was performed using gradient elution

(Table 1) with a flow rate of using 0.2 (% v/v) phosphoric acid as mobile phase A and HPLC grade methanol as mobile phase B, respectively. The UV detector was set at 280 nm and injection volume was 20 µl. The chromatograms were processed with Chromeleon 7, version 7.2.0.3765 software (Thermo Scientific, U.S.A.).

Table 1: UHPLC gradient programme for the HPLC fingerprinting study.

Time (minute)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	1	100	0
10	1	100	0
20	1	90	10
30	1	70	30
40	1	50	50
50	1	30	70
60	1	10	90
70	1	0	100

2.10. FTIR

Fourier transform infrared (FTIR) spectra of the different extracts of *D. volubilis* were performed in ATR mode using Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA) with a total of 30 scans at a resolution of 4 cm^{-1} in the wave number range between

4000 cm^{-1} to 525 cm^{-1}. Background spectrum of a clean ATR crystal was collected immediately before collecting the spectrum of the plant extracts. The extracts were then placed in the ATR accessory and pressed for acquiring the FTIR spectra of the samples. The spectral acquisitions were processed with OMNIC software supplied by the manufacturer. FTIR study provided the qualitative information on the types of functional groups and chemical bonds present in the phytochemicals of the extracts by analyzing the peak values (cm^{-1}) of the spectra (Tarantilis et al., 2008).

2.11. Determination of UV-VIS spectra

The different extracts of *D. volubilis* were diluted with respective solvents at a concentration of 0.05 % (w/v) and scanned between 200 nm to 800 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA) and the spectra were recorded (Cheng et al., 2014).

2.12. Determination of pH

The shade dried powdered material of flowers of *D. volubilis* was mixed with water at a concentration of 1% (w/v), 2% (w/v) and 10% (w/v) and kept in a water bath for 20 minute. It was then filtered through Whatman filter paper No. 1 and the pH of the filtrate was measured using a pH meter (Model: 3200P, Agilent Technologies, USA) at 25 °C (Wahab et al., 2012).

2.13. Determination of protein content

The protein content of the sample was determined according to Bradford method with some modifications (Sharma et al., 2014). 0.5 gm powder of *D. volubilis* flower was mixed with

10 ml of water and the mixture was shaken for 10 minutes followed by filtration using Whatman filter paper No. 1. 0.2 ml of the sample solution was mixed with 5 ml of Bradford's reagent (0.1 g of coomassie brilliant blue G250 was dissolved in 50ml of ethanol followed by addition of 100 ml 85% (v/v) phosphoric acid and volume was made up to 1L). The reaction mixture was kept for 10 minute for development of colour completely. The absorbance of the reaction mixtures were measured at 595 nm against a blank using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). Protein present in the sample was quantified from calibration curves of absorbance versus concentration in µg/ml of bovine serum albumin which was used as a standard.

2.14. Determination of carbohydrate content

The total carbohydrate content of the dried powdered material of flowers of *D. volubilis* was determined by Anthrone method with slight modifications (Baloch et al., 2015). A standard stock solution (10 mg/ml) containing glucose was prepared in Milli-Q water and different concentrations (20, 40, 60, 80, 90 and 100 µg/ml) of standard solutions were prepared by diluting the stock solution for calibration curve. 100 mg of dried sample was hydrolyzed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 (N) HCl and cooled down to room temperature. It was then neutralized with solid sodium carbonate until effervescence ceased and volume of the solution made up to 100 ml with Milli-Q water. It was then centrifuged at 2000 rpm and the supernatant was collected. 1 ml each of standard solutions and sample solution was added to 4 ml of anthrone reagent (0.2% (w/v) anthrone in ice cold concentrated sulphuric acid) and heated for eight minutes in boiling water bath and cooled to room temperature. A blank solution was prepared by adding 1 ml

Milli-Q water to 4 ml of anthrone reagent. The absorbance of the reaction mixtures were measured at 630 nm against the blank using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA).

2.15. Determination of free sugar composition

The free sugars were determined by ultra fast liquid chromatography (UFLC) using a Prominence UFLC system (Shimadzu, Japan) equipped with a LC-20AT Solvent Delivery Unit, SIL-20A UFLC version Auto-Sampler, RID-10A refractive index (RI) detector (Heleno et al., 2009). A mixed standard stock solution (10 mg/ml) containing rhamnose, xylose, fructose, glucose, trehalose, and maltose was prepared in Milli-Q water and different concentrations (0.625, 1.25, 2.5, 5 and 7.5 mg/ml) of standard solutions were prepared by diluting the mixed standard stock solution for calibration curves to quantify the sugars present in the sample. 1 gm of dried sample was extracted with 40 ml of 80 % (v/v) aqueous ethanol at 80 °C for 30 minutes. It was then centrifuged at 15000 g for 10 minutes and the supernatant was concentrated at 60 °C under reduced pressure. The concentrated sample was defatted three times with 10 ml of diethyl ether. The defatted material was concentrated at 40 °C and dried. The dried sample was dissolved in Milli-Q water to a final volume of 5 ml. The solution was filtered through a 0.45 μm syringe filter (Millex, Merck, Germany) which was injected as a test solution and Milli-Q water was injected as blank. Chromatographic separations of the sugars was performed using a NH_2 column (250 mm × 4.6 mm i.d.) with a particle size of 5 μm and pore size of 100 Å, Luna NH_2 (Phenomenex, U.S.A.) and column oven temperature was maintained at 40 °C. A solvent mixture consisting of seven volumes of acetonitrile and three volumes of Milli-Q water was used as

mobile phase. The chromatographic separation was performed with a flow rate of 1.0 ml min^{-1} with run time of 15 minute and injection volume was 10 µl.

2.16. Statistical analysis

All experiments were performed in triplicate and the results of the quantitative studies are presented as mean ± standard error of mean (SEM). The statistical analyses were performed with GraphPad PRISM6 software, USA.

3. Results and discussion

3.1. Macroscopic evaluation

The *D. volubilis* flowers were numerous, green or pale green in colour, sweet scented and bitter in taste. Inflorescences were in lateral drooping umbellate cymes. 2.5- 5 cm long slender peduncles were arising from between the petioles. 1- 2.5 cm long slender calyx dividing nearly to the base was ovate to oblong, obtuse and ciliolate. Corolla was deeply divided and glabrous outside. Lobes were broadly ovate, obtuse and veined overlapping to right. Stamina column aroused from the base of the corolla and anther tips were membranous, broadly ovate, oblong and obtuse. Pollen masses were attached to the pollen carriers by very short caudicles (Fig. 1). The macroscopic evaluation of a crude drug is used for its authentication by comparing the diagnostic characters with the prescribed standards of the standard drug. The macroscopic feature helps to evaluate the basic differentiating characteristics between the various species within a single genus (Chanda, 2014). The parameters of the macroscopic evaluation are mostly subjective and there is a chance of existence of adulterants which are closely resembles the genuine drug. The microscopic and

physico-chemical analyses are more authentic studies to check whether the parameters of the crude drugs conform to the standard or not. The parameters of the macroscopic evaluation for the flowers of *D. volubilis* can be served as reference diagnostic characters for the identification and authentication of the drug.

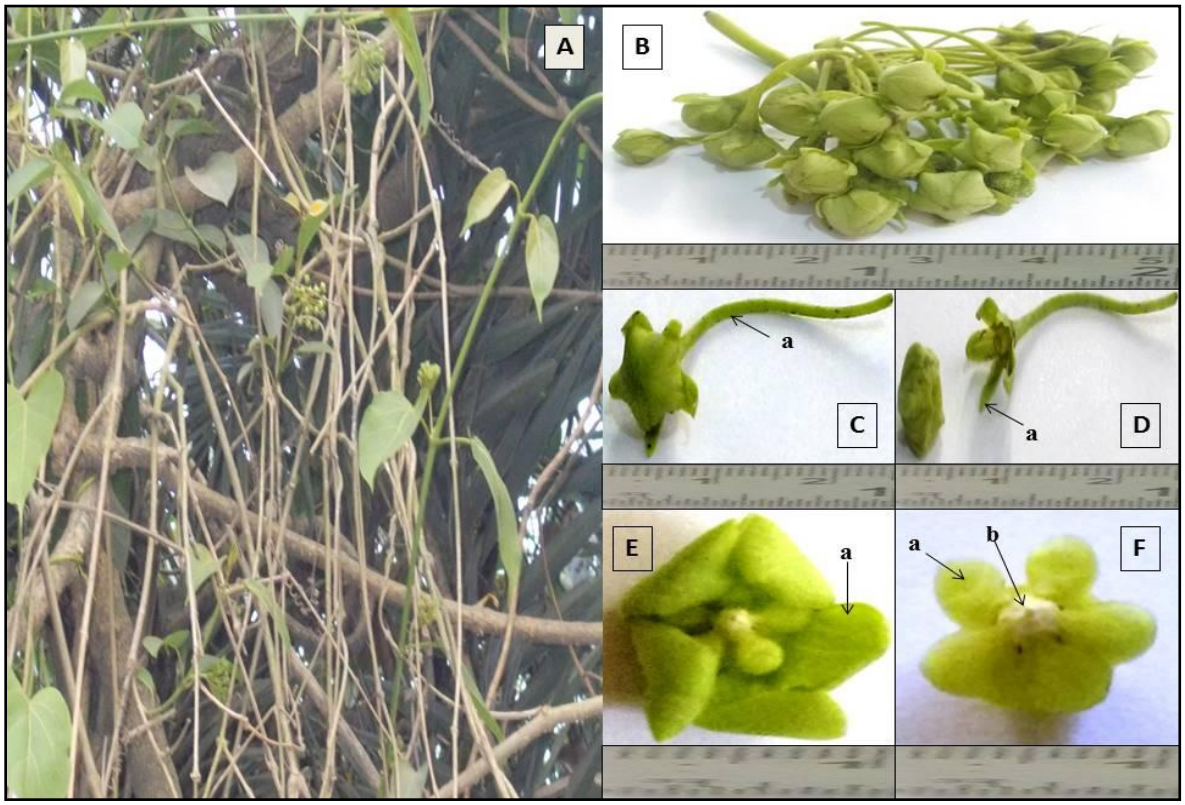


Fig. 1. A. *D. volubilis* in its natural habitat. B. Pale green flower in dense drooping umbels. C. Individual flower of *D. volubilis* showing pedicel (a). D. *D. volubilis* flower part showing sepal (calyx) (a). E. *D. volubilis* flower part showing petal (corolla) (a). F. *D. volubilis* flower part showing androecium (a) and gynoecium (b).

3.2. Microscopic evaluation

The T. S of the different floral parts of flower (Fig. 2) showed the following observations under microscope.

Thalamus

The T. S of thalamus showed that epidermal layer was composed of a single layer of compactly arranged tabular cells with cuticularised outer walls. The cortex was composed of 2-3 layers of collenchyma, known as hypodermis. Several layers of thin walled parenchyma cells, known as parenchymatous zone, were present next to the hypodermis in the cortex. The vascular bundles were collateral and open and each bundle was composed of external phloem and internal xylem. Pith was made of parenchyma cells. Trichomes were uniseriate, multicellular with blunt tip. Cluster crystals of calcium oxalate were present.

Stalk

The T. S of stalk showed that the epidermis was single layered outermost zone consisting of compactly set tabular living cells, outer walls of which were cuticularised. Cortex was distinctive consisting of hypodermis which was composed of 2-3 layers of collenchyma cells. General cortex was present next to the hypodermis which was composed of several layers of thin walled parenchyma cells. Vascular bundles were collateral and open consisting of external phloem and internal xylem. Pith was very distinct and large, situated in the centre consisting of thin walled, oval or polygonal parenchyma cells with abundant intercellular spaces between them. Cluster crystals of calcium oxalate were found to be present. Trichomes were uniseriate, multicellular with blunt tip.

Calyx

The T. S of sepal showed that there were two epidermal layers e.g. upper and lower epidermis. Both the epidermal layers were uniseriate and composed of compactly arranged tabular cells, the outer walls of which were cuticled. The mesophyll was made of parenchyma cells lying between two epidermal layers. The mesophyll was differentiated into (a) upper closely packed, tubular chloroplast containing cells, known as palisade parenchyma, and (b) lower loosely arranged, more or less rounded cells, called spongy parenchyma. Oil globules and cluster crystals of calcium oxalate were also present. Trichomes were uniseriate, multicellular with blunt tip. Stomata were anomocytic.

Corolla

The T. S of petal showed that the epidermal layer was uniseriate and composed of compactly arranged tabular cells, the outer walls of which were cuticled. Trichomes were uniseriate, multicellular with blunt tip. Anomocytic Stomata and oil globules were found to be present.

Androecium

The T. S showed that the epidermal layer was uniseriate and composed of compactly arranged tabular cells, the outer walls of which were cuticled. The cluster crystals of calcium oxalate were found to be present.

Gynoecium

The T. S showed the presence of cluster crystals of calcium oxalate.

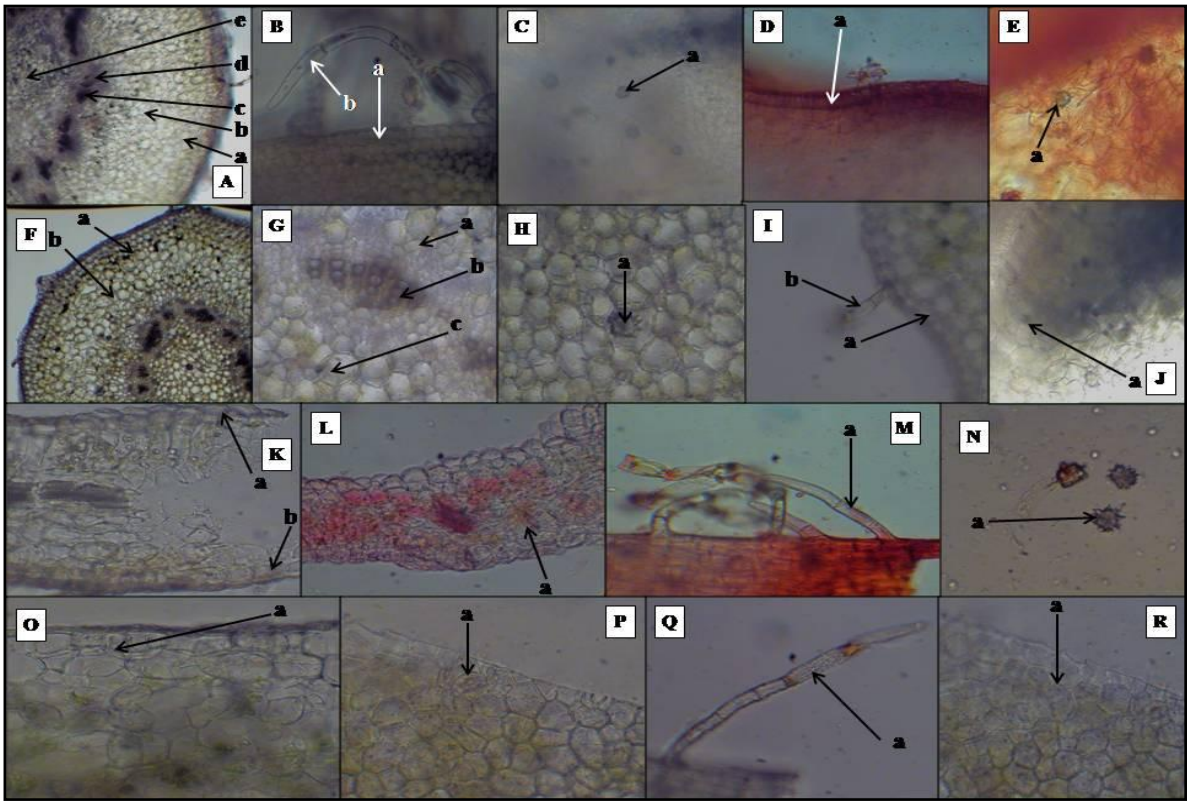


Fig. 2. Transverse section (T. S) of different parts of *D. volubilis* flower. A. T. S of thalamus showing hypodermis (a), parenchymatous zone (b), xylem (c), phloem (d) and pith (e). B. Epidermis (a) and trichome (b) in thalamus. C. Cluster crystals of calcium oxalate (a) in thalamus. D. T. S of androecium showing epidermis (a). E. Cluster crystals of calcium oxalate (a) in androecium. F. T. S of stalk showing hypodermis (a) and general cortex (b). G. Phloem (a), xylem (b) and pith (c) in stalk. H. Cluster crystals of calcium oxalate (a) in stalk. I. Epidermis (a) and trichome (b) in stalk. J. T. S of gynoecium showing cluster crystals of calcium oxalate. K. T. S of sepal showing upper epidermis (a), lower epidermis (b). L. Stomata in sepal (a). M. Trichome in sepal (a). N. Cluster crystals of calcium oxalate (a) in sepal. O. T. S of petal showing epidermis (a). P. Stomata in petal (a). Q. Trichome in petal (a). R. Oil globules in petal (a).

The detailed microscopic examination of the flowers of *D. volubilis* can be used as a reference to identify the crude drug by comparing the known histological characters. The

microscopic examination is an easiest and finest way to set standard parameters depending upon the internal anatomy of the plant (Aslam and Afridi, 2018). The microscopic study alone cannot provide complete evaluation profile for the herbal drugs. The histological characters of the drug along with other analytical parameters can be utilized to set the standardization specifications for the evaluation of the herbal drug.

3.3. Powder characteristics

The powder microscopy showed the presence of fibers, cluster crystals of calcium oxalate and epidermal trichomes. Some fragments consisted of groups and parts of parenchyma cells and epidermal cells. The observations of the study on the powder of *D. volubilis* flowers can serve as useful parameters for the proper identification of the drug. The dried sample in the powdered form gives characteristic features of the drug under microscope after proper treatments. The microscopical examination of epidermal trichomes and calcium oxalate crystals are extremely valuable for authentication of crude drugs (Rabinarayan et al., 2018).

3.4. Quantitative Standards

The total ash, acid insoluble ash and water soluble ash were found to be 11.767 ± 0.130 % (w/w), 1.287 ± 0.106 % (w/w) and 9.140 ± 0.344 % (w/w) respectively. The water and alcohol soluble extractive values were found to be 21.600 ± 0.133 % (w/v) and 9.603 ± 0.104 % (w/v) respectively. Loss on drying was 14.110 ± 0.061 % (w/w). Therapeutic efficacy of herbal drugs can be ensured by determining the quantitative standards. The ash of crude drugs is consisted of nonvolatile inorganic materials which can be used to set quality control parameter to check the contamination of crude drugs. A high content of ash

value beyond the standard limit gives an indication of contamination, substitution or adulteration (Kokate et al., 2006). The content of active constituents in a given amount of crude drug is estimated by extractive value in a particular solvent. The extractive values give valuable information regarding the quality of the crude drug whether it is exhausted or not. The high extractive value is an indicative parameter of better extraction of phytoconstituents from crude drugs and it is also helpful for proper selection of solvent that will provide maximum yield (Folashade et al., 2012). The physical and physicochemical state of the interior of cell depends upon the loss of water. The enzymes present in the cell are responsible for different chemical reactions like oxidation, hydrolysis and polymerization of the phytoconstituents present in the plant material when the enzymes come in contact with the active substances during the process of drying. Most of the enzymes present in plant material need sufficient water to act leading to decomposition reactions of the crude drugs. Moisture present in the crude drugs helps in microbial growth leading to degradation of it. It is desirable to keep the water content of the crude drugs at low level to deactivate the enzyme activity as well as to retard microbial degradation to such an extent that the storage stability of the crude drugs are guaranteed (Madhav et al., 2011). These standardization parameters are essential to ensure the quality of herbal drugs. Quantitative standards can be applied for the evaluation of crude drugs. These parameters can be utilized for maintaining the identity, purity, and quality of crude drugs. Purity depends on the absence of foreign matter in the crude drugs. Quality depends on the concentration of the active constituents present in the crude drugs that exerts health beneficiary properties.

3.5. Fluorescence analysis

The fluorescence analysis of the powdered drug showed various colours after treatment with different chemical reagents and observed visually under daylight, short wavelength ultra violet light (254 nm) and long wavelength ultra violet light (366 nm). The results are shown in Table 2.

Table 2: Fluorescence analysis of powdered flowers of *D. volubilis*.

Sl. No.	Treatment	Daylight	UV light	
			254 nm	366 nm
1	Powder as such	Light brown	Dark green	Reddish brown
2	Powder + Acetic acid	Brown	Dark brown	Brownish black
3	Powder + Ferric chloride (5 % w/v)	Greenish brown	Brownish black	Black
4	Powder + Conc. Hydrochloric acid (HCl, 5N)	Yellowish brown	Brown	Bluish black
5	Powder + Conc. Nitric acid (HNO_3)	Reddish brown	Brownish black	Black
6	Powder + Conc. Sulphuric acid (H_2SO_4)	Brownish black	Brown	Brownish black
7	Powder + Iodine Solution (1 % w/v)	Reddish yellow	Brown	Brownish black
8	Powder + Methanol	Light brown	Dark brown	Brownish black
9	Powder + Picric acid	Yellowish brown	Brownish yellow	Brownish black
10	Powder + NaOH Solution (1 N)	Reddish yellow	Dark brown	Brownish black
11	Powder + Distilled water	Yellowish brown	Dark brown	Brownish black
12	Powder + Liquid Ammonia (NH_3)	Deep brown	Dark brown	Brownish black
13	Powder + Conc. HNO_3 + NH_3	Reddish brown	Dark brown	Brownish black
14	Powder + Dilute HNO_3	Yellowish brown	Brown	Brownish black
15	Powder + Potassium dichromate solution (10 % w/v)	Deep yellow	Dark brown	Black
16	Powder + Acetone	Brown	Yellowish brown	Bluish black

3.6. Preliminary phytochemical studies

The results of preliminary qualitative phytochemical studies of the different extracts of flowers of *D. volubilis* are presented in Table 3.

Table 3: Preliminary phytochemical analysis of *D. volubilis* flower extracts.

Phytochemicals	Petroleum benzine extract	Chloroform extract	Methanol extract	Aqueous extract
Carbohydrates	-	-	+	+
Proteins	-	-	+	+
Amino acids	-	-	+	+
Steroids	+	+	-	-
Glycosides	-	+	+	+
Alkaloids	-	-	+	+
Tannins and Phenolics	-	-	+	+
Flavonoids	-	-	+	+

The TLC studies of the different extracts were performed in different solvent systems on trial and error method. The retention factor (R_f) values of the different extracts are presented in Table 4. The phytochemical studies of the different extracts of *D. volubilis*

flower showed the presence of different types of plant metabolites which are responsible for the medicinal values of the plant.

Table 4: Thin layer chromatography of *D. volubilis* flower extracts.

Sl. No.	Solvent system	Extracts	No. of spots (254 nm)	R_f values (254 nm)	No. of spots (366 nm)	R_f values (366 nm)
1	Chloroform : Ethyl acetate (6 : 4)	Aqueous	1	0.48	-	-
		Methanol	4	0.04, 0.09, 0.13, 0.47	1	0.25
		Chloroform	16	0.04, 0.06, 0.10, 0.12, 0.19, 0.30, 0.35, 0.41, 0.48, 0.56, 0.61, 0.72, 0.79, 0.95, 0.96, 0.98	9	0.08, 0.16, 0.30, 0.35, 0.41, 0.93, 0.95, 0.97, 0.98
		Petroleum benzine	7	0.07, 0.23, 0.48, 0.56, 0.72, 0.91, 0.96	3	0.84, 0.92, 0.96
2	Chloroform : Ethyl acetate (8 : 2)	Aqueous	2	0.04, 0.15	-	-
		Methanol	4	0.02, 0.06, 0.10, 0.16	3	0.01, 0.05, 0.14
		Chloroform	9	0.02, 0.05, 0.08, 0.10, 0.15, 0.16, 0.88, 0.91, 0.96	4	0.06, 0.09, 0.90, 0.96
		Petroleum benzine	12	0.10, 0.20, 0.24, 0.29, 0.52, 0.64, 0.67, 0.79, 0.86, 0.89, 0.91, 0.97	3	0.66, 0.89, 0.96
3	Chloroform : Methanol : Glacial acetic acid (4 : 5 : 1)	Aqueous	1	0.69	2	0.10, 0.73
		Methanol	7	0.13, 0.17, 0.24, 0.53, 0.66, 0.76, 0.90	5	0.24, 0.69, 0.78, 0.90, 0.97
		Chloroform	2	0.84, 0.93	2	0.74, 0.87
		Petroleum benzine	3	0.46, 0.48, 0.72	4	0.46, 0.72, 0.78, 0.90
4	Chloroform : Ethyl acetate : Glacial acetic acid (4 : 5 : 1)	Aqueous	3	0.06, 0.17, 0.88	1	0.88
		Methanol	8	0.03, 0.04, 0.07, 0.12, 0.19, 0.33, 0.71, 0.87	5	0.04, 0.13, 0.16, 0.70, 0.96
		Chloroform	8	0.02, 0.04, 0.42, 0.55, 0.86, 0.90, 0.94, 0.95	4	0.04, 0.85, 0.94, 0.96
		Petroleum benzine	1	0.94	1	0.94
5	Chloroform : 2-Propanol : Glacial acetic acid (5 : 4 : 1)	Aqueous	1	0.05	1	0.05
		Methanol	9	0.03, 0.12, 0.19, 0.48, 0.57, 0.62, 0.72, 0.92, 0.96	7	0.05, 0.13, 0.21, 0.48, 0.62, 0.69, 0.92
		Chloroform	4	0.12, 0.72, 0.88, 0.94	1	0.97
		Petroleum benzine	1	0.94	1	0.97
6	Chloroform : Ethanol : Triethylamine (6 : 3 : 1)	Aqueous	3	0.06, 0.08, 0.11	-	-
		Methanol	12	0.05, 0.13, 0.16, 0.19, 0.31, 0.37, 0.41, 0.47, 0.61, 0.65, 0.90, 0.96	5	0.06, 0.89, 0.92, 0.95, 0.97
		Chloroform	3	0.44, 0.84, 0.95	2	0.91, 0.96
		Petroleum benzine	2	0.81, 0.90	3	0.82, 0.89, 0.96
7	Chloroform : Methanol : Glacial acetic acid (5 : 4 : 1)	Aqueous	6	0.08, 0.12, 0.38, 0.50, 0.69, 0.88	2	0.52, 0.69
		Methanol	11	0.04, 0.09, 0.16, 0.34, 0.44, 0.62, 0.68, 0.78, 0.87, 0.95, 0.98	7	0.16, 0.40, 0.65, 0.79, 0.83, 0.95, 0.98
		Chloroform	2	0.80, 0.92	3	0.79, 0.87, 0.93
		Petroleum benzine	2	0.80, 0.96	2	0.87, 0.96

The extraction with different solvents gives rise to separation of medicinally active portions of the plant according to the polarity of the solvents. The purpose of the standardized extracts is to obtain the therapeutically active compounds and to eliminate unwanted materials by treatment with a selective solvent known as menstrum. The extracts of the crude drugs can be considered as good source of useful drugs (Yadav and Agarwala, 2011). The various types of phytochemicals present contribute medicinal as well as physiological properties to the plants. The TLC analysis of the different extracts was carried out for the development of characteristic fingerprint profile which may be used as a reference for the quality evaluation and standardization of the drug. The bands of the different extracts in the TLC plates were obtained at different R_f values which can be used as identifying markers (Borah et al., 2014). The extracts can be utilized as medicinal agents after standardization in different dosage forms of pharmaceutical interest. The preliminary phytochemical studies are of great importance in the field of standardization of crude drugs.

3.7. HPLC fingerprinting

The different phytoconstituents present in the sample were separated on C18 column using UHPLC (Fig. 3). The different peaks along with their retention times (minute), area (mAU*min), height (mAU), relative area (%) and relative height (%) are presented in Table 5. HPLC plays an important role as an important analytical tool for the quality control of drugs (Farooqui et al., 2014). Natural products have a unique chemical diversity which results in diversity in their biological activity leading to the development of lead compounds which will play an important role in the discovery of drugs for treating various ailments. The modern analytical technique (HPLC) with high power of separation and reproducibility can be used to separate multidimensional chemical structures present in the

plant materials. The peak number 7 and 26 with retention time of 4.912 minute and 34.855 minute respectively are the two more intense peaks among the others in the chromatogram generated after HPLC study. The peak number 7 accounts for 27.54 % and the peak number 26 accounts for 23.92 % relative area.

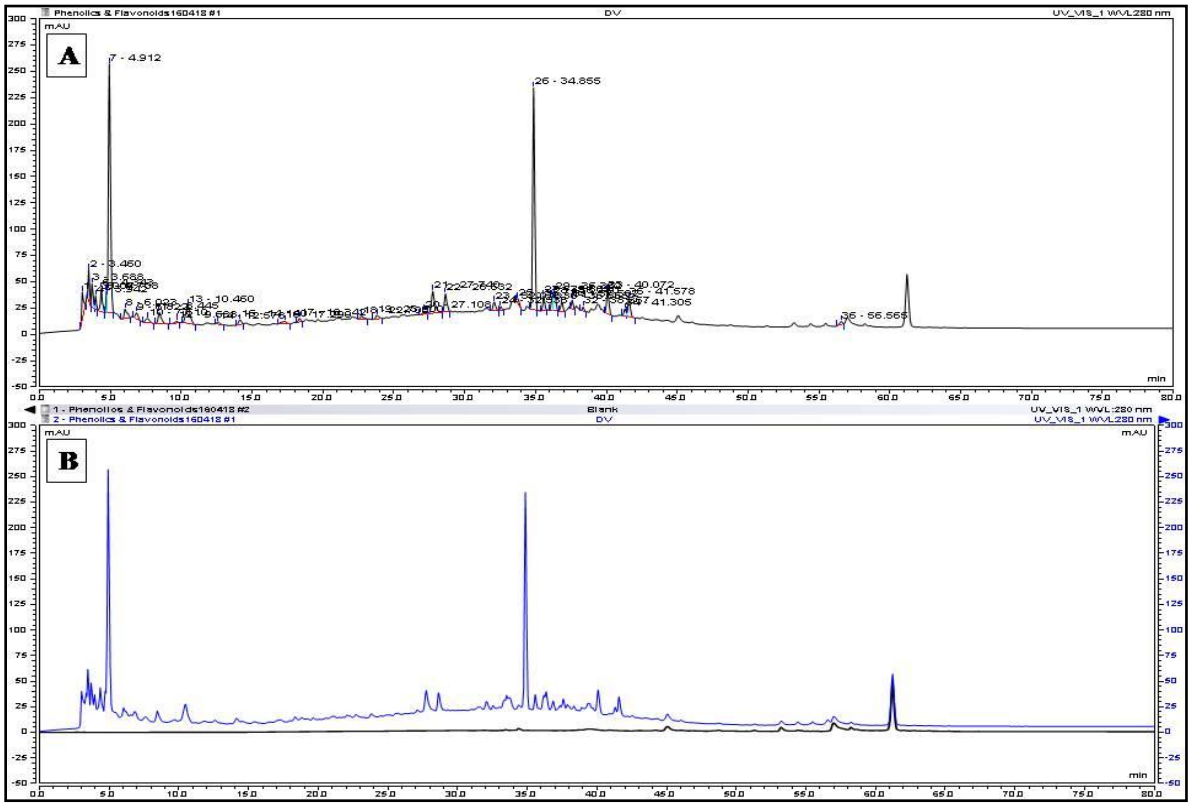


Fig. 3. UHPLC chromatograms of a sample solution of the flower of *D. volubilis* (A) and an overlapped chromatogram (black coloured chromatogram represents blank solution and blue coloured chromatogram represents sample solution) (B) as detected at 280nm.

The chromatograms generated after HPLC study can be used to establish reference HPLC fingerprints of flower of *D. volubilis* against which raw materials can be evaluated and finished products containing the plant material can be analyzed.

Table 5: HPLC peaks present in the sample solution of the flower of *D. volubilis* as detected at 280 nm.

Sl. No. of HPLC Peaks	Retention Time (min)	Area (mAU*min)	Height (mAU)	Relative Area (%)	Relative Height (%)
1	3.007	3.639	26.758	2.52	3.24
2	3.460	2.992	29.535	2.07	3.58
3	3.688	2.887	22.366	2.00	2.71
4	3.942	1.443	13.231	1.00	1.60
5	4.343	3.521	22.164	2.44	2.68
6	4.708	1.774	19.838	1.23	2.40
7	4.912	39.758	236.210	27.54	28.60
8	6.023	2.299	9.147	1.59	1.11
9	6.822	1.329	5.662	0.92	0.69
10	7.610	1.339	4.054	0.93	0.49
11	8.445	2.820	10.939	1.95	1.32
12	9.688	0.501	1.924	0.35	0.23
13	10.460	5.894	17.178	4.08	2.08
14	12.578	0.595	2.365	0.41	0.29
15	14.140	1.131	4.393	0.78	0.53
16	17.290	1.123	2.568	0.78	0.31
17	18.342	0.735	3.604	0.51	0.44
18	22.708	0.772	3.080	0.53	0.37
19	23.800	0.936	3.735	0.65	0.45
20	27.108	0.644	3.177	0.45	0.38
21	27.740	6.010	21.011	4.16	2.54
22	28.632	3.555	17.369	2.46	2.10
23	32.073	1.885	8.404	1.31	1.02
24	32.538	0.623	4.004	0.43	0.48
25	33.720	0.911	4.209	0.63	0.51
26	34.855	34.533	210.94	23.92	25.54
27	35.565	2.167	14.434	1.50	1.75
28	36.180	2.312	14.081	1.60	1.71
29	36.352	3.113	17.537	2.16	2.12
30	36.855	1.667	8.962	1.15	1.09
31	37.582	1.252	8.645	0.87	1.05
32	38.357	0.592	4.046	0.41	0.49
33	40.072	4.243	21.997	2.94	2.66
34	41.305	1.173	6.969	0.81	0.84
35	41.578	3.454	18.030	2.39	2.18
36	56.565	0.749	3.239	0.52	0.39
Total:		**144.370**	**825.81**	**100**	**100**

3.8. FTIR

The FTIR spectrums of the different extracts of *D. volubilis* flowers are presented in Fig. 4. The petroleum benzine and chloroform extracts exhibited characteristic bands for the asymmetrical stretching vibrations of the C–H bonds in CH_2 and CH_3 groups between 2980 and 2810 cm^{-1}, C–H bending vibrations between 1480 and 1400 cm^{-1}, C=O stretching vibrations between 1870- 1540 cm^{-1}, interactions of O–H bending and C–O stretching in the C–O–H group between 1390 and 1350 cm^{-1} and the secondary C–O vibrations in the C–O–H group between 1060 and 1025 cm^{-1} which are characteristics of phytosterols (Ubeyitogullari and Ciftci, 2016). The aqueous, chloroform and methanol extracts showed characteristic bands for the O–H stretching vibrations between 3550 and 3200 cm^{-1}, C–O stretching vibration band between 1060-1000 cm^{-1} and O–H bending vibration band between 1420-1330 cm^{-1} which are due to the presence of phenolic compounds. The extracts also exhibited characteristic O–H stretching vibration band near 3000 cm^{-1} and C=O stretching vibration band near 1700 cm^{-1}, C–O stretching vibration band between 1320-1210 cm^{-1} and O–H bending vibration band between 1440-1395 cm^{-1} which are due to presence of phenolic acids. All the extracts showed characteristic absorption bands for the out-of-plane bending of ring C–H bonds between 900-675 cm^{-1}, in-plane bending bands between 1300-1000 cm^{-1}, C–C stretching band within the ring between 1600-1585 cm^{-1} and 1500-1400 cm^{-1} which are due to the presence of mononuclear and polynuclear aromatic hydrocarbons (Silverstein et al., 2005). The characteristic absorption bands of the different extracts of the plant material confirms the presence of different types of phytoconstituents in *D. volubilis* flowers. FTIR is a useful analytical tool in the field of standardization of

drugs. FTIR spectrum is useful in the identification of drugs by comparing the spectrum of the test material with that of the reference material.

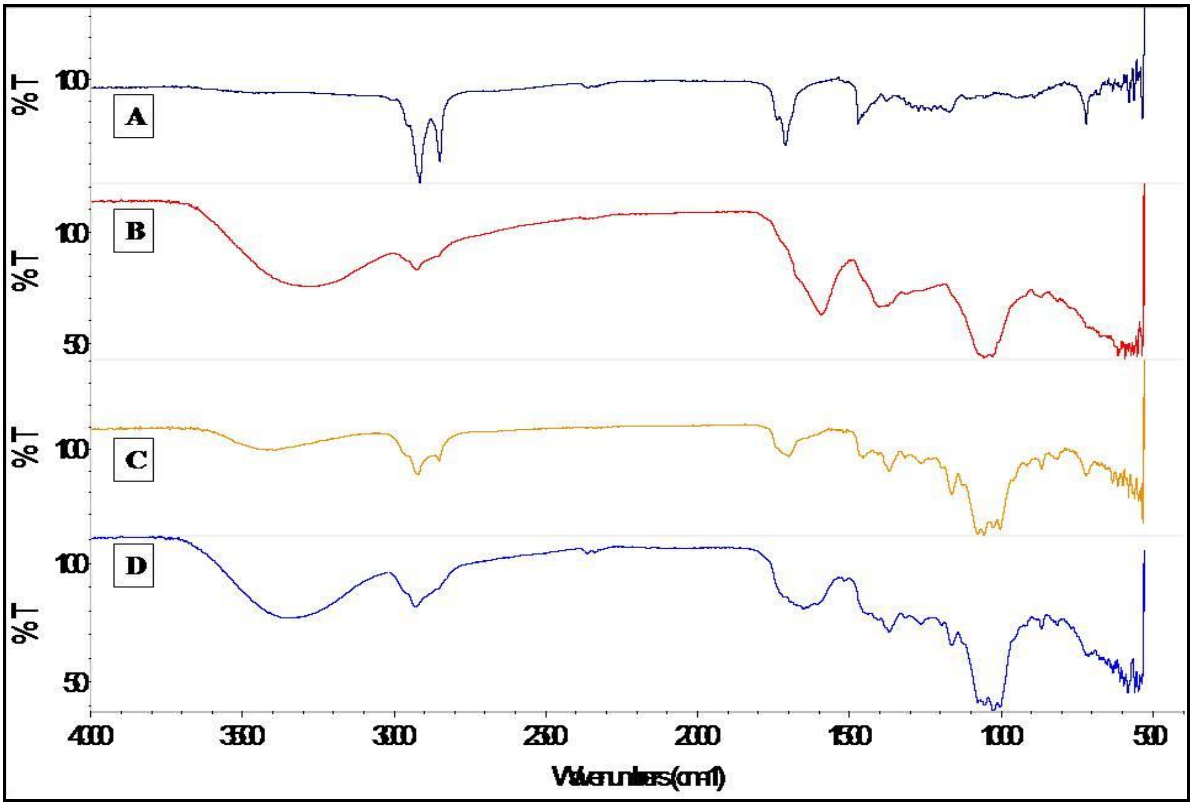


Fig. 4. Fourier transform infrared spectra of the petroleum benzine extract (A), aqueous extract (B), chloroform extract (C) and methanol extract (D) of the flower of *D. volubilis*.

The FTIR spectra of the different extracts can be served as reference FTIR fingerprints of *D. volubilis* flowers for the quality control of raw materials and finished products containing it. The study also provides qualitative information on the types of chemicals present in the different extracts of the flowers of *D. volubilis.*

3.9. Determination of UV-VIS spectra

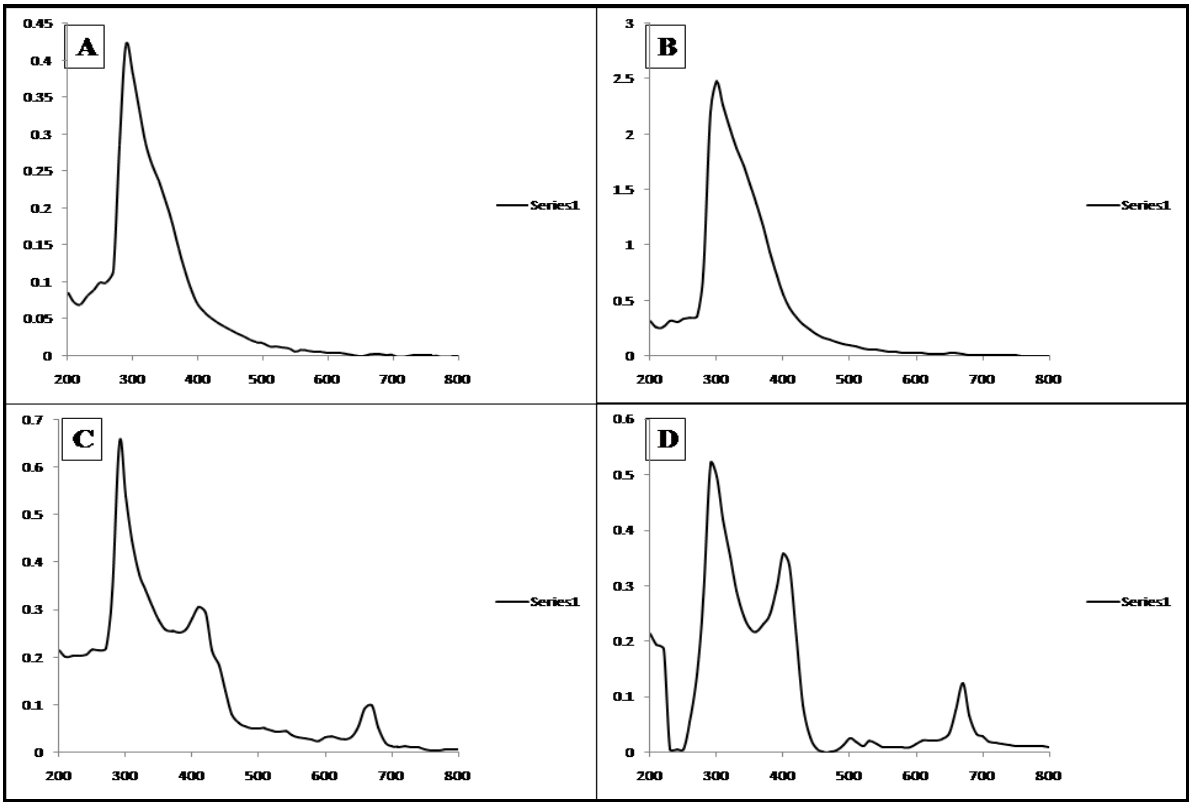


Fig. 5. UV-Visible spectra of the aqueous extract (A), methanol extract (B), chloroform extract (C) and petroleum benzine extract (D) of the flower of *D. volubilis*.

The UV-VIS spectrums of different extracts of *D. volubilis* flowers are presented in Fig. 5. The aqueous extract showed the peaks at 250 nm, 290 nm (λ_{max}); the methanol extract showed the peaks at 230 nm, 300 nm (λ_{max}); the chloroform extract showed the peaks at 250 nm, 300 nm (λ_{max}), 410 nm, 510 nm, 540 nm, 670 nm and the petroleum benzine extract showed the peaks at 300 nm (λ_{max}), 400 nm, 500 nm, 530 nm, 670 nm. UV-VIS spectrum plays an important role for identification and quantification of many drugs. This analytical tool is very useful for the quality control of drugs (Gad et al., 2013). The UV-VIS spectrum

of the different extracts of *D. volubilis* flowers can be served as a reference spectrum for quality control of drugs.

3.10. Determination of pH

The pH of the 1% (w/v), 2% (w/v) and 10% (w/v) aqueous solutions of the dried powdered material were found to be 5.02 ± 0.02, 4.89 ± 0.02 and 4.74 ± 0.02 respectively. pH can also serve as a quality control tool for the identification of the drugs. A change in the value of pH from the standard value indicates the deterioration of the quality of the product. The aqueous solution of *D. volubilis* flower was found to be acidic in nature. The pH value is of great importance in product development to estimate stability and dissolution of the product. The pH value is also helpful in the development of suitable extraction procedure for the phytoconstituents from the plant (Aslam and Afridi, 2018).

3.11. Determination of protein content

Proteins are important biomolecules with multiple functions within organ is differing from one another primarily in their sequence of amino acids. The protein content of the sample was found to be 2.112 ± 0.058 mg/gm of the sample. The protein content of the powder of *D. volubilis* flower can be served as a quantitative parameter for standardization of the plant material. Any deviation from the standard value of protein content reflects the changes in the quality of the crude drug. Estimation of protein of herbal drugs plays a crucial role in assessing nutritional significance and health effects (Hussain et al., 2009). The parameter can be used as a reference for the quality control of the crude drugs.

3.12. Determination of carbohydrate content

The carbohydrate content of the dried powdered material of *D. volubilis* flowers was found to be 124.243 ± 3.573 mg/gm of the sample. Carbohydrate is one of the most widely used substances in nature and is a main ingredient of food. The quantitative analysis for the estimation of carbohydrate content of crude drugs can be considered as a quality control parameter for assessing the crude drugs (Zhao et al., 2018).

3.13. Determination of free sugar composition

The UFLC analysis (Fig. 6) was performed to identify and quantify the free sugars present in the *D. volubilis* flowers. The retention times of rhamnose, xylose, fructose, glucose, trehalose, and maltose were found to be 4.982, 5.456, 5.734, 6.198, 7.351 and 8.032 minute, respectively. The sugars present in the sample were identified by comparing the retention times of the standards with that of the sample. Xylose and trehalose were not detected in the crude drug. The study showed that the flower contained rhamnose (103.229 ± 4.994 µg/gm), fructose (738.670 ± 25.714 µg/gm), glucose (285.532 ± 24.465 µg/gm) and maltose (49.082 ± 5.206 µg/gm). Characterization of sugars present in the crude drugs is very important for their quality control (Zhao et al., 2018). The sugars present in the flower can be considered as markers for the standardization of the crude drug.

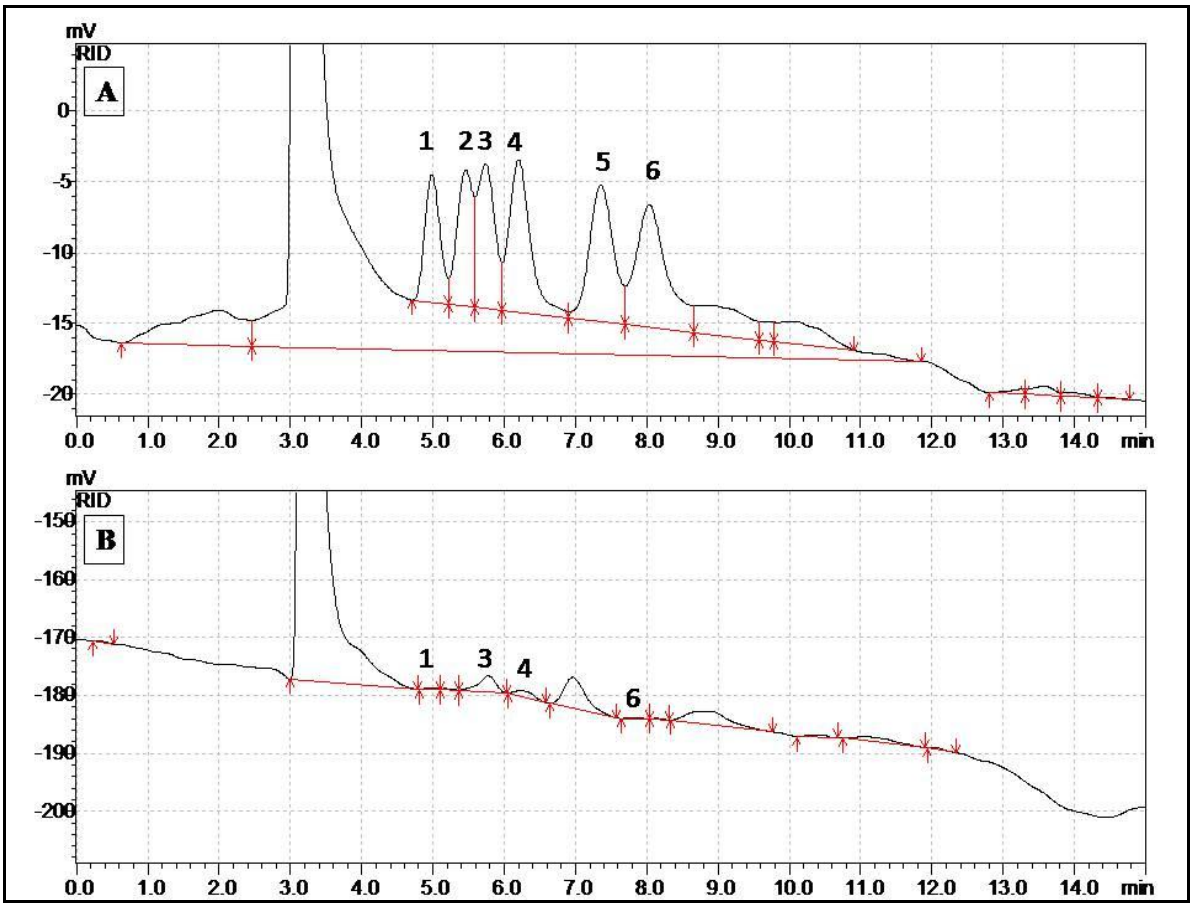


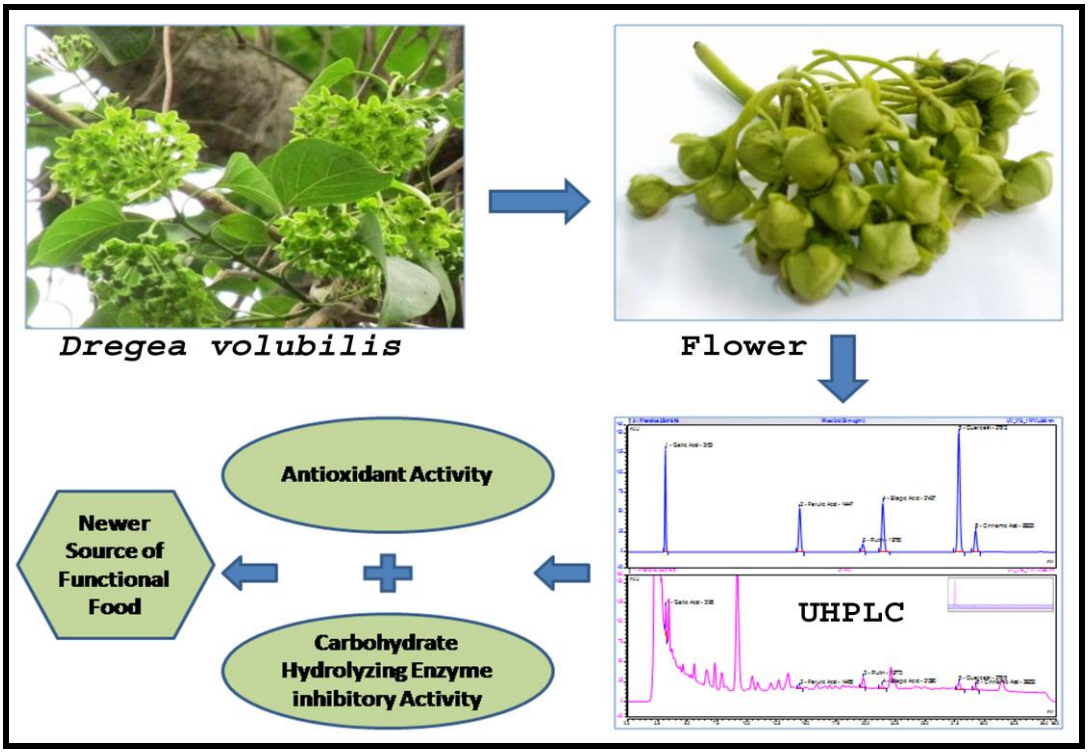
Fig. 6. HPLC chromatograms of mixed standard of sugars (A) and the sample solution of flower of *D. volubilis* (B) as detected with RI detector (1: rhamnose, 2: xylose, 3: fructose, 4: glucose, 5: trehalose, 6: maltose).

4. Conclusion

Herbal drugs are subjected to variability in quality as they derived from heterogeneous sources. The main concerned area is that the activity of the plant material may vary and even inferior quality material may be produced which may impart quality impact on the products of the pharmaceutical industry. Standardization of herbal drugs is an important

topic of great concern. The present study of pharmacognostical evaluation on the flowers of *D. volubilis* has laid down standard parameters for proper identification, authentication and for distinguishing the material from its adulterants and substituents. The detailed study also set the parameters which can be utilized as a Pharmacopoeial reference for recognition of its distinctiveness, genuineness and quality. The study also contributes to the documentation of the nutritional composition on the flowers of *D. volubilis* which are consumed as vegetable.

Chapter 2



Antioxidant and antidiabetic studies on *Dregea volubilis* flower

1. Introduction

India is one of the richest nations in the world in terms of exhibiting a wide range of plant diversity and many plants are consumed as food or in the form of traditional medicine (Kala et al., 2006). Many of the plants are scientifically unexplored. Medicinal plants contain diverse chemical entities with excellent biological activities (Dias et al., 2012). A number of scientific works have focused on exploration of health promoting effects of edible plants (Savran et al., 2016). There has been a correlation between lower risk of diseases like cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases with consumptions of natural products like vegetables, fruits and herbs (Pandey and Rizvi, 2009). Thus, scientific studies on unexplored edible plants or plant parts give us valuable information for the development of nutraceuticals.

Non-insulin dependent diabetes mellitus, commonly called type 2 diabetes, is a chronic metabolic disease characterized by hyperglycemia which results from insufficient or inefficient insulin secretion. The increasing prevalence of diabetes mellitus has become a major health problem worldwide, reaching epidemic proportions. Controlling postprandial hyperglycemia through the inhibition of α-amylase and α-glucosidase (carbohydrate hydrolyzing enzymes) present in the gastro-intestinal tract is one of the major management therapies. To maintain biological processes, oxidation is necessary in living organisms for the production of energy (Shukla et al., 2016). Oxygen derived free radicals and other reactive oxygen species (ROS), which are produced uninterruptedly in vivo, are responsible for cell death and tissue damage (Ozsoy et al., 2008). Oxygen radicals have shown to be involved in various diseases like aging, cancer, cardiovascular diseases, diabetes etc

(Halliwell and Gutteridge, 1999). Antioxidants play an important protective role in cell injury promoted by free radical-induced oxidative stress (Kurutas, 2016). Foods containing natural antioxidants, e.g., polyphenols, which might assist to save living body systems against oxidative damage, have taken a great deal of attention to researchers (Baret et al., 2013; Hooshmand et al., 2015; Dey and Lakshmanan, 2013). Polyphenols have been reported to have antioxidant and hypoglycaemic property with ability to inhibit digestive enzymes such as α-amylase and α-glucosidase (Ang et al., 2015). α-Amylase and α-glucosidase inhibitors can retard utilization of dietary carbohydrates into absorbable monosaccharides and suppress postprandial hyperglycemia, making them applicable for treating type 2 diabetes (Wojdylo et al., 2016). Synthetic enzyme inhibitors used to control postprandial hyperglycemia are undesirable for long term usage because of gastrointestinal side effects and are costly too (Poovitha and Parani, 2016). Natural resources enriched with α-amylase and α-glucosidase inhibitors can be utilized as an effective therapy for treating postprandial hyperglycemia with minimal adverse effects.

The present study investigated the total phenolic content, total flavonoid content, antioxidant effects, α-amylase and α-glucosidase inhibitory activities of flowers of *D. volubilis* in order to establish their medicinal and nutritional potentials.

2. Materials and methods

2.1. Chemicals

Gallic acid, ferulic acid, rutin, ellagic acid, quercetin, cinnamic acid, ascorbic acid, acarbose, α-glucosidase, α-amylase, aluminium chloride, PNPG (*p*-nitrophenyl-α-D-glucopyranoside), DPPH (1,1-diphenyl-2-picryl-hydrazyl) and TPTZ (2,4,6 tripyridyl- S-

triazine) were purchased from Sigma-Aldrich, St Louis, MO, USA. Folin–Ciocalteu reagent, thiobarbituric acid, 2- deoxy-D-ribose and nitro blue tetrazolium were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Sodium carbonate, phosphoric acid, acetic acid, hydrochloric acid, sulfuric acid, ethylenediaminetetraacetic acid, sodium acetate trihydrate, ferric chloride, ferrous sulphate, hydrogen peroxide, sodium hydroxide, trichloroacetic acid, hydroxylamine hydrochloride, sodium phosphate, ammonium molybdate, sodium nitroprusside, NED (N-(1-naphthyl)-ethylenediamine dihydrochloride), sodium chloride, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, starch, sodium potassium tartrate, DNS (3, 5-dinitrosalicylic acid) and methanol for liquid chromatography were procured from Merck Life Sciences Private Limited, Mumbai, India. The water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used for all tests. All other reagents used were of analytical grade.

2.2. Preparation of plant extract

Flowers of *D. volubilis* were shade dried at room temperature and the dried plant material was powdered using mechanical grinder. 100 g of powdered material was extracted with 1000 ml of a hydroalcoholic solvent mixture prepared by mixing 70 volume methanol and 30 volume water using soxhlet apparatus. The crude hydroalcoholic flower extract (DVHA) was filtered through Whatman filter paper No. 1 and concentrated by rotary evaporation under vacuum and then the material was evaporated to dryness with a percentage yield of 12.6 % (w/w). Extract (DVHA) was stored in the dark at a temperature of 10° C for future use.

2.3. Total phenolic content

The content of soluble phenolics in DVHA was determined using Folin-Ciocalteu assay (Vongsak, et al., 2013) with slight modifications and the volumes of the reagents added were scaled down to fit the microtiter plate volume. Folin-Ciocalteu reagent was prepared with ten-fold of dilution in water. Briefly, 40 µl of DVHA (1000 µg/ml) was mixed with 100 µl of Folin-Ciocalteu reagent in microtiter plate and 160 µl sodium carbonate (7.5%, w/v) was added to the mixture. It was allowed to left for 30 minute at room temperature with occasional shaking. The absorbance was read at 765 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA) against reagent blank containing water in place of sample. Gallic acid was used as standard to estimate total phenol content and a calibration curve (absorbance vs. µg/ml) was constructed using different concentrations of gallic acid (20-100 µg/ml). The values for TPC are presented in mg gallic acid equivalents (GAE)/g dried extract.

2.4. Total flavonoids content

The aluminium chloride colorimetric method (Pothitirat et al., 2009) was adapted for the determination of total flavonoids with minor changes. An aliquot of 120 µl of DVHA (1000 µg/ml) was mixed with an equal volume of a solution of 2% (w/v) aluminium chloride solution in a microtiter plate. The absorbance of the mixture was measured at 415 nm after 10 min of incubation against a blank without aluminium chloride solution using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). Result was expressed as mg of quercetin equivalents (QE)/g of dried extract with a calibration curve of quercetin (20-100 µg/ml).

2.5. Fourier transform infrared spectroscopy analysis

Fourier transform infrared (FTIR) spectra of DVHA were recorded in ATR mode using Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA) with a total of 30 scans at a resolution of 4 cm^{-1} in the wave number range between 4000 cm^{-1} and 525 cm^{-1} (Tarantilis et al., 2008). A background spectrum was obtained by collecting the spectrum of the clean ATR crystal immediately before acquiring the spectrum of sample. DVHA was then placed on the ATR accessory and pressed for collecting the FTIR spectrum of the sample. The study was used to acquire information on the nature of the functional groups and chemical bonds present in phytochemicals of DVHA by studying their peak values (cm^{-1}). The spectral acquisitions were done using OMNIC software supplied from the manufacturer of the spectrometer. The analysis represented qualitative information regarding the types of phytoconstituents present in the flowers of *D. volubilis.*

2.6. Identification and quantification of phenolic compounds by UHPLC

The phenolic compounds present in DVHA were identified and quantified by ultra high performance liquid chromatography using a UHPLC+ focused system consisting of a Dionex Ultimate 3000 Pump, a Dionex Ultimate 3000 auto sampler column compartment and a Dionex Ultimate 3000 variable wavelength detector (Karaman et al., 2010). A mixed standard stock solution (1 mg/ml) containing gallic acid, ferulic acid, rutin, ellagic acid, quercetin and cinnamic acid was prepared in HPLC grade methanol and subsequently different concentrations **(**6, 10, 20, 30, 40 and 50 µg/ml**)** of standard solutions were prepared by diluting the mixed standard stock solution for calibration curves in order to quantify the phenolic compounds present in DVHA. All solutions were filtered through a

0.45 µm membrane filter. Separations of phenolic compounds were performed using a C18 column (250 mm × 4.6 mm i.d.) with a particle size of 5 µm, Hypersil GOLD (Thermo Fisher Scientific, U.S.A) and column oven temperature was maintained at 25°C. The chromatographic separation was carried out using gradient elution as presented in Table 1 with a flow rate of 1.0 ml min^{-1} using 0.2 (% v/v) phosphoric acid as mobile phase A and HPLC grade methanol as mobile phase B, respectively. The injection volume was 5 µl and a wavelength of 280 nm was used for the analysis. The phenolic compounds present were quantified from calibration curves of standards. Data processing of the chromatograms was performed using Chromeleon 7, version 7.2.0.3765 software (Thermo Scientific, U.S.A).

Table 1: UHPLC gradient programme for the identification and quantification of phenolic compounds in DVHA.

Time (minute)	Flow rate (ml/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	1.0	70	30
5.00	1.0	70	30
32.00	1.0	43	57
33.00	1.0	70	30
36.00	1.0	70	30

2.7. DPPH radical scavenging activities

Antioxidant activity of DVHA was evaluated by determining its scavenging ability against DPPH radical (Yang et al., 2015). 50 µl of different concentrations (20-400 µg/ml) of DVHA were mixed with 100 µl of 0.1 mM ethanolic solution of DPPH in a microtiter plate and were recorded as A_s. Absorbance was measured after 30 min of incubation in dark at room temperature at 517 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). A control experiment was performed applying the same procedure using 50 µl water in place of sample and 100 µl of DPPH solution and the absorbance was recorded as A_c. Sample colour contribution was corrected by measuring absorbance of sample colour blank containing 50 µl sample and 100 µl of water. Ascorbic acid was used as standard. The free radical scavenging activity was calculated as percent inhibition according to the following equation:

$$\% \text{ inhibition} = ((A_c - A_s) / A_c \times 100)$$

IC_{50} value is the concentration of sample required to produce a 50% decrease in absorbance of initial DPPH concentration. IC_{50} value was calculated for expressing antioxidant activity of DVHA.

2.8. Ferric reducing antioxidant power

The reducing capability of antioxidants can be assessed by ferric reducing antioxidant power (FRAP) assay (Pascu et al., 2014) with slight modifications to accommodate microtiter plate volume which measures the absorbance at 593 nm of a blue colour solution of ferrous form that is generated from a reaction of ferric tripyridyltriazine (Fe^{3+}-TPTZ)

complex with antioxidants. Antioxidant compounds break radical chain reaction by giving hydrogen atom to the ferric complex and thus act as reducing agent. The FRAP reagent was prepared freshly by mixing 5 ml of 300 mM acetate buffer (pH 3.6) with 0.5 ml of 10 mM TPTZ (2,4,6 tripyridyl- S-triazine) in 40 mM of HCl and 0.5 ml of 20 mM of $FeCl_3.6H_2O$. The solution was incubated at 37°C. Then 10 µl of DVHA (0.5 mg/ml) was mixed with 190 µl of FRAP reagent in a microtiter plate and the mixture was allowed to stand for 30 minute in dark. Absorbance readings were measured at 593 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). Sample colour contribution was also corrected. A standard curve of $FeSO_4.7H_2O$ (0–1000 µmol/L) was plotted for determining ferric reducing activity. Fe^{2+} content in DVHA is evaluated as mean ± SEM (n=3) and results are expressed as µmol Fe^{2+}/g dried extract and compared with ascorbic acid.

2.9. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined using 2- deoxy-D-ribose oxidation method (Hajiaghaalipour et al., 2015). Stock solutions of ferric chloride ($FeCl_3$, 100 mM), hydrogen peroxide (H_2O_2, 1.25 mM), 2- deoxy-D-ribose (2.5 mM), ascorbic acid (AA, 100 mM), 0.5% thiobarbituric acid (TBA) diluted with sodium hydroxide (0.025 M), 2.8% trichloroacetic acid (TCA), DVHA (20-400 µg/ml) were prepared. The volumes of reagents added were minimized to accommodate microtiter plate. The experiment was performed by adding 40 µl of $FeCl_3$, 40 µl of H_2O_2, 40 µl of 2- deoxy-D-ribose and 40 µl of AA to 40 µl of different concentrations of DVHA in a microtiter plate and it was incubated at 37°C for one hour. The incubated mixtures were mixed with 20 µl of TBA and 20 µl of TCA and it was maintained at 100°C for 30 minute. It was then cooled down to room

temperature and absorbances of the pink coloured mixtures developed were measured at 532 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA) and were recorded as A_s. A control experiment was performed using 40 µl of water in place of DVHA and the absorbance of the solution at 532 nm was recorded as A_c. The samples colour contributions were also corrected by measuring absorbances of solutions containing 40 µl extract (DVHA) and 200 µl water at 532nm. Ascorbic acid was used as standard. The hydroxyl radical scavenging activity was calculated as percent inhibition according to the following equation:

$$\% \text{ inhibition} = ((A_c - A_s) / A_c \times 100)$$

IC_{50} value of DVHA was calculated and compared with ascorbic acid using the formula.

2.10. Superoxide radical scavenging activity

Nitro blue tetrazolium (NBT) method was used for evaluation of superoxide radical scavenging activity of DVHA (Shukla et al., 2009). Hydroxylamine hydrochloride generates superoxide radical (O_2^-) through auto oxidation in presence of NBT, which gets reduced to nitrile. Nitrile in presence of ethylenediaminetetraacetic acid (EDTA) shows a colour that is measured at 560 nm. Volumes of the reagents were scaled down to accommodate with the volume of microtiter plate. 50 µl of different concentrations of DVHA (20-400 µg/ml) were separately mixed with a reaction mixture consisting of 50 µl of sodium carbonate (50mM), 20 µl of NBT (24mM), 20 µl of EDTA (0.1mM) in a microtiter plate and absorbance readings were measured immediately at 560 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). 20 µl of hydroxylamine hydrochloride was then added to the reaction mixtures to start the

reaction and it was incubated at 25°C for 15 minute and the reduction of NBT was measured at 560 nm. Ascorbic acid was used as standard. The hydroxyl radical scavenging activity was calculated as percent inhibition according to the following equation:

% inhibition = $((A_c-A_s) / A_c \times 100)$, where A_c was the absorbance of control reaction mixture (50 µl water was used in place of sample) and A_s was the absorbance of sample reaction mixture at 560 nm. The samples colour contributions were also corrected by measuring absorbances of solutions containing 50 µl extract (DVHA) and 110 µl water at 560 nm. IC_{50} value of DVHA for superoxide radical scavenging activity was determined and compared with ascorbic acid using the formula.

2.11. Total antioxidant capacity

The total antioxidant capacity (TAC) of DVHA was evaluated by phosphomolybdenum method (Hammi et al., 2015) with slight modifications and the volumes of reagents added were adjusted to accommodate microtiter plate volume. In the method Mo(VI) is reduced to Mo(V) by the sample and a green phosphate/Mo(V) complex formed at acidic pH is measured at 695 nm. 20 µL of DVHA (500 µg/ml) was mixed with 200 µL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the mixture was incubated at 95°C for 90 min and cooled down to room temperature. The absorbance of the solution was measured at 695 nm against a blank. The antioxidant activity was expressed as mg gallic acid equivalents (GAE)/g dried extract.

2.12. Nitric oxide radical scavenging activity

Antioxidant activity of DVHA was evaluated by determining its scavenging ability against nitric oxide radical (Hajiaghaalipour et al., 2015). Sodium nitroprusside, at physiological pH, spontaneously produces nitric oxide which reacts with oxygen to produce nitric ions that can be measured by the Greiss reaction. Nitric oxide scavengers compete with oxygen leading to reduced production of nitric oxide radical. 50 µl of various concentrations (20-400 µg/ml) of DVHA was mixed with 50 µL of sodium nitroprusside (5 mM) in a microtiter plate and incubated for 60 minute. 100 µL of Greiss reagent was added to the mixtures and incubated for 5 minute. Griess reagent was prepared by mixing equal amount of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NED). The absorbance of the chromophores formed in the diazotization reaction of nitrile with sulphanilamide and subsequent coupling with NED were measured at 532nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The hydroxyl radical scavenging activity was calculated as percent inhibition according to the following equation:

% inhibition = $((A_c-A_s) / A_c \times 100)$, where A_c was the absorbance of control reaction mixture (50 µl water was used in place of sample) and A_s was the absorbance of sample reaction mixture at 532 nm. The samples colour contributions were also corrected by measuring absorbances of solutions containing 50 µl extract (DVHA) and 150 µl water at 532 nm. Ascorbic acid was used as a standard. IC_{50} value of DVHA and ascorbic acid were calculated.

2.13. α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity of DVHA is based on hydrolysis of chromogenic substrate *p*-nitrophenyl-α-D-glucopyranoside (PNPG) by α-glucosidase to yellow coloured *p*-nitrophenyl (Shukla et al., 2016). 50 µl of different concentrations (0.5-5 mg/ml) of DVHA was mixed with 100 µl of phosphate buffered saline (PBS, 100 mM, pH 6.9) containing α-glucosidase (1 unit/ml) in a microtiter plate and incubated at 25°C for 10 minute. 50 µl of PNPG (5 mM) prepared in PBS (100 mM, pH 6.9) was mixed to the each reaction mixture wells in the microtiter plate and incubated for 5 minute at 25°C. The absorbances of mixtures were measured at 405 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The α-Glucosidase inhibitory activity was presented as percent inhibition according to the following equation:

% inhibition = $((A_c-A_s) / A_c \times 100)$, where A_c was the absorbance of control reaction mixture (50 µl PBS was used in place of sample) and A_s was the absorbance of sample reaction mixture at 405 nm. The samples colour contributions were also corrected by measuring absorbances of solutions containing 50 µl extract (DVHA) and 150 µl PBS at 405 nm. Acarbose was used as a standard. IC_{50} value of DVHA and acarbose for enzyme inhibition were calculated.

2.14. α-Amylase inhibitory activity

DVHA was evaluated for α-amylase inhibitory activity (Yu et al., 2015). Stock solutions of starch (1% w/v) and α-amylase (0.5 mg/ml) were prepared in 20 mM of phosphate buffered saline with pH 6.9, containing 6 mM sodium chloride. 25 µl of different concentrations (0.5-5 mg/ml) of DVHA were mixed with 25 µl of starch solution in a microtiter plate and

incubated for 10 minute at 25 °C. 25 µl of α-amylase solution was then added to the mixtures and incubated at 25°C for 10 minute. The reaction was terminated by adding 50 µl of dinitrosalicylic acid colour reagent and the reagent mixtures were immediately heated in boiled water for 10 min and cooled down to room temperature. All the solutions in the microtitre plate were diluted by adding 50 µl of distilled water and the absorbences were measured at 540 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The α-amylase inhibitory activity was calculated as percent inhibition according to the following equation:

% inhibition = $((A_c-A_s) / A_c \times 100)$, where A_c was the absorbance of control reaction mixture (20 µl water was used in place of sample) and A_s was the absorbance of sample reaction mixture at 540 nm. Acarbose was used as a standard. IC_{50} value of DVHA and acarbose for enzyme inhibition were calculated.

2.15. Statistical analysis

All the experiments were performed in triplicate. The results are presented as mean ± standard error of the mean (SEM). GraphPad PRISM 6 software, USA was used for statistical analysis.

3. Results and discussion

3.1. Total phenolic content

The total amount of phenolic content present in DVHA was quantified as gallic acid equivalents (GAE). Total phenolic content of *D. volubilis* was 39.82 ± 1.22 mg GAE/g of DVHA (Table 2).

Table 2: Total phenolic content, total flavonoids content, ferric reducing antioxidant power and total antioxidant capacity of hydroalcoholic flower extract of *D. volubilis*.

Extract	Total phenolic content (mg GAE/g of DVHA)	Total flavonoids content (mg QE/g of DVHA)	Ferric reducing antioxidant power (µmol Fe^{2+}/g of DVHA)	Total antioxidant capacity (mg GAE/g of DVHA)
DVHA	39.82 ± 1.22	27.50 ± 0.87	176.47 ± 3.18	39.68 ± 1.62

DVHA: Hydroalcoholic flower extract of *D. volubilis.*

GAE: Gallic acid equivalent.

QE: Quercetin equivalent.

Results were expressed as mean ± SEM (n = 3).

The antioxidant and α-amylase, α-glucosidase inhibitory activity of the plant were due to the presence of phenolic phytoconstituents. Plant polyphenols are therapeutically very useful phytoconstituents having hydroxyl groups which exhibit scavenging activity (Shukla et al., 2009). Phenolic compounds are associated with antioxidative action and can inactivate α-amylase, α-glucosidase through non-specific binding to enzymes (Yu et al., 2015). Plant polyphenolic compounds are gaining much attention to food and pharmaceutical industry because they help to improve quality and nutritional aspects of food with the ability to inhibit oxidative degradation of lipids as well as health promoting and disease preventing properties (Wojdyło et al., 2007; Tan et al., 2017).

3.2. Total flavonoids content

The content of total flavonoids present in DVHA was presented as quercetin equivalents (QE). Total flavonoids content of *D. volubilis* was 27.50 ± 0.87 mg QE/g of DVHA (Table 2). Flavonoids are low molecular weight polyphenolic secondary metabolites having C_6-C_3-C_6 carbon frame which are widely distributed in green plant kingdom (Samanta et al., 2011). Flavonoids have antioxidant activity and hydroxyl groups in flavonoids are accountable for free radical scavenging activity (Kumkrai et al., 2015) and they also possess inhibitory activity on carbohydrate hydrolyzing enzymes (Olaokun et al., 2013). Flavonoids are widely present in plants and the flowers of *D. volubilis* can be considered as an important flavonoid-rich food which can protect human beings from diseases related to oxidative stress.

3.3. Fourier transform infrared spectroscopy analysis

A Fourier transform infrared (FTIR) spectrum of DVHA is presented in Fig. 1. The presence of O-H stretching vibration band at 3266 cm^{-1}, C-O stretching vibration band at 1027 cm^{-1} and O-H bending vibration band at 1393 cm^{-1} are due to the phenolic compounds (Silverstein et al., 2005). The characteristic O-H stretching vibration band at 2927 cm^{-1} and C=O stretching vibration band at 1700 cm^{-1}, C-O stretching vibration band at 1304 cm^{-1} and O-H bending vibration band at 1430 cm^{-1} are due to presence of phenolic acids. The FTIR spectra (Fig. 1) show the presence of two bands of strong intensity at 2855 cm^{-1} and 2960 cm^{-1}, characteristic of C-H bonds of aliphatic CH_2 and CH_3 groups, respectively. The spectrum shows the presence of aromatic hydrocarbons due to the presence of low frequency range bands between 900-675 cm^{-1} representing out-of-plane bending of ring C-

H bonds and in-plane bending bands in the region of 1300-1000 cm^{-1}, C-C stretching band within the ring in the region of 1600-1585 cm^{-1} and 1500-1400 cm^{-1}. The band at 1625 cm^{-1} is due to conjugation of an alkene double bond with an aromatic ring. The FTIR spectrum of extract may serve as a quality control tool for identification of the plant material. The Fourier transform infrared spectroscopy study reveals that the characteristic absorption bands in the spectrum of DVHA confirms the richness of the extract of flower of *D. volubilis* in phenolic compounds.

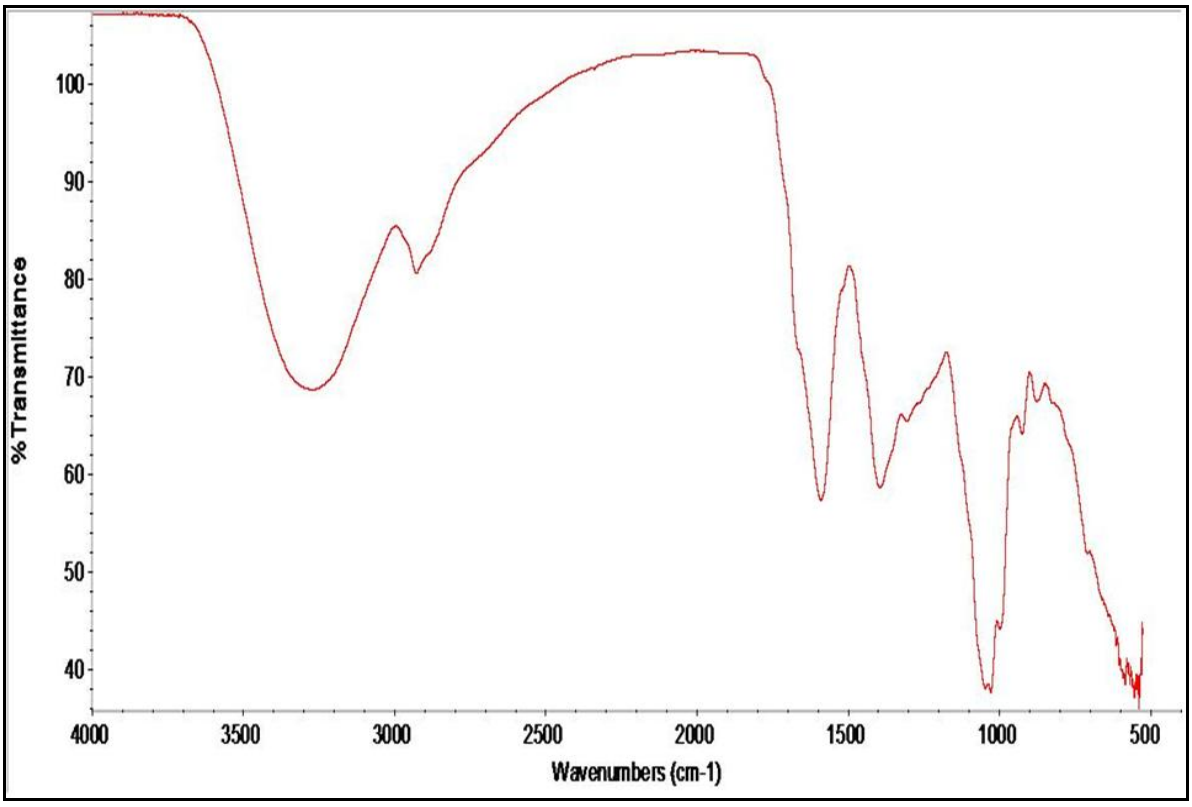


Fig. 1: Fourier transform infrared spectra of the extract of flower of *Dregea volubilis*.

3.4. Identification and quantification of phenolic compounds by UHPLC

UHPLC analysis was performed to identify and quantify the phenolic phytochemicals present in flower of *D. volubilis.* The phenolic compounds present in the plant can be considered as markers in the quality control of the herbal extract. The flower of the plant is known to contain rutin and quercetin (Panda et al., 2006). The present study shows that the DVHA contains gallic acid, ferulic acid, ellagic acid and cinnamic acid.

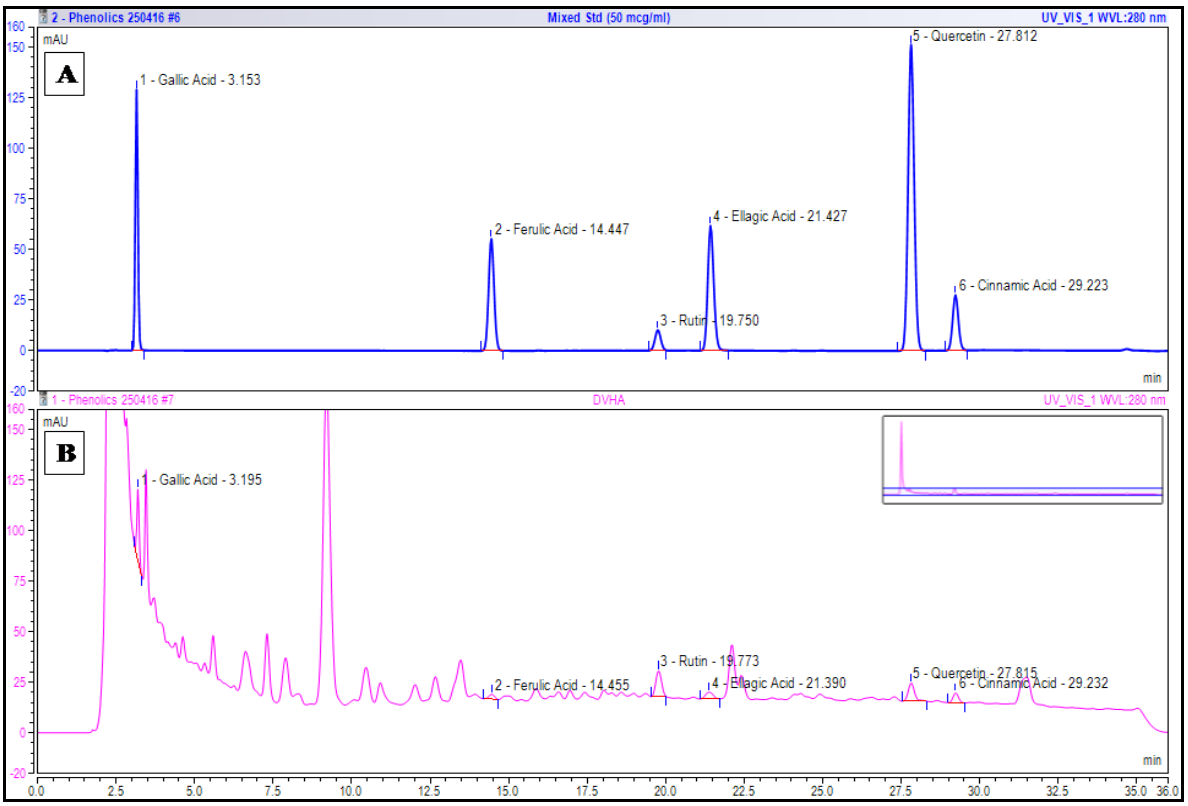


Fig. 2: UHPLC chromatograms of a mixed standard of phenolic compounds (A) and the extract of flower of *Dregea volubilis* (B) as detected at 280 nm (1: Gallic acid, 2: Ferulic acid, 3: Rutin, 4: Ellagic acid, 5: Quercetin, 6: Cinnamic acid).

The phenolic compounds present in DVHA were identified by comparing the retention times of the standards with that of the sample and the retention times of gallic acid, ferulic acid, rutin, ellagic acid, quercetin and cinnamic acid were found to be 3.153, 14.447, 19.750, 21.427, 27.812 and 29.223 minute, respectively (Fig. 2). The contents of the phenolic compounds present in DVHA were presented in Table 3. The phenolic compounds can act as antioxidants and OH groups present in phenolic compounds form strong hydrogen linkages with the catalytic residues of the enzymes involved in type-2 diabetes (Ang et al., 2015). It was not possible to analyse all the peaks in the chromatogram of sample due to unavailability of corresponding standards for the HPLC analysis. The flower of *D. volubilis* can be considered as a valuable natural source of polyphenolics with medicinal values.

Table 3: The content of phenolic constituents (µg/g of DVHA) detected and quantified in the flower of *D. volubilis* using UHPLC.

Extract	Gallic acid	Ferulic acid	Rutin	Ellagic acid	Quercetin	Cinnamic Acid
DVHA	412.36 ± 2.29	162.72 ± 0.89	386.25 ± 2.00	208.80 ± 2.00	306.85 ± 2.24	213.71 ± 2.14

DVHA: Hydroalcoholic flower extract of *D. volubilis* (values in µg/g of DVHA).

Results were expressed as mean ± SEM (n = 3) on dry weight basis.

3.5. DPPH radical scavenging activities

The decrease in absorbance of DPPH solution at 517 nm implies the reduction of DPPH to yellow coloured diphenylpicrylhydrazine which is initiated by antioxidants and the radical scavenging activity of DVHA was compared with ascorbic acid and the IC_{50} values were found to be 237.86 ± 1.05 µg/ml and 27.18 ± 0.15 µg/ml for DVHA and ascorbic acid, respectively (Table 4). DPPH is a stable lipophilic free radical which becomes a diamagnetic molecule by taking an electron or hydrogen radical at room temperature (Shukla et al., 2009). A high level of antioxidants in the extract is associated with greater radical scavenging activity and it is related to lower IC_{50} value.

Table 4: Radical scavenging and carbohydrate hydrolyzing enzymes inhibitory activities of hydroalcoholic flower extract of *D. volubilis*.

	IC_{50} value					
	DPPH radical scavenging activity	**Hydroxyl radical scavenging activity**	**Superoxide radical scavenging activity**	**Nitric oxide radical scavenging activity**	**α-Glucosidase inhibitory activity**	**α-Amylase inhibitory activity**
DVHA	237.86 ± 1.05	170.67 ± 0.98	219.07 ± 1.25	196.38 ± 1.49	3780.09 ± 21.19	360.68 ± 1.26
Ascorbic acid	27.18 ± 0.15	89.64 ± 0.50	59.33 ± 0.44	48.79 ± 0.26	-	-
Acarbose	-	-	-	-	489.38 ± 1.09	42.62 ± 0.23

DVHA: Hydroalcoholic flower extract of *D. volubilis* (values in µg/ml).

Results were expressed as mean ± SEM (n = 3)

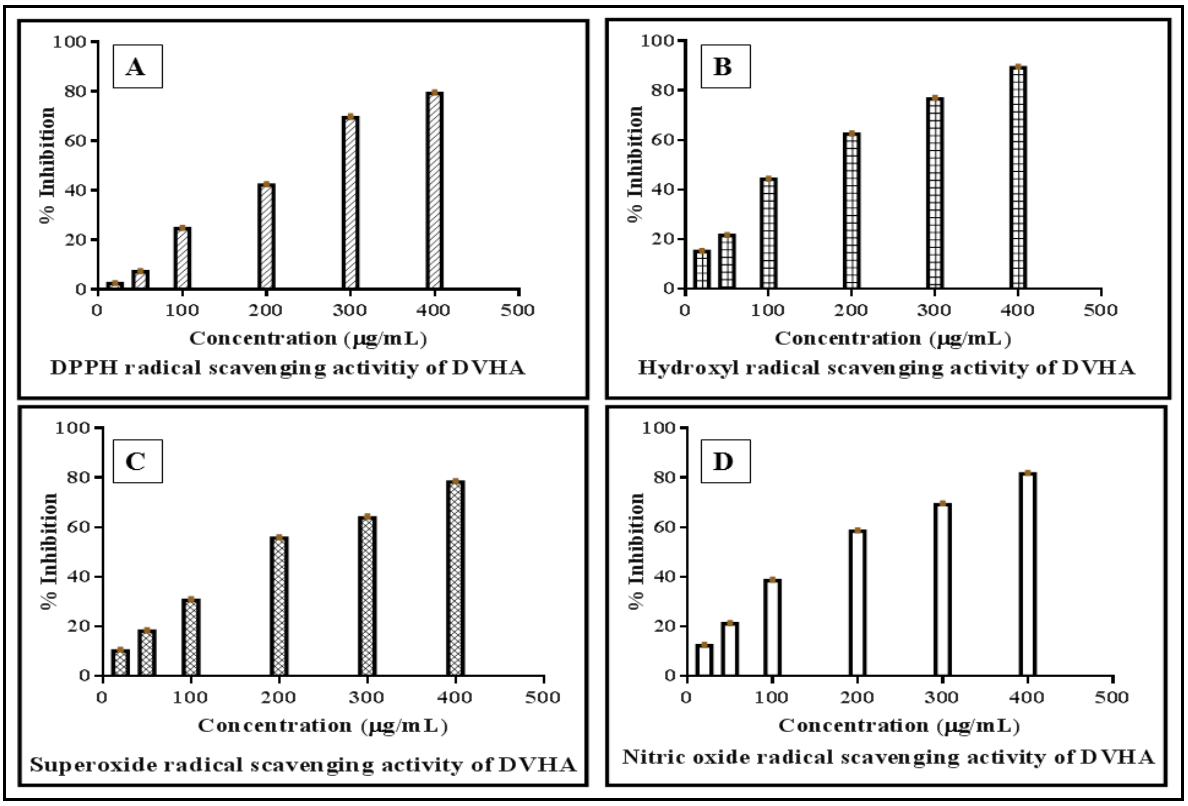


Fig. 3: Free radical scavenging activity (A, DPPH radical; B, hydroxyl radical; C, superoxide radical; D, nitric oxide radical) of the flower extract of *Dregea volubilis.*

DVHA in different concentrations (20, 50, 100, 200, 300 and 400 µg/ml) range revealed DPPH radical scavenging activity in a dose dependent manner (2.32%, 7.16%, 24.50%, 42.12%, 69.32% and 79.12%) in the study (Fig. 3).

3.6. Ferric reducing antioxidant power

FRAP assay procedure is a novel method for evaluation of antioxidant activity. Antioxidants form an intense blue coloured ferrous-tripyridyltriazine complex through ferric to ferrous ion reduction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex (Shen et al., 2016). Antioxidants contained in various foods act as reductants in the redox linked colorimetric reaction and the antioxidant potential of DVHA and ascorbic acid were found

to be 176.47 ± 3.18 μmol Fe^{2+}/g and 2316.63 ± 23.71 μmol Fe^{2+}/g, respectively (Table 2). The high values of total phenolics and flavonoids present in DVHA are responsible for the antioxidant activity of flowers of *D. volubilis*. FRAP assay method is a useful technique for quick estimation of total antioxidant activity of various antioxidants in vegetables.

3.7. Hydroxyl radical scavenging activity

Hydroxyl radical is a highly reactive free radical formed in biological system in a state of oxidative stress. The free radical is responsible for the pathogenesis of various chronic diseases. The radical causes joining of nucleotides in DNA leading to DNA strand breaking which is responsible for carcinogenesis, mutagenesis and cytotoxicity (Shukla et al., 2009). Oxygen radicals have capability to damage DNA by attacking either at sugar or the base of DNA and causes free radical pathology. The inhibitory effect of DVHA on free radical mediated deoxyribose damage was evaluated through iron (II) dependent DNA damage assay. DVHA was capable of scavenging hydroxyl radical at different concentrations (20, 50, 100, 200, 300 and 400 μg/ml) in a dose dependent manner (15.12%, 21.52%, 44.18%, 62.39%, 76.58%, 89.12%) and the IC_{50} values for DVHA and ascorbic acid were found to be 170.67 ± 0.98 μg/ml and 89.64 ± 0.50 μg/ml, respectively (Table 4). The capability of DVHA to scavenge hydroxyl radical is associated with its antioxidant activity and thus DVHA can control process of lipid peroxidation. Phenolic compounds present in DVHA are responsible for free radical scavenging activity due to their high molecular weight, proximity of many aromatic rings and hydroxyl groups.

3.8. Superoxide radical scavenging activity

Superoxide radical is a reactive oxygen species which is known to be harmful to cellular components and DNA leading to diverse ailments (Shukla et al., 2009). A number of methods are now available for the generation of superoxide radicals and thus superoxide radical scavenging activity of antioxidants are evaluated. In the study auto oxidation of hydroxylamine produced superoxide radicals in presence of NBT and the decrease of absorbance due to reduction of NBT in presence of antioxidants was measured at 560nm. DVHA at different concentrations (20, 50, 100, 200, 300 and 400 μg/ml) showed superoxide radical scavenging activity in a dose dependent manner (10.23%, 18.16%, 30.58%, 55.62%, 63.78% and 78.17%) in the experiment (Fig. 3). The IC_{50} values for DVHA and ascorbic acid were found to be 219.07 ± 1.25 μg/ml and 59.33 ± 0.44 μg/ml, respectively (Table 4). The superoxide radical scavenging activity of DVHA may be due to presence of bioactive constituents.

3.9. Total antioxidant capacity

A green phosphate/Mo (V) complex with maximal absorption at 695 nm was formed in the phosphomolybdenum method and the absorbance value was proportional to antioxidant power of DVHA. The reducing capacity or electron donating power of the antioxidants to molybdenum was evaluated in the assay. The reducing ability of a chemical entity may play as a remarkable indicator of its potential antioxidant activity. The total antioxidant capacity of *D. volubilis* was found to be 39.68 ± 1.62 mg GAE/g of DVHA (Table 2). The finding obtained in phosphomolybdenum assay indicates that the phytoconstituents present in DVHA act as antioxidants.

3.10. Nitric oxide radical scavenging activity

Nitric oxide, a reactive oxygen species, causes oxidative damage to the membrane lipid peroxidation, DNA fragmentation and lipoprotein oxidation which plays an important role in various types of diseases (Taira et al., 2015). Various concentrations of DVHA (20, 50, 100, 200, 300 and 400 μg/ml) showed 12.31%, 21.12%, 38.53%, 58.41%, 69.12% and 81.51% inhibition, respectively (Fig. 3). The results showed that the plant extract exhibited nitric oxide radical scavenging activity in a dose dependent manner. IC_{50} values for DVHA and ascorbic acid were found to be 196.38 ± 1.49 μg/ml and 48.79 ± 0.26 μg/ml, respectively (Table 4). Phenolic compounds are free radical scavengers due to specific structural features and catechol group present in the molecule is required for excellent nitric oxide radical scavenging activity. Flavonoids exert their bioactivity through scavenging of nitric oxide radical. Polyphenols present in DVHA plays an important role for nitric oxide radical scavenging activity.

3.11. α-Glucosidase inhibitory activity

A fruitful approach to manage diabetes mellitus, especially the non-insulin-dependent Type II diabetes, involves control over excessive rise of the blood glucose level by inhibiting the starch digestive enzymes (Tan et al., 2017). α-Glucosidase, a membrane bound carbohydrate hydrolyzing enzyme present in the epithelium of small intestines, catalyzes hydrolysis of complex carbohydrates and disaccharides into absorbable monosaccharides and subsequent influx of glucose from the intestinal tract to blood vessels is responsible for postprandial hyperglycaemia (Olaokun et al., 2013). Many scientific studies have reported that vegetables and herbal extracts have the capability to inhibit activity of α-glucosidase

leading to control of blood glucose level which suggests that food resources can be utilized as dietary antidiabetic agents for controlling postprandial hyperglycaemia (Wojdylo et al., 2016; Yu et al., 2015). Natural food resources enriched with phenolic compounds strongly interacts with proteins and inhibits the enzymatic activities by forming complexes with enzymes and changing their biological structures (Yu et al., 2015). In the study, DVHA at different concentrations (500, 1000, 2000, 3000, 4000 and 5000 μg/ml) exhibited 7.21%, 11.85%, 22.93%, 44.70%, 53.15% and 64.65% inhibition of α-glucosidase, respectively (Fig. 4). The extract of flower of *D. volubilis* showed the enzyme inhibitory activity in a concentration dependent manner. The half-inhibitory concentration (IC_{50}) of DVHA and acarbose were estimated to be 3780.09 ± 21.19 μg/ml and 489.38 ± 1.09 μg/ml, respectively (Table 4). The α-glucosidase inhibitory activity of DVHA might be credited to its high content of bioactive phenolic compounds. Therefore, flower of *D. volubilis* can be regarded as a valuable food resource with health-promotion effects.

3.12. α-Amylase inhibitory activity

Natural sources with α-amylase inhibiton activity have taken great attention to researchers due to lower side effects of natural substitutes to synthetic enzyme inhibitors such as acarbose, metformin and orlistat. α-Amylase enzyme catalyze the cleavage of α-D-(1-4) glycosidic linkages of starch, amylose, amylopectin, glycogen and various maltodextrins into shorter oligosaccharides leading to production of absorbable monosaccharides and subsequent intestinal absorption (Yu et al., 2015). Plant phenolics have ability to bind with the reactive sites of α-amylase and can modify the catalytic activity of α-amylase enzyme and hence exhibit hypoglycemic effect (Kunyanga et al., 2012). The inhibitors of α-amylase retard breakdown and digestion of starch and other complex carbohydrates present in diet in

gastrointestinal tract and therefore make them useful in dietary management of Type II diabetes.

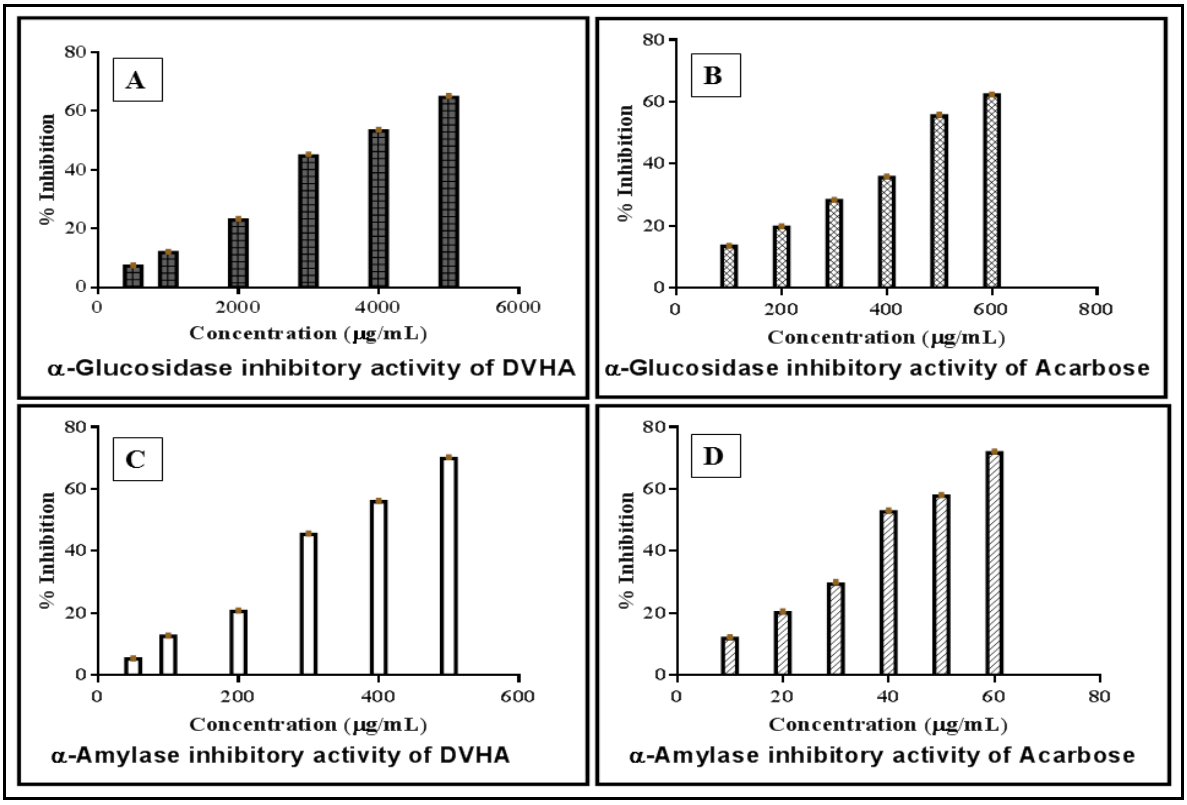


Fig. 4: Enzyme inhibition activity (A, α-glucosidase inhibitory activity of DVHA; B, α-glucosidase inhibitory activity of acarbose; C, α-amylase inhibitory activity of DVHA; D, α-amylase inhibitory activity of acarbose).

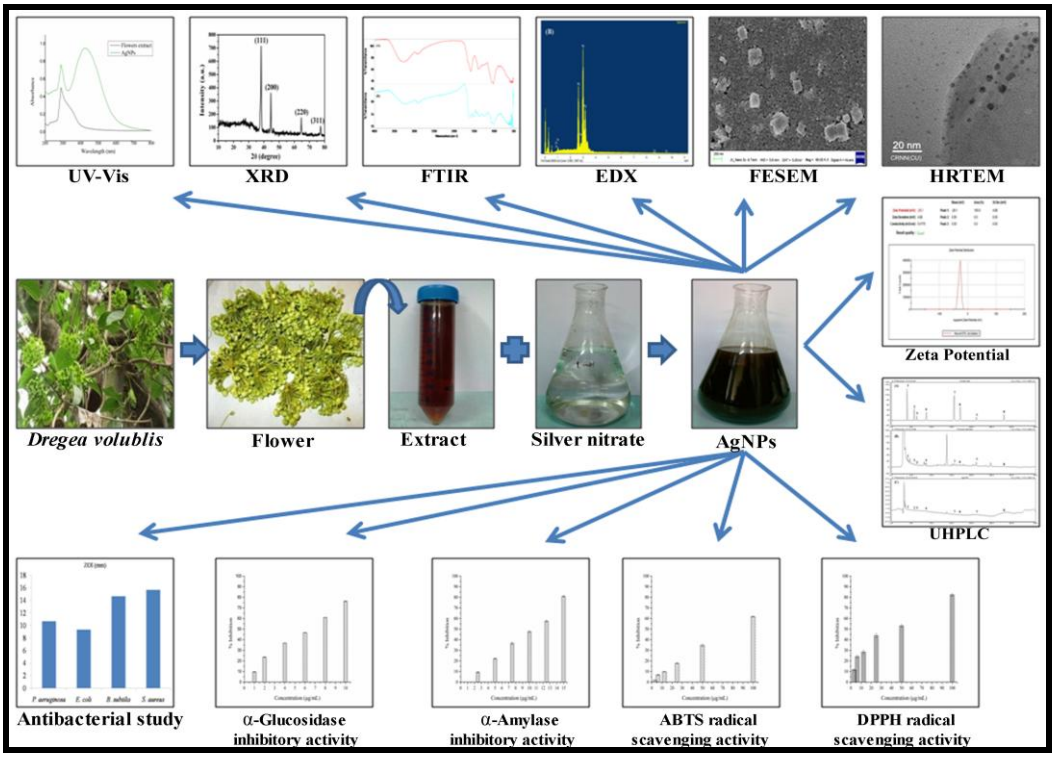
The α-amylase inhibitory activity of DVHA at various concentrations (50, 100, 200, 300, 400 and 500 µg/ml) were found to be 5.23%, 12.56%, 20.58%, 45.39%, 55.87% and 69.86% inhibition of α-amylase, respectively (Fig. 4). IC_{50} of DVHA and acarbose were found to be 360.68 ± 1.26 µg/ml and 42.62 ± 0.23 µg/ml, respectively (Table 4). The phenolics present in flower of *D. volubilis* may be attributed to its enzyme inhibition

activity. Flower of *D. volubilis* may be consumed as a promising natural source with therapeutic benefits.

4. Conclusion

This study has revealed that DVHA of the flower of *D. volubilis* exerts promising antioxidant potential towards different systems *in vitro* and α-glucosidase and α-amylase inhibiting activities *in vitro*. The phytochemical profiling of the edible vegetable provides data relating to phenolic characterization of DVHA from the view of being a source of bioactive compounds with health beneficiary effects. The flower of the plant is therefore recognized as powerful antioxidant as well as carbohydrate hydrolyzing enzymes inhibitor which is helpful in the field of nutrition and medicine. The biological activities might be due to the presence of the phenolic compounds in the flower of the plant which were identified via UHPLC. To the best of our knowledge, this is the first study in evaluating its health promoting effects. In conclusion, flower of *D. volubilis* can be considered as a nutraceutical because of their health beneficial properties which deserve to be further researched and developed in food and pharmaceutical industries.

Chapter 3



Application of *Dregea volubilis* flower in the biofabrication of silver nanoparticles

1. Introduction

Nanotechnology is a branch of science with interdisciplinary approach which has received considerable attention in recent years to researchers from different fields like life sciences, material sciences, biomedical engineering, chemistry, and physics (Pandian et al., 2016). Nanotechnology has the advantages of imparting unique and considerable changed physical, chemical and biological properties to the nanoscale materials compared to their macroscale counterparts. The synthesis of nanoscale materials and structures of different shapes and sizes has received increasing recognition due to their versatile uses in several technological and medical applications (David et al., 2014). Nanoparticles usually ranging from 1 to 100 nanometer (nm) are of great interest due to their extremely diminutive size and large surface to volume ratio with contrasting physicochemical properties compared to their bulk materials of an identical chemical composition (Iravani, 2011). Silver is a well recognized metal and its use can be traced back to times even before Neolithic revolution whereas the medicinal use of silver was recorded during 8th Century (Ravindran et al., 2013). Among the noble metal nanopaticles silver nanoparticles (AgNPs) has gained more scientific attention due to its extraordinary features of exceptional magnetic, optical and electronic properties for which AgNPs has gained extensive applications in various fields of science and technology such as catalysis, optoelectronics, photonics, pharmaceuticals, biomedical, antimicrobial products, electronics, sensing, therapeutics, and so on (Maddinedi et al., 2017). Nanoparticles can be synthesized through various physicochemical methods which produce huge number of unsafe byproducts resulting in contamination of the environment. There is a need to develop a clean, nontoxic, environmentally friendly and

cost effective method for synthesis of nanoparticles so as to remove the negative effects faced. The environmentally benign biological methods using nontoxic materials as green synthesis of metal nanoparticles are aimed at protecting the health of planet by preventing pollution, and moving towards sustainability. Several routes of synthesis of metal nanoparticles have been developed using biological materials such as microorganisms, whole plants, different parts of plants, plant extracts and marine algae from salts of the corresponding metals (Mittal et al., 2013). The biogenic synthesis of metal nanoparticles using plant extracts could be advantageous over other environmentally benign biological routes of synthesis by quashing the laborious process of maintaining cell cultures. Plants contain thousands of biologically active compounds such as alkaloids, tannins, phenolics, saponins, terpenoids, polysaccharides, proteins and vitamins which can act both as reducing agents and stabilizing agents in the synthesis of metal nanoparticles (Sharma et al., 2009). Phytochemicals with functional groups such as carbonyl, hydroxyl, and amine have the ability of reducing metal ions and capping the newly formed metal nanoparticles during their growth processes (Bar et al., 2009). Plant extracts derived from various species and parts of plants such as leaves, stems, bark, pods, flowers, and fruits, may be exploited as a key resource for the green synthesis of AgNPs which has attracted considerable attention within the last 30 years, can serve as an alternate for the existing chemical, physical, and even microbial methods (Mittal et al., 2013). Reduction of silver ions (Ag^{+}) in aqueous solution yields colloidal silver of several nanometers in diameter which are of great interest because of its distinctive properties. Multidrug resistant bacterial strains arising due to mutation, pollution and changing environmental conditions are a major problem in human life and metal nanoparticles especially AgNPs occupy an important place among such

nanoparticles as antimicrobial agents to curb such a situation (Siddiqi et al., 2018). Silver has long been used as an effective antimicrobial agent with low toxicity in human beings (Dipankar and Murugan, 2012). Oxidation is essential in living organisms to maintain biological processes for the production of energy. Oxygen derived free radicals and other reactive species, which are responsible for cell death and tissue damage, are produced continuously *in vivo* (Ozsoy et al., 2008). Excess free radicals generated in the body are responsible for many degenerative diseases. Antioxidants are capable of deactivating free radicals which play protective role in cell injury promoted by free radical induced oxidative stress. AgNPs possess a wide range of biological activities such as antioxidant and antidiabetic properties (Balan et al., 2016).

New and innovative experimental processes are of potential interest for the synthesis of silver nanoparticles (AgNPs) which are used in the field of science, technology and medicine. Bio-inspired methodologies for synthesis of nanoparticles are receiving significant attention in nanobiotechnology. Plants are an attractive resource for the biosynthesis of the AgNPs due to the presence of a wide verity of secondary metabolites which have the potentials to act as reducing and capping agents. The green synthesis of AgNPs is based on green chemistry perspectives which include selection of solvent medium, environmentally benign reducing agent, and nontoxic substances for the stability of AgNPs. Based on this approach, the present study is aimed to biosynthesize and characterizes AgNPs using aqueous extract of *D. volubilis* flowers along with investigation of its antioxidant, antidiabetic, and antibacterial properties.

2. Materials and Methods

2.1. Chemicals

Gallic acid, catechin, protocatechuic acid, para hydroxybenzoic acid, para coumaric acid, ferulic acid, rutin, cinnamic acid, quercetin, ascorbic acid, acarbose, α-glucosidase, α-amylase, aluminium chloride, PNPG (p-nitrophenyl-α-D-glucopyranoside), DPPH (1,1-diphenyl-2-picryl-hydrazyl) and ABTS were purchased from Sigma-Aldrich, St Louis, MO, USA. Folin-Ciocalteu reagent, silver nitrate, Mueller Hinton Agar and Mueller Hinton Broth were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Sodium carbonate, phosphoric acid, sulfuric acid, sodium phosphate, ammonium molybdate, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, sodium chloride, starch, sodium potassium tartrate, DNS (3, 5- dinitrosalicylic acid) and methanol for liquid chromatography were procured from Merck Life Science Private Limited, Mumbai, India. The water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used throughout all the experiments. The other reagents utilized were of analytical grade.

2.2. Preparation of aqueous flowers extract of D. volubilis

Accurately weighed 5 g of dried powdered flowers was mixed with 100 ml of Milli-Q water in a beaker and the mixture was boiled for 15 min in a water bath at 100 °C. The mixture was allowed to cool at room temperature followed by filtration using Whatman filter paper No. 1. The aqueous solution of extract was used as reducing and capping agent for silver nano particle synthesis.

2.3. Synthesis of silver nanoparticles

Silver nitrate (1 mM) solution and aqueous flowers extract of *D. volubilis* were mixed together in a ratio of 40:1 in a 1000 ml conical flask followed by autoclaving at 121 °C with pressure of 15 psi for 20 min for the biofabrication of AgNPs. The coloured reaction mixture obtained after the process of autoclaving was kept at room temperature to cool down and then centrifuged (HERAEUS BIOFUGE STRATOS Centrifuge, Thermo Fisher Scientific, USA) at 12000 rpm for 20 min to remove the unreacted reductants. The AgNPs was washed thrice with Milli-Q water and the pellet of the AgNPs thus obtained was lyophilized and stored at 10 °C until further use.

2.4. Characterization

The surface Plasmon resonance property of the AgNPs and the UV-Vis spectral analysis of the samples were confirmed by recording the spectra in the range 200-800 nm with a resolution of 1 nm using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA) against Milli-Q water as a blank (Mandal et al., 2016). Crystallinity and phase of the AgNPs were characterized by X-ray diffractometer (Ultima-III, Rigaku, Japan) (Nadagouda et al., 2014). Fourier transform infrared (FTIR) spectra of the samples were recorded in ATR mode using Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA) with a total of 30 scans at a resolution of 4 cm^{-1} in the wave number range between 4000 cm^{-1} and 525 cm^{-1} (Tarantilis et al., 2008). The morphology and size of the synthesized AgNPs was analyzed using field emission scanning electron microscope (FESEM, Supra VP35, Carl Zeiss, Germany) and high resolution transmission electron microscopy (HRTEM, JEM-

2100, JEOL, Japan) (Nisha et al., 2014; Cumberland and Lead, 2013). The elemental composition of the biogenic AgNPs was studied using energy-dispersive X-ray (EDX) spectrometry (JEOL JSM 6360) (Mandal et al., 2016). The zeta potential of the biosynthesized AgNPs was performed using Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) (Maddinedi et al., 2017).

2.5. Determination of total phenolic content

The total phenolic content (TPC) present in the sample was estimated by Folin-Ciocalteu assay (Vongsak, et al., 2013). Folin-Ciocalteu reagent was prepared by diluting it ten times in water. Briefly, 40 µl of the AgNPs (200 µg/ml) was added to 100 µl of Folin-Ciocalteu reagent in microtiter plate and 160 µl of sodium carbonate (7.5% w/v) was mixed to the reaction mixture at room temperature. The absorbance of the reaction mixture was measured at 765 nm using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA) against reagent blank containing water in place of sample after 30 min of incubation. Gallic acid was used as a standard to determine TPC of the *D. volubilis* flowers extract and AgNPs by constructing a calibration curve (absorbance vs. µg/ml) using different concentrations of gallic acid (20-100 µg/ml). The TPC are presented in mg of gallic acid equivalent (GAE)/g AgNPs.

2.6. Determination of total flavonoids content

The aluminium chloride colorimetric method was followed to quantify the total flavonoids content (TFC) present in the sample (Pothitirat et al., 2009). An aliquot of 120 µl of AgNPs (200 µg/ml) was added to 120 µl of a solution of 2% (w/v) aluminium chloride in a

microtiter plate and the absorbance of the reaction mixture was measured at 415 nm using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA) against a reagent blank containing water in place of sample after 10 min of incubation. Quercetin was taken as a standard to estimate the TFC present in the AgNPs and the result was expressed in mg of quercetin equivalent (QE)/g of the AgNPs.

2.7. Identification and quantification of phenolic compounds by UHPLC

Ultra high performance liquid chromatography (UHPLC) analysis was performed for the identification and quantification of phenolic compounds present in *D. volubilis* flowers extract and AgNPs using a UHPLC+ focused system comprising of a Dionex Ultimate 3000 Pump, a Dionex Ultimate 3000 auto sampler column compartment and a Dionex Ultimate 3000 variable wavelength detector (Karaman et al., 2010). A stock solution (1 mg/ml) containing gallic acid, catechin, protocatechuic acid, para hydroxybenzoic acid, para coumaric acid, ferulic acid, rutin and cinnamic acid was prepared and different concentrations of the mixed standard solutions were prepared by diluting the stock solution for the preparation of calibration curves in order to quantify the phenolic compounds present in the samples. The sample solution for AgNPs (1 mg/ml) was prepared in Milli-Q water and sonicated (Equitron Ultrasonic Bath, Medica Instrument Mfg. Co., Mumbai, India) for one min. The solutions used in the study were filtered through 0.45 μm syringe filter (Millex, Merck, Germany). A C18 column (250 mm × 4.6 mm i.d.) with a particle size of 5 μm, Hypersil GOLD (Thermo Fisher Scientific, U.S.A.) was used as stationary phase for the separations of phenolic compounds and column oven temperature was maintained at 25 °C.

Table 1: Gradient programme used in UHPLC study for the identification and quantification of phenolic compounds.

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	1.0	70	30
5.00	1.0	70	30
32.00	1.0	43	57
33.00	1.0	70	30
36.00	1.0	70	30

A gradient elution programme as presented in Table 1 was used for the chromatographic separation using 0.2% (v/v) phosphoric acid as mobile phase A and HPLC grade methanol as mobile phase B, respectively. The injection volume of sample was 5 µl and the wavelength was set at 280 nm for the analysis. The chromatograms were processed using Chromeleon 7, version 7.2.0.3765 software (Thermo Scientific, U.S.A.).

2.8. In vitro antioxidant assays

2.8.1. DPPH free radical scavenging activity

Antioxidant potential of the biogenic AgNPs was evaluated by determining DPPH radical scavenging ability (Yang et al., 2015). Ethanolic solution of DPPH (0.1 mM) was used for the determination of DPPH radical scavenging potential. An aliquot of 50 µl of different concentrations (3.125-100 µg/ml) of AgNPs was mixed with 100 µl of the DPPH solution in a microtiter plate and the plate was kept in dark for 30 min. The absorbance was measured as A_s at 517 nm using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA). Absorbance of reaction control containing 50 µl of water and 100 µl of DPPH solution was recorded as A_c. Absorbance of sample colour blank containing 50 µl sample and 100 µl of water was measured to correct the colour contribution of sample. Ascorbic acid was taken as standard. The scavenging of free radicals was determined by the following formula:

% inhibition = $((A_c - A_s)/A_c \times 100)$.

IC_{50} value is the sample concentration needed to produce a 50% decrease in absorbance of the reaction control. IC_{50} value was determined for expressing antioxidant activity of AgNPs.

2.8.2. ABTS radical scavenging activity

The AgNPs was evaluated for ABTS radical scavenging activity to determine antioxidant activity (Khlifi et al., 2013). ABTS solution was prepared by mixing 7 mM of ABTS and

2.5 mM potassium persulfate in a buffer at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4 and 154 mM NaCl). The reagent mixture was stored for 16 h before use in the dark at room temperature. It was further diluted up to an absorbance value of 0.70 ± 0.02 at 734 nm. Briefly, 20 µl of different concentrations (3.125-100 µg/ml) of AgNPs was mixed with 180 µl of ABTS solution in a microtiter plate followed by incubation for 6 min. The absorbance was recorded as A_s at 734 nm against a blank using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA). Absorbance of reaction control containing 20 µl of water and 180 µl of ABTS solution was recorded as A_c. Sample colour contribution was also corrected and ascorbic acid was used as standard. The ABTS radical scavenging activity was calculated using the following formula:

% inhibition = $((A_c - A_s)/A_c \times 100)$.

IC_{50} value of AgNPs was calculated and compared with standard using the formula.

2.8.3. Total antioxidant capacity

The biofabricated AgNPs was evaluated by phosphomolybdenum method (Hammi et al., 2015) for the determination of the total antioxidant capacity (TAC) wherein Mo(VI) was reduced to Mo(V) by the sample resulting in the formation of a green phosphate/Mo(V) complex at acidic pH which was recorded at 695 nm. An aliquot of 20 µl of AgNPs (200 µg/ml) was added to 200 µl of reagent solution comprising of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The reaction mixture was kept at 95 °C for 90 min and allowed to cool down to room temperature and the absorbance was measured at 695 nm against a blank using a microplate reader (SpectraMax M5, Molecular

Devices, CA, USA). Gallic acid was used as standard to determine TAC of the AgNPs by constructing a calibration curve (absorbance vs. µg/ml) using different concentrations of gallic acid (6.25-100 µg/ml) and the TAC was presented as mg GAE/g AgNPs.

2.9. In-vitro antidiabetic activity

2.9.1. Inhibition of α-amylase enzyme

The green synthesized AgNPs was evaluated for antidiabetic activity by determining α-amylase inhibitory activity (Yu et al., 2015). Solutions of starch (1% w/v) and α-amylase (0.5 mg/ml) were prepared in phosphate buffered saline (PBS, 20 mM, pH 6.9, containing 6 mM sodium chloride). An aliquot of 25 µl of different concentrations (2.5-15 µg/ml) of AgNPs were added to 25 µl of α-amylase solution in a microtiter plate and kept at 25 °C for 10 min followed by addition of 25 µl of starch solution which was further incubated at 25 °C for 10 min. Dinitrosalicylic acid colour reagent (50 µl) was added to the reaction mixtures to terminate the reaction and heated in boiled water for 10 min. It was allowed to cool down to room temperature and the reagent mixtures were diluted with 50 µl of distilled water. The absorbance was measured as A_s at 540 nm against a blank using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA) and absorbance of control reaction mixture was recorded as A_c. The colour contributions of the sample were also corrected and acarbose was taken as standard. The α-amylase inhibitory activity was calculated using the following equation:

% inhibition = $((A_c - A_s)/A_c \times 100)$.

IC_{50} values were calculated for the samples.

2.9.2. Inhibition of α-glucosidase enzyme

The biogenic AgNPs were investigated for its ability to inhibit the α-glucosidase enzyme in order to establish antidiabetic activity. The study is based on hydrolysis of PNPG by α-glucosidase to yellow coloured p-nitrophenyl (Shukla et al., 2016). Solutions of α-glucosidase (1 unit/ml) and PNPG (5mM) were prepared in PBS (100mM, pH 6.9). An aliquot of 100 µl of the α-glucosidase was mixed with 50 µl of various concentrations (1-10 µg/ml) of AgNPs in a microtiter plate and incubated for 10 min at 25 °C. The absorbance were noted as A_s at 405 nm against a blank using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA) and acarbose was taken as standard. The absorbance of control reaction mixture was recorded as A_c. The α-glucosidase inhibitory activity was calculated using the following equation:

% inhibition = $((A_c - A_s)/A_c \times 100)$.

IC_{50} values of AgNPs and acarbose for enzyme inhibition were calculated.

2.10. Antibacterial screening

2.10.1. Bacterial pathogens and their growth conditions

The biosynthesized AgNPs was evaluated for *in vitro* antibacterial activity against four different pathogenic bacterial strains of both Gram-negative like *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* ATCC 25923 and Gram-positive like *Bacillus subtilis* ATCC 6673, *Staphylococcus aureus* ATCC 29737. The bacterial strains were maintained on nutrient agar slants at 4 °C in the laboratory (Dipankar and Murugan, 2012).

2.10.2. Preparation of inoculums

A loopful of the bacterial suspension from the stock cultures were transferred to Mueller-Hinton broth (MHB) tubes to get the active cultures of the bacterial strains for studying antibacterial screening which were then incubated without agitation for 24 h at 37 °C. The cultures were diluted with fresh MHB for adjusting to 0.5 McFarland standards (Dipankar and Murugan, 2012).

2.10.3. Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the green synthesized AgNPs was the lowest concentration of the AgNPs which completely inhibited the growth of bacteria i.e. no visible formation of colony as judged by the naked eye. The MIC of the green synthesized AgNPs was determined using a microtiter plate (Bolivar et al., 2011). The diluted bacterial strains of 0.5 McFarland standards were mixed with different concentrations (10-100 µg/ml) of the AgNPs which were evaluated in a final volume of 200 µl/well in the microtiter plate. The microtiter plate containing sample was incubated for 24 h at 37 °C. The MICs were the concentrations of the AgNPs at which no turbidity in the well was observed for the respective bacterial strains.

2.10.4. Determination of zone of inhibition

The antibacterial activity of green synthesized AgNPs was performed by determining zone of inhibition (ZOI). Agar well diffusion method was followed to determine ZOI wherein Mueller-Hinton agar plates were prepared and 100 µl of bacterial cell suspension (2×10^6 CFU/ml) was spread on solid agar plates using a sterile spreader. A sterile cork borer is

used aseptically for boring the plates to produce wells of 6 mm in diameter by carefully taking out the agar plugs without disturbing the surrounding medium and 100 µl of each of the tested compounds at their respective MIC was placed into the wells. The plates were allowed to stand for 1 h for the perfusion of the samples and were kept at 37 °C for 24 h for incubation. The Petri dishes were evaluated for antibacterial activity which was measured in terms of the diameter (in mm) of ZOI. Deionized water was used as negative control and amoxicillin was used as positive control (Dipankar and Murugan, 2012).

2.11. Statistical analysis

All the experiments were performed in triplicate and the results are presented as mean ± standard error of mean (SEM). The statistical analyses were executed with GraphPad PRISM6 software, USA.

3. Results and Discussion

3.1. Characterization

3.1.1. UV-Visible spectroscopy

There are various techniques applied for characterization of nanoparticles wherein the simplest way is to observe the change in colour of the reaction solution due to surface plasmon resonance vibration of the synthesized nanoparticles, which is the first qualitative indication of the formation of nanoparticles (Mandal et al., 2016). The UV−Visible absorption spectroscopy is one of the most widely used simple and sensitive techniques to examine the formation of the metal nanoparticles as the peak positions and shapes are

sensitive to particle size of the nanoparticles (Ashokkumar et al., 2014). The bioreduction of silver ions present in the silver nitrate solution (1 mM) with the biomolecules present in the *D. volubilis* flowers extract was observed by the UV−Visible spectroscopy after allowing the reaction mixture to cool down to room temperature (Ashokkumar et al., 2014). The absorption spectra of the biogenic AgNPs and the flowers extract are showed in Fig. 1 wherein the surface plasmon resonance band at~ 420 nm confirmed spherical AgNPs formation (Ajitha et al., 2016).

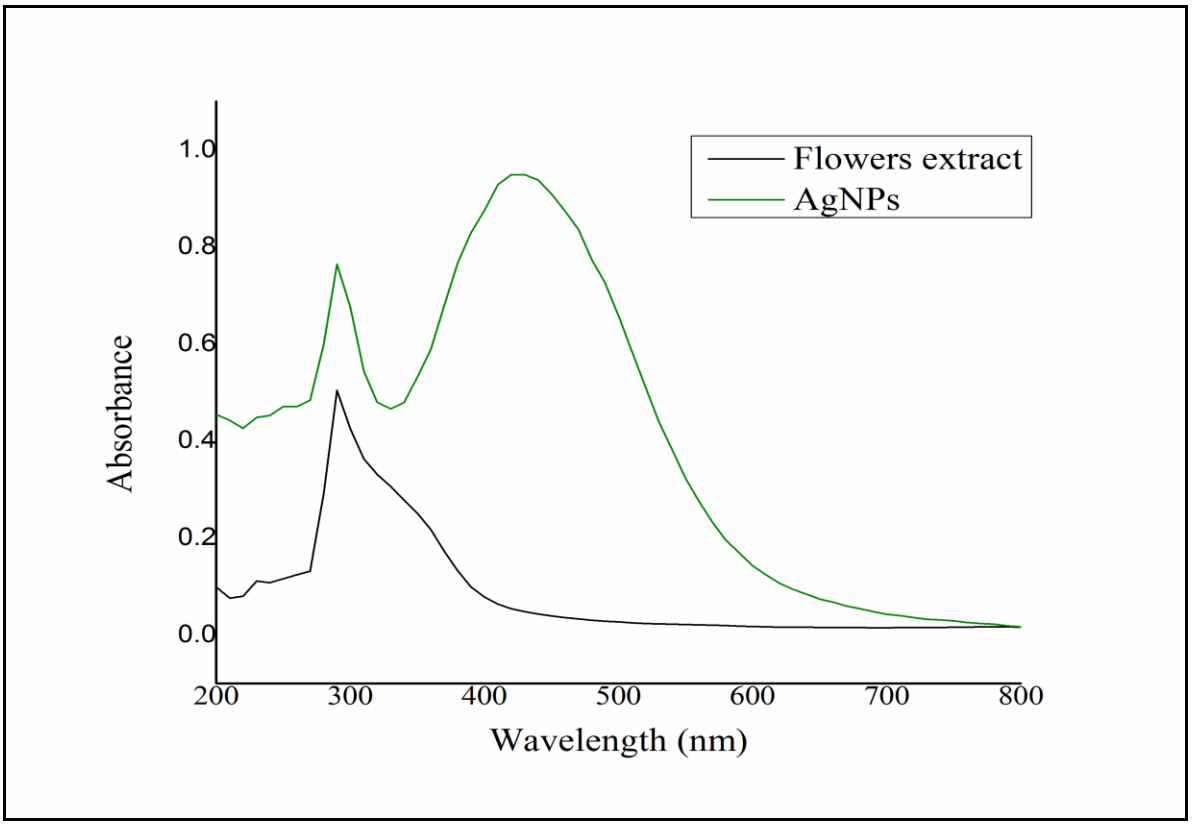


Fig. 1. UV-Vis spectroscopy absorbance spectra of *D. volubilis* flowers extract and green synthesized AgNPs solution.

The surface plasmon resonance band for the AgNPs was due to oscillation of the conducting electrons present on the AgNPs surface in resonance with certain wavelengths upon interaction with incident electromagnetic radiation (AbdelHamid et al., 2013). The broad absorbance spectrum for surface plasmon resonance band of the biofabricated AgNPs may possibly be associated with diverse class of capping agents deriving from varied groups of secondary metabolites of the flowers extract present in the reaction mixture for the biosynthesis of AgNPs. The absorbance spectra of the flowers extract treated to identical reaction condition as that of the AgNPs was recorded to conclude that there was no absorbance at ~ 420 nm wherein the strong absorbance peak at ~ 300 nm of the extract indicated the presence of polyphenols which act both as reducing and capping agent (Wang et al., 2014).

3.1.2. X-ray Diffraction Analysis

For estimation of phase purity, crystallinity of as synthesized AgNPs X-ray diffraction was carried out. Fig. 2 depicted X-ray diffraction pattern of as synthesized AgNPs using *D. volubilis* flowers extract. Four Bragg's reflections with 2θ values of 38.2^{o}, 44.6^{o}, 64.65^{o} and 77.35^{o} were observed which were readily be indexed to (111), (200), (220) and (311) planes of face centred cubic crystal (fcc) silver (JCPDS file no. PDF# 030921) which were further consistent with SAED pattern of as prepared AgNPs. Absence of any other peak easily validated phase purity of as synthesized sample. The sharp and strong peaks unambiguously revealed highly crystalline nature of as synthesized silver. From figure it is clearly evident that the intensity gets reduced for (200), (220) and (311) planes successively from that of (111) plane. Thus it can be concluded that the as synthesized silver samples are (111) plane

oriented. It is noteworthy that the relative intensity of (200) to (111) peaks in Fig. 2 (0.504) was higher than its conventional value (0.45). Thus it can be concluded that the as synthesized AgNPs were enriched in {100} facets (Sun and Xia, 2002). The average crystallite size calculated from Debye-Scherrer's formula: D = ($0.9\ \lambda / \beta\ cos\theta$) where D is the crystallite size, λ = 1.5406 A˚, β and θ represent full width at half maxima (FWHM) and Bragg's diffraction angle respectively, was 56.8 nm.

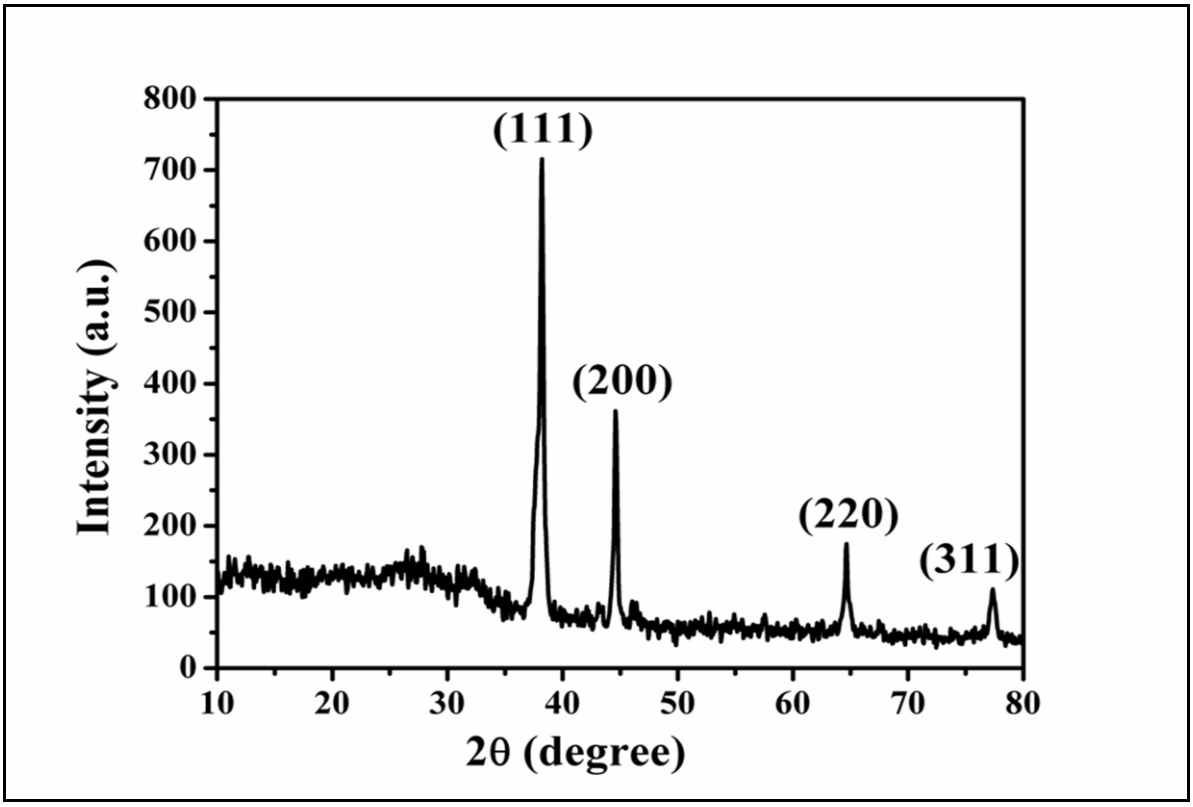


Fig. 2. XRD pattern of as synthesized AgNPs.

3.1.3. Fourier transform infrared spectroscopy

FTIR analysis was carried out to investigate the role of possible functional groups of the biomolecules present in *D. volubilis* flowers extract responsible for bioreduction of the

metal precursors as well as capping of the biogenic AgNPs leading to efficient stabilization (Zayed et al., 2012). The FTIR spectrum of the AgNPs showed significant changes in comparison to the FTIR spectrum of the flowers extract as showed in Fig. 3.

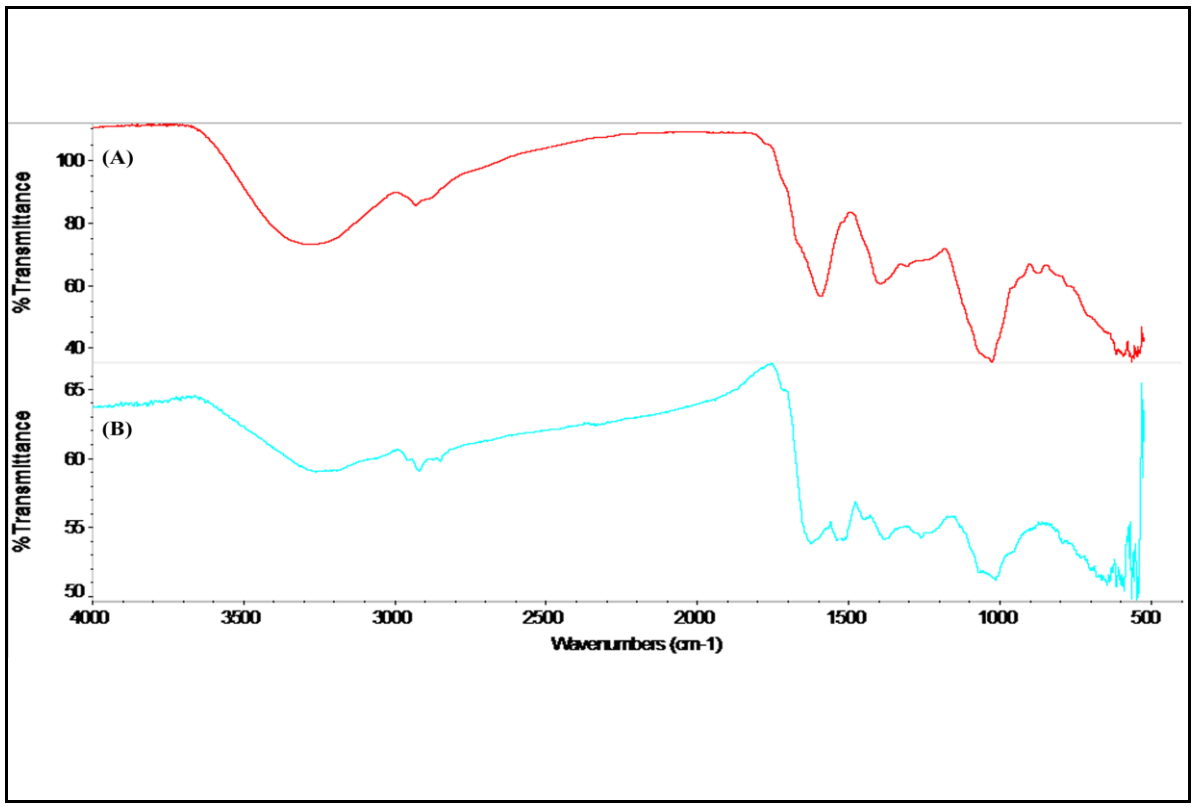


Fig. 3. FTIR spectra of (A) *D. volubilis* flowers extract and (B) biosynthesized AgNPs.

The O-H stretching vibration band at 3274 cm^{-1} was due to the presence of polyphenols in the flowers extract whereas the vibration band shifted towards shorter frequency at 3260 cm^{-1} during the formation of AgNPs establishing the involvement of the functional group (or polyphenols) as reducing agent for the biosynthesis of the AgNPs (Patra et al., 2015). The O-H stretching vibration band at 2930 cm^{-1}, C=O stretching vibration band at 1700 cm^{-1}, C-O stretching vibration band at 1308 cm^{-1} and O-H bending vibration band at 1430 cm^{-1}

present in the spectrum of extract were due to presence of phenolic acids which were also shifted in the spectrum of AgNPs confirming the involvement of the functional groups (or phenolic acids) as reducing as well as capping agents. The C-OH stretching vibration bands at 1016 cm^{-1} and 1020 cm^{-1} were observed in the FTIR spectra, which are typical for polysaccharides (Ashokkumar et al., 2014). The vibration band at 1056 cm^{-1} was assigned to the hydroxyl group in carbohydrate (Lu et al., 2014). The vibration band at 1232 cm^{-1} was attributed to C-O group of polyols such as hydroxyl flavones (Ashokkumar et al., 2014). The band at 1454 cm^{-1} was due to the methylene scissoring vibrations of the proteins (Bar et al., 2009). The band at 1612 cm^{-1} was assigned to stretching vibration of C-OH bond from proteins (Yan-yu et al., 2016). The amide I band (1654 cm^{-1}) and amide II band (1535 cm^{-1}) were found to be present in the FTIR spectrum of extract which were shifted in the spectrum of AgNPs confirming the involvement of proteins for the synthesis of AgNPs (Mandal et al., 2016; Ashokkumar et al., 2014; Patra et al., 2015). These changes in the spectrum of AgNPs suggested that the reduction and stabilization of the nanoparticles proceed via the coordination between N of the amide group and silver ions. The FTIR study supports the presence of polyphenols, protein, saccharides in the extract which play a key role in reducing, capping and subsequent formation of metallic nanoparticles. The FTIR studies have confirmed the fact that these biomolecules have the stronger ability to bind with metal leading to formation of layer covering the AgNPs to prevent agglomeration (Zayed et al., 2012). The aqueous extract of *D. volubilis* flowers played complex roles in the bioreduction by donating electrons to the silver ions present in the precursors and shape evolution of the nanoparticles (Raj et al., 2018).

3.1.4. Field emission scanning electron microscopy

Field emission scanning electron microscopy (FESEM) analysis was performed to study the topography of the green synthesized AgNPs. FESEM micrographs were taken at 1 µm (low resolution) and 200 nm (high resolution) as depicted in Fig. 4 (A)-(B).

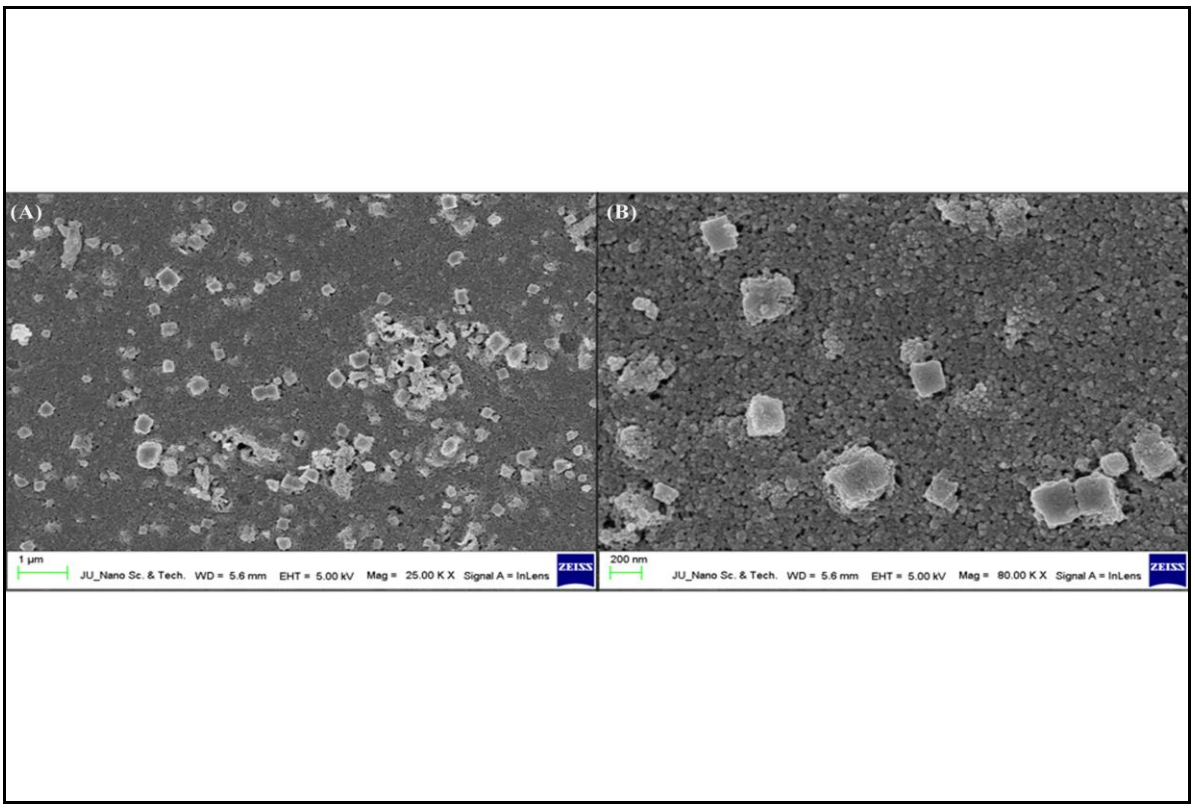


Fig. 4. (A)-(B) FESEM images of the biofabricated AgNPs at different magnifications.

The average particle size of the biogenic AgNPs was found to be 13.50 ± 0.40 nm ranging from 8.59 to 19.18 nm and it was observed that larger particles were also formed due to aggregation of nanoparticles during sample preparation (Raj et al., 2018). Homogeneous distribution of AgNPs with spherical morphology was observed . The shape of the biogenic

nanoparticles has correlated with surface plasmon resonance band at~ 420 nm. The green synthesized AgNPs were uniform in size with smooth surface.

3.1.5. Energy dispersive X-ray study

The elemental composition of the biofabricated AgNPs using flowers extract was analyzed by means of EDX spectrometry which was showed in Fig. 5 wherein the strong signals of silver was certainly due to the formation of AgNPs. The optical absorption peak was observed at 3keV which was typically for metallic silver that might be due to surface plasmon resonance (Ajitha et al., 2016). The silver detection limit was much higher than other trace elements present in the sample which indicated the prevalence of Ag spots in the elemental mapping (Ajitha et al., 2016). The EDX spectrum reflected the uniform distribution of silver throughout the as prepared sample (Ajitha et al., 2016). The presence of C and O signals in the EDX spectrum were attributed to the polyphenols and other C-containing molecules in the *D. volubilis* flowers extract which suggested that the biogenic AgNPs were bonded to phytoconstituents of the extract (Wang et al., 2014). The weak signal of Zn might be due to the biomolecules that are capping the biogenic AgNPs (AbdelHamid et al., 2013). The analysis of the EDX spectrum also revealed that the nanoparticles were in metallic form without formation of Ag_2O in it and freed from any other impurities (Raj et al., 2018). The surface of AgNPs in the FESEM images looked cloudy even under adequate resolution conditions that might be attributed to the biomolecules present as capping agent which could be correlated with the EDX spectrum (Wang et al., 2014).

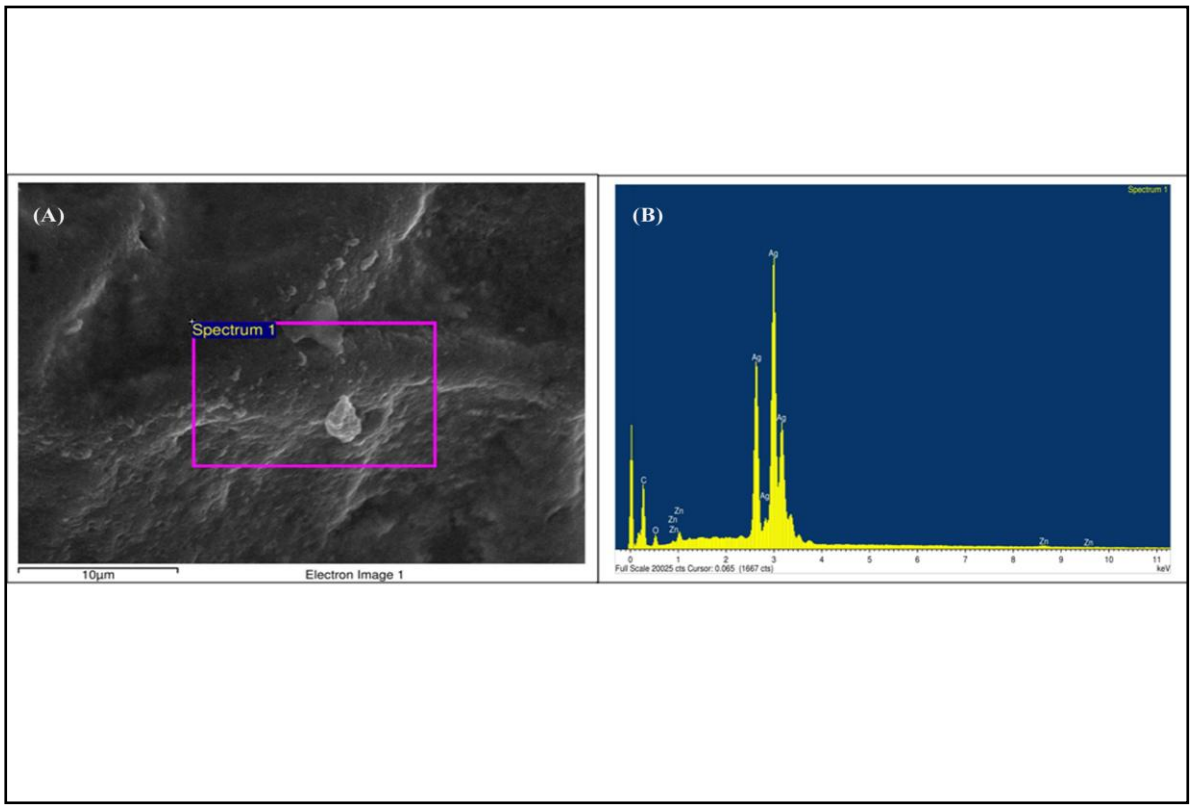


Fig. 5. (A)-(B) EDX spectrum of biogenic AgNPs.

3.1.6. High resolution transmission electron microscopy

HRTEM measurements of as prepared sample were conducted in order to estimate the particle size, size distribution and crystallinity of the biologically synthesized AgNPs using the flowers extract of *D. volubilis*. Fig. 6 (A)-(C) show the HRTEM micrographs of AgNPs at different magnifications. Fig. 6 (B) exhibits individual AgNPs along with number of aggregates with spherical morphology. It was observed that the highly monodispersed spherical AgNPs were uniformly distributed in the sample with no significant aggregation (Fig. 6 (A)). The nanoparticles were embedded in a dense matrix comprising of

biomolecules of *D. volubilis* flowers extract (Mandal et al., 2016). Careful inspection revealed that the nanoparticles were well segregated even within the aggregates (Fig. 6 (B)).

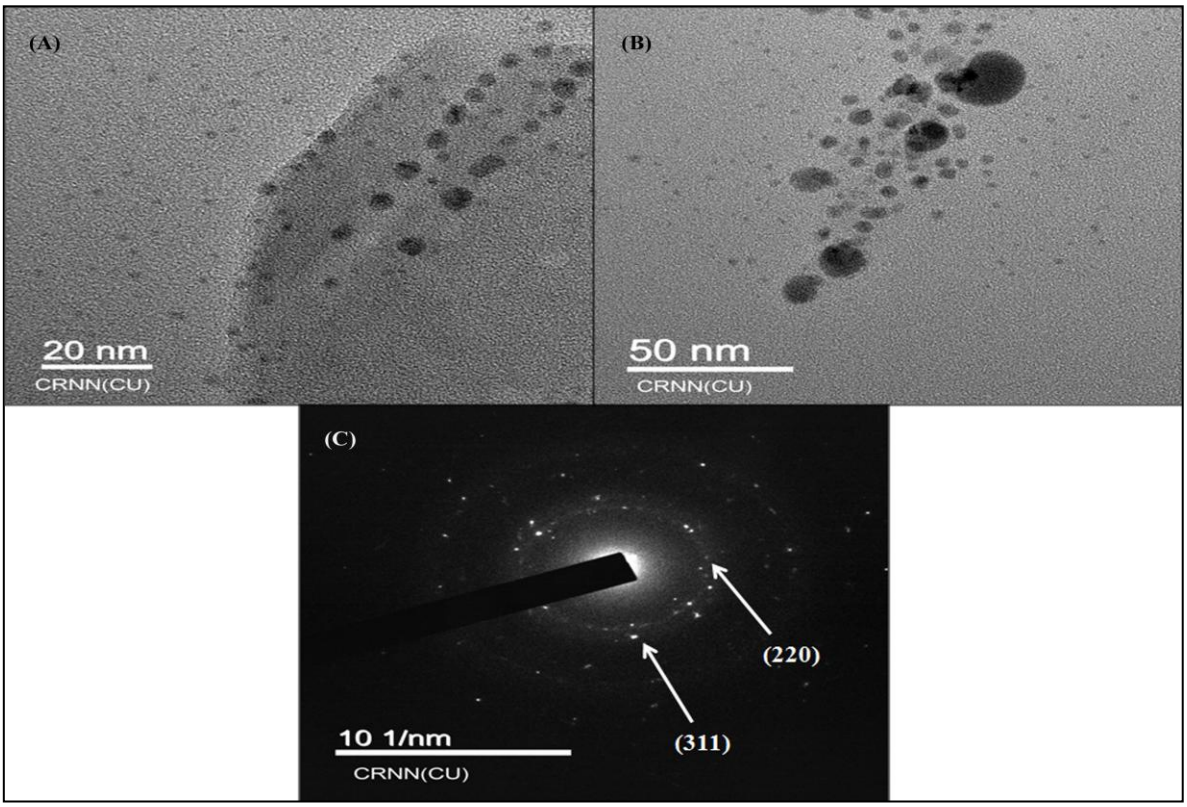


Fig. 6. (A)-(B) HRTEM micrographs of as synthesized AgNPs from *D. volubilis* flowers extract at different magnifications; (C) SAED pattern of as prepared AgNPs clearly reveals polycrystalline AgNPs.

The separation between the AgNPs within the aggregates seen in the HRTEM image could be due to good capping effect of flowers extract. The extract was utilized as protective agents by hindering the agglomeration of AgNPs through interaction with initial nanoparticles (Zayed et al., 2012). The size of the AgNPs as gathered from HRTEM images

was found to be 2.22-5.39 nm and the mean diameter of the nanoparticles was 3.45 ± 0.16 nm. In rare cases, larger diameters AgNPs were also observed in the sample and were vastly outnumbered by those with smaller particles in the HRTEM images (Kahrilas et al., 2014). Such difference in particle size between HRTEM and XRD measurement is attributed to quick agglomeration of individual AgNPs due to difference in sample preparation and highly polycrystalline nature of AgNPs as evident from (Fig. 6 (C)). The selected area electron diffraction (SAED) pattern (Fig. 6 (C)) with spots superimposed on rings revealed polycrystalline nature of AgNPs. Careful observation reveals that the diffraction rings with d-spacing of 0.1401, and 0.12, could readily be indexed as (220) and (311) reflections respectively.

3.1.7. Zeta potential study

The zeta potential of nanoparticles provides valuable information regarding surface charge and stability of nanoparticles (Zayed et al., 2012). The zeta potential analysis of the biogenic AgNPs showed a sharp peak at -27.43 ± 0.34 mV (Fig. 7) indicating the negatively charged surface of the nanoparticles. The negative zeta potential of the AgNPs might be due to the oxidized biomolecules that were capped on the surface of nanoparticles, which further generated the electrostatic repulsive forces between the neighbouring AgNPs (Maddinedi et al., 2017). The high negative charge on the surface of the biogenic nanoparticles could form a repulsive barrier that might help physical separation of the AgNPs (AbdelHamid et al., 2013). The repulsive forces between the AgNPs in the solution could help to avoid aggregation of AgNPs and high dispersity of the nanoparticles resulting in improvement of the stability of the silver nanocolloids (Yan-yu et al., 2016).

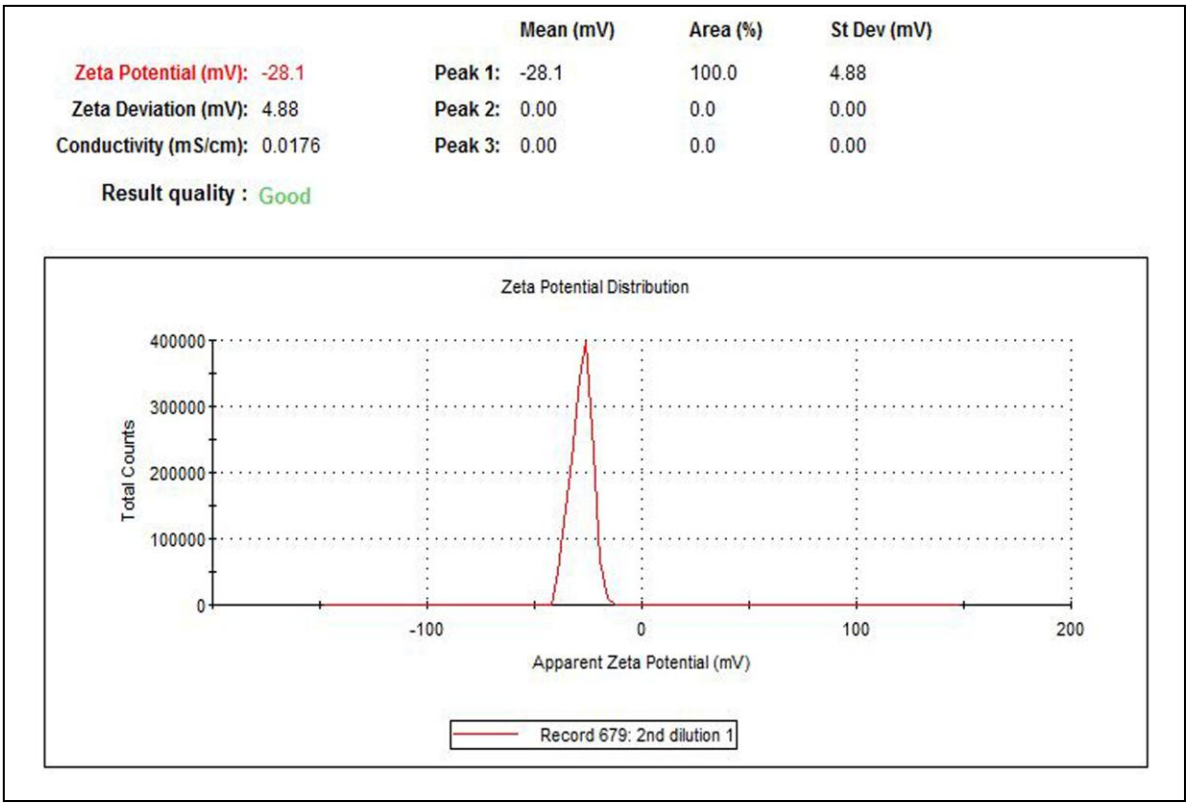


Fig. 7. Zeta potential analysis of biosynthesized AgNPs.

3.2. Determination of total phenolic content

The content of soluble phenolics in the shed dried flowers and AgNPs were found to be 9.73 ± 0.20 and 42.33 ± 1.00 mg GAE/g, respectively. Plant polyphenols are naturally occurring secondary metabolites found widely in the fruits, vegetables, cereals and beverages. Diets rich in polyphenols are therapeutically very useful as the biomolecules may protect cell components against oxidative damage leading to protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Pandey and Rizvi, 2009). The antioxidant activity of phenolic

compounds is due to their capability to donate an electron or hydrogen from phenolic hydroxyl groups wherein relatively stable phenoxy radicals are formed which are less reactive due to electron delocalization in the aromatic ring of the phenolic compounds resulting in replacement of the free radicals with higher reactivity with the less reactive species of phenoxy radicals (Bhutto et al., 2018). The phenolic compounds ranges from simple to highly polymerized structures. The radical scavenging activity of phenolic compounds depends upon the degree of hydroxylation in the aromatic ring wherein higher degree of hydroxylation in the chemical structures of phenolic compounds reveal a higher radical scavenging activity and higher propensity to reduce Ag+ to AgNPs. (Bhutto et al., 2018). The result suggested that *D. volubilis* flowers contained high content of phenolic compounds which could act as reducing agents in the formation of AgNPs and the presence of quantifiable amount of phenolic compounds in AgNPs demonstrated that the phenolic compounds could act as capping agent to prevent agglomeration of the biogenic AgNPs. The results are in good agreement with the results obtained by FTIR spectroscopy.

3.3. Determination of total flavonoids content

The total flavonoids content in the shed dried flowers and AgNPs were found to be 3.7 ± 0.04 and 117.59 ± 1.96 mg QE/g, respectively. Flavonoids are large molecular weight polyphenols consisting of diphenylpropane ($C_6C_3C_6$) skeleton with a varying number of hydroxyl groups (-OH) attached to the skeleton (Bhutto et al., 2018). The antioxidant activities of flavonoids depend on the number and arrangement of the phenolic hydroxyl groups and the degree of hydroxylation or extent of alkylation and glycosylation within each group. Flavonoids are natural antioxidants due to their redox properties with health

beneficiary effects. The result showed that the flavonoids present in *D. volubilis* flowers could act as reducing agents in the biofabrication of AgNPs. The presence of flavonoids in AgNPs suggested that the flavonoids could act as capping and stabilizing agent in the biosynthesis of AgNPs.

3.4. Identification and quantification of phenolic compounds by UHPLC

The shed dried flowers of *D. volubilis* and the biogenic AgNPs were found to contain phenolics and flavonoids and the UHPLC study was aimed to investigate the profile of phenolic compounds to have an insight on its bioactive compounds. The present study showed the presence of gallic acid, catechin, protocatechuic acid, para hydroxybenzoic acid, para coumaric acid, ferulic acid, rutin and cinnamic acid in both of the shed dried flowers of *D. volubilis* and the biogenic AgNPs. Identification of the bioactive phenolic metabolites was determined by comparing the retention times of the standard with that of the sample and the retention times were found to be 3.902, 5.643, 6.295, 8.708, 15.645, 17.187, 21.221 and 28.072 min, respectively (Fig. 8). Structurally different plant derived phenolic compounds were investigated in earlier work for antioxidant activities and correlated with their capacities to form biofabricated AgNPs which was assigned to the differences in substitution pattern and the degree of hydroxylation in the aromatic ring of the biomolecules (Bhutto et al., 2018). The antioxidant ability of a phenolic compound increases with the degree of hydroxylation, especially on the ortho or para position and the polyphenolic compounds are higher in reactivity in the synthesis of the AgNPs than the monophenols.

Phenolic compounds with multiple electron-donating groups have low anodic peak potentials and higher antioxidant activities than mono substituted phenols (Bhutto et al., 2018). Gallic acid and rutin are fast reacting as compared to other phenolic acids as reducing agents in the biogenesis of AgNPs. The contents of the phenolic compounds were presented in Table 2 which showed that the flowers of *D. volubilis* is a rich source of phenolic compounds and the presence of the biomolecules in the biogenic AgNPs suggested that the phenolic compounds played important role both as reducing and capping agents in the biosynthesis of AgNPs. The observations are in good agreement with the results obtained by FTIR and UV-Visible spectroscopy which supports the presence of phenolic phytoconstituents in the samples.

Table 2: The content of phenolic biomolecules detected using UHPLC in the shed dried flower of *D. volubilis* and biogenic AgNPs.

Sample	Gallic acid	Catechin	Protocatechuic acid	para Hydroxybenzoic acid	para Coumaric acid	Ferulic acid	Rutin	Cinnamic acid
***D. volubilis* flowers (µg/100g)**	234.08 ± 2.09	25.22 ± 0.43	107.22 ± 5.27	162.47 ± 3.84	82.10 ± 1.45	64.30 ± 2.33	663.53 ± 12.99	63.99 ± 4.80
AgNPs (µg/g)	122.79 ± 0.35	94.56 ± 0.99	98.89 ± 0.73	180.50 ± 1.34	100.52 ± 0.10	61.82 ± 0.34	255.87 ± 0.98	110.09 ± 0.42

Results were represented as mean ± SEM (n= 3).

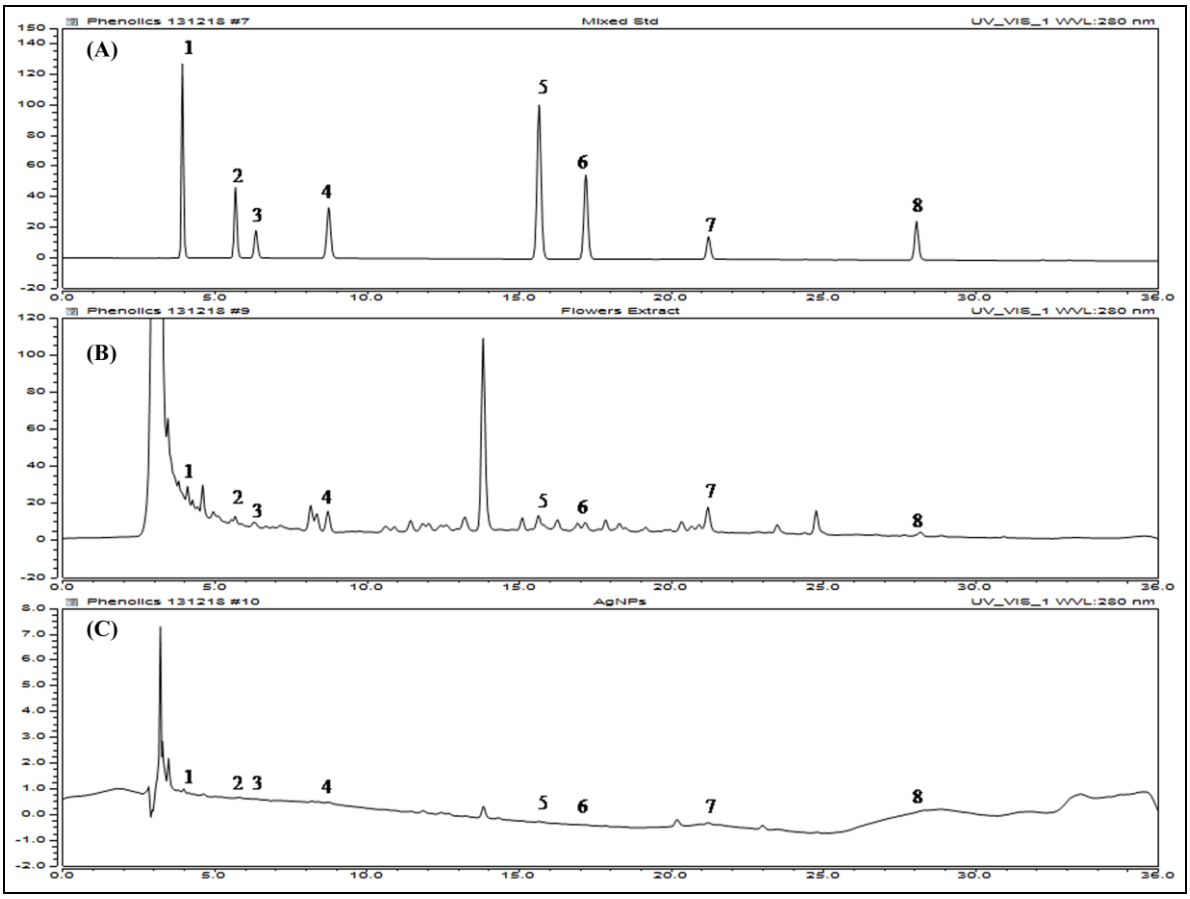


Fig. 8. UHPLC chromatograms of (A) standard of phenolic compounds, (B) the aqueous extract of *D. volubilis* flowers and (C) the biogenic AgNPs (1: gallic acid, 2: catechin, 3: protocatechuic acid, 4: para hydroxybenzoic acid, 5: para coumaric acid, 6: ferulic acid, 7: rutin, 8: cinnamic acid).

3.5. In vitro antioxidant assays

3.5.1. DPPH free radical scavenging activity

Free radicals are reactive chemical species containing one or more unpaired electrons which contribute to its high degree of reactivity and the free radicals derived from oxygen, also

known as reactive oxygen species (ROS), are the most important class of radical species (Fatehi-Hassanabad et al., 2010). ROS are beneficial at low concentration and play important physiological roles in host defense mechanism (against infectious agents) and in a number of cellular signaling systems. The harmful effects of ROS, known as oxidative stress, are due to the overproduction of ROS and /or deficiency of antioxidant mechanisms. The oxidative stress has been implicated in the pathogenesis of many diseases including cardiovascular diseases, cancer, inflammatory disease, ageing and diabetes. It is widely recognized that hyperglycemia-induced ROS contribute to cell and tissue dysfunction in diabetes and the antioxidants may alleviate the development of diabetic complications. There is a growing interest in antioxidants, particularly in the prevention of presumed deleterious effects of free radicals in the human body and to prevent the deterioration of fats and other constituents of foodstuffs. Antioxidants and radical scavengers are beneficial dietary supplements which could protect the human health against various degenerative diseases. There is a preference for antioxidants from natural rather than from synthetic sources and there is a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants (Molyneux, 2004). Since the reducing power of compounds is directly proportional to antioxidant activity, antioxidant activity of the biogenic AgNPs was determined in terms of their hydrogen donating or radical-scavenging ability using the DPPH radical scavenging assay which is a reliable method for the evaluation of antioxidant capacity of natural or synthetic compounds. The molecule of 1,1-diphenyl-2-picrylhydrazyl is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour

with an absorption band in ethanol solution at about 517 nm. The DPPH radical scaveging assay is based on the reduction of alcoholic DPPH solution to the non-radical yellow coloured diphenyl-picryl hydrazine in the presence of an antioxidant (Saratale et al., 2017). The colour of DPPH solution was changed from deep violet to pale yellow in the presence of AgNPs and the absorbance of the DPPH solution was gradually decreased with increase of concentrations of AgNPs which further confirmed the free radical scavenging activity of AgNPs. The radical scavenging activity of AgNPs was compared with ascorbic acid and the IC_{50} values were found to be 40.45 ± 5.06 μg/ml and 15.15 ± 1.00 μg/ml for AgNPs and ascorbic acid, respectively. A high level of antioxidant activity of the sample is associated with greater radical scavenging activity and it is related to lower IC_{50} value. AgNPs in different concentrations (3.125, 6.25, 12.5, 25, 50 and 100 μg/ml) range revealed enhanced bit by bit DPPH radical scavenging activity in a dose dependent manner (11.66 ± 0.99 %, 24.08 ± 1.49 %, 28.45 ± 1.73 %, 43.85 ± 2.22 %, 53.04 ± 1.46 % and 82.37 ± 1.38 %) in the study (Fig. 9). It was assumed that various phytoconstituents specially polyphenols could be adsorbed onto the active surfaces of AgNPs as capping agent which increased the surface area, and thereby interacted and scavenged these free radicals efficiently (Saratale et al., 2017). Thus, the enhanced free radical scavenging activity of the AgNPs could be due to the synergistic effect of both the AgNPs and the bioactive compounds of the aqueous flowers extract of *D. volubilis* present in the surface of nanoparticles (Mohanty and Jena, 2017). This antioxidant behavior of the biosynthesized AgNPs establishes its potential applications in the therapy of many diseases including diabetes caused by oxidative stress.

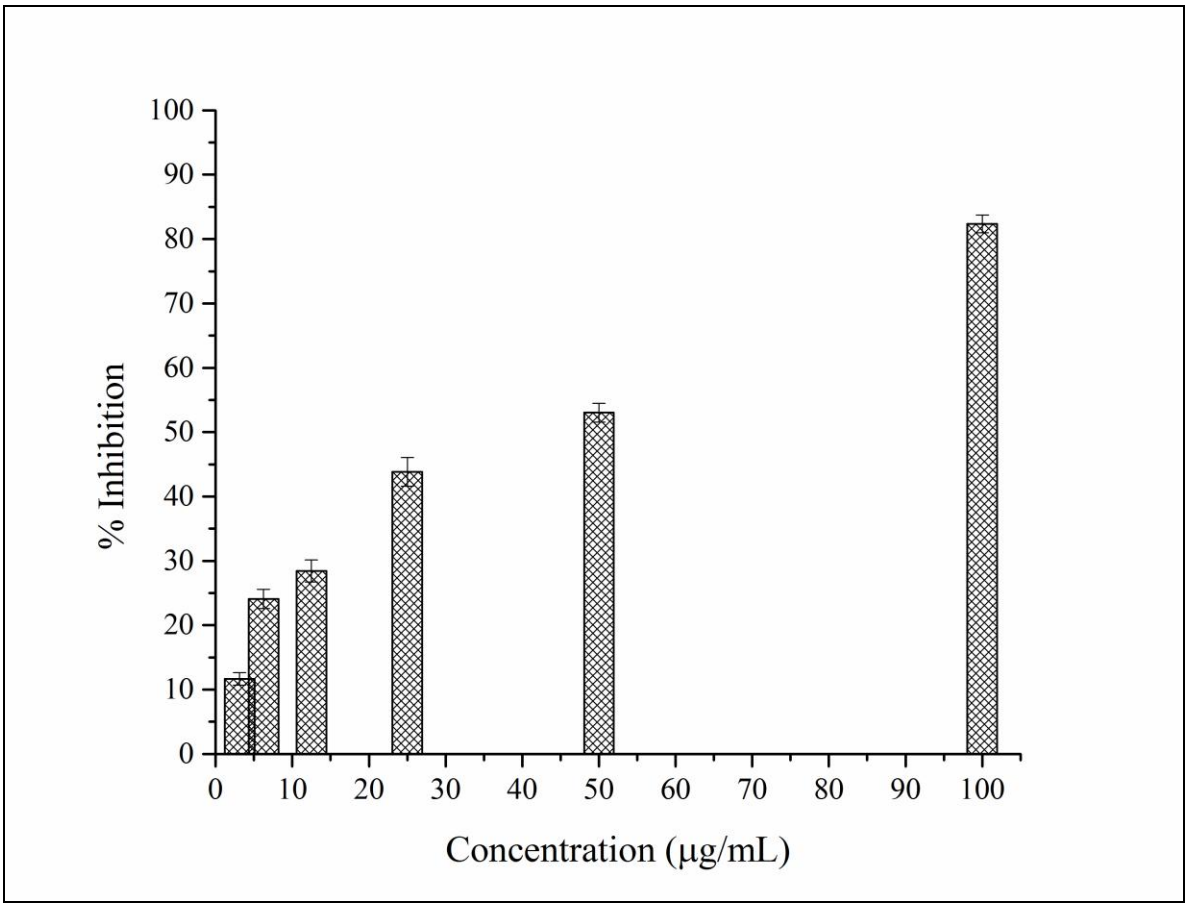


Fig. 9. DPPH free radical scavenging activity of biofabricated AgNPs.

3.5.2. ABTS radical scavenging activity

This method determines the relative antioxidant ability of the AgNPs to scavenge the preformed radical monocation of 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) which is generated by oxidation of ABTS with potassium persulfate. The assay involves the production of blue chromophore of ABTS radical cation through the reaction between ABTS and potassium persulfate and the blue coloured radical forms the basis of one of the spectrophotometric methods that have been applied for determination of antioxidant activity of substances (Re et al., 1999). The AgNPs was capable of scavenging

the ABTS radical at different concentrations (3.125, 6.25, 12.5, 25, 50 and 100 µg/ml) in a dose dependant manner (1.83 ± 0.13 %, 6.89 ± 0.59 %, 9.91 ± 0.18 %, 17.77 ± 0.31 %, 34.79 ± 1.39 % and 61.85 ± 0.29 %) (Fig. 10) and the IC_{50} values for AgNPs and ascorbic acid were found to be 78.49 ± 1.41 µg/ml and 55.84 ± 0.13 µg/ml, respectively. Free radicals are generated in the body when cells utilize oxygen to produce energy in term of ATP and the ROS are produced as by-products from the cellular redox (reduction-oxidation) process (Zaidun et al., 2018). The free radicals and oxidants are being produced in the body in excessive quantity in case of some pathophysiological conditions which disturb the normal body functions resulting in oxidant and antioxidant imbalance and eventually cause oxidative stress. Oxidative stress is considered as the biggest contributor in the pathogenesis of diabetes mellitus, cardiovascular disease, neurodegenerative diseases, and obstructive lungs disease. Free radicals are produced endogenously or exogenously in the body. Excess ROS can result in damage of cellular DNA, lipids, and protein leading to the inhibition of their normal function. Cellular enzymatic systems are potential sources of ROS, including NADPH oxidase, xanthine oxidase, uncoupled endothelial nitric oxide synthase, arachidonic acid metabolizing enzymes including cytochrome P-450 enzymes, lipoxygenase and cyclooxygenase, and the mitochondrial respiratory chain (Fatehi-Hassanabad et al., 2010). The biological antioxidants are the substances that, when present at a lower concentration compared to that of an oxidizable substrate, are able to either delay or prevent the oxidation of the substrate (Pisoschi and Pop, 2015). Antioxidant functions imply lowering of oxidative stress, DNA mutations, malignant transformations, as well as other parameters of cell damage. The antioxidant defense systems comprising of enzymatic and non-enzymatic substances developed against oxidative damage are those that prevent

ROS occurrence and block the free radicals that are formed. The capacity of the biosynthesized AgNPs to scavenge the ABTS radical is associated with its antioxidant activity. The high content of TPC and TFC of the AgNPs are responsible for the radical scavenging activity of the biofabricated metal nanoparticles.

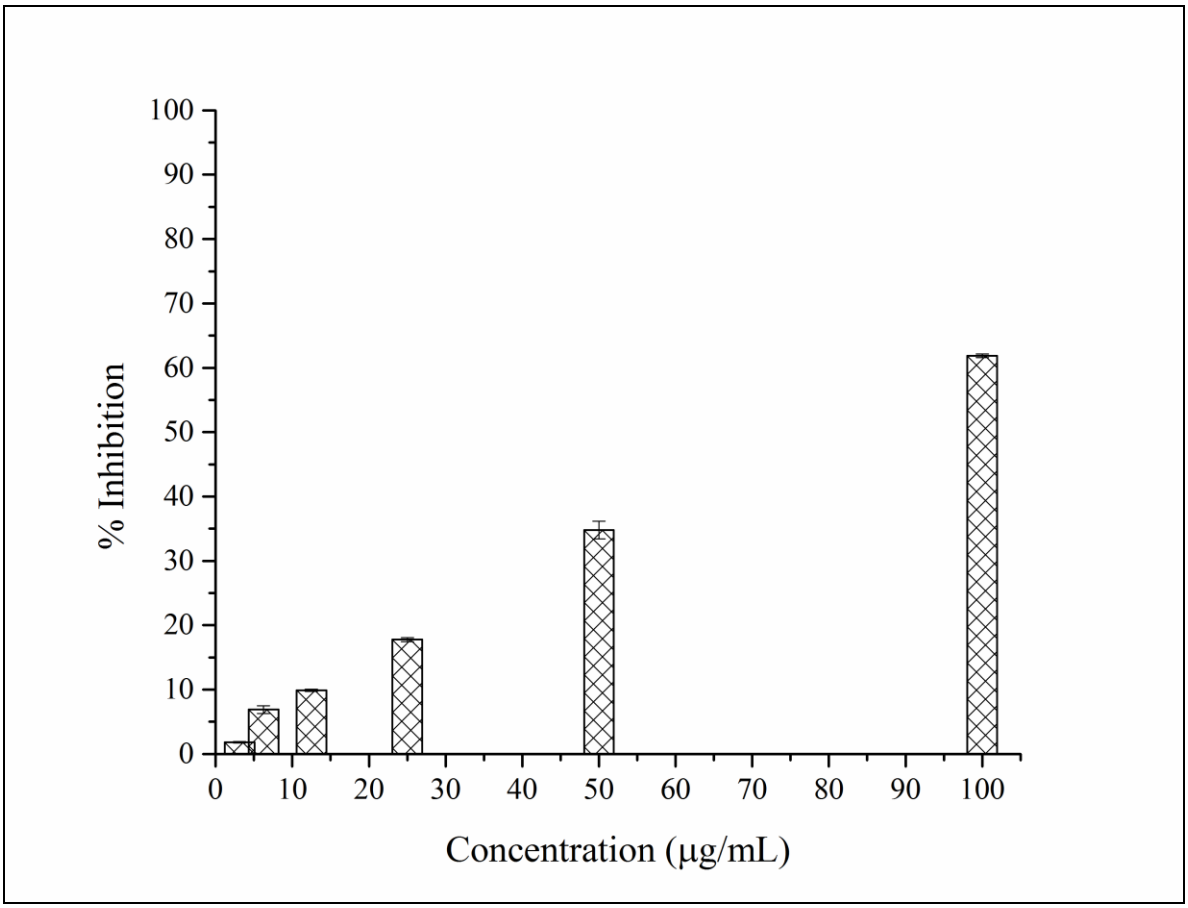


Fig. 10. ABTS radical scavenging activity of biogenic AgNPs.

3.5.3. Total antioxidant capacity

The reducing capacity or electron donating power of antioxidants to molybdenum was investigated in the assay. A green phosphate/Mo (V) complex with maximal absorption at

695 nm was produced in the phosphomolybdenum method and the absorbance value was proportional to antioxidant power of the biogenic AgNPs (Hammi et al., 2015). The TAC of the AgNPs was found to be 148.83 ± 2.99 mg GAE/g of AgNPs. The result obtained in phosphomolybdenum assay indicates that the AgNPs have remarkable antioxidant activity which is due to both of AgNPs and the phenolics which are present as capping material in the biosynthesized AgNPs. The biosynthesize *D. volubilis* flowers mediated AgNPs have the potential to become potent antioxidants for controlling ROS and thereby the AgNPs can play a crucial role in the prevention of diabetes mellitus.

3.6. In-vitro antidiabetic activity

3.6.1. Inhibition of α-amylase enzyme

Inhibition of α-amylase enzyme is one of the important strategies for the treatment of diabetes mellitus, especially the non-insulin-dependent type 2 diabetes, which involves control over excessive rise of the blood glucose level by inhibiting the starch digestive enzymes. α-Amylase enzyme catalyses the cleavage of α-D-(1-4) glycosidic linkages of starch, amylose, amylopectin, glycogen and various maltodextrins into shorter oligosaccharides leading to formation of monosaccharides for absorption. Increased oxidative stress and hyperglycaemia can directly lead to overproduction of ROS which is believed to be the underlying cause of cellular injury, tissue damage or organ dysfunctions in diabetic patients as diabetic complications and glucose has been considered as a factor that induces cell death through a free radical mediated mechanism (Zhang et al., 2016). ROS is responsible for insulin resistance, β-cell dysfunction and both the microvascular and

macrovascular long-term complications of diabetes (Fatehi-Hassanabad et al., 2010). Until it is feasible to readily achieve tight control of blood glucose in diabetic patients at the time of diagnosis, adjunct therapy to reduce oxidative stress may be beneficial. Antioxidants may reduce the development of diabetic complications by inhibiting formation of ROS. The α-amylase inhibitory activity of the AgNPs at various concentrations (2.5, 5, 7.5, 10, 12.5 and 15 μg/ml) were found to be 9.36 ± 0.73%, 22.16 ± 0.68 %, 36.55 ± 1.17 %, 47.59 ± 0.97 %, 57.56 ± 0.87 % and 80.92 ± 0.81 % inhibition of α-amylase, respectively (Fig. 11). The IC_{50} values of AgNPs and acarbose were found to be 10.62 ± 0.22 μg/ml and 51.17 ± 1.99 μg/ml, respectively. The result suggested that the AgNPs showed the α-amylase enzyme inhibitory activity in a concentration dependent manner and the polyphenolics present as capping agent in the biosynthesized AgNPs might contribute to the enzyme inhibitory activity of the nanoparticles as the phenolics have ability to bind with the reactive sites of α-amylase leading to modification of the catalytic activity of enzyme and hence exhibit hypoglycemic effect. Silver nanoparticles are known to cause the formation of protein corona, protein unfolding, and altered protein function and these properties might be attributed to the enzyme inhibitory activity of the biofabricated AgNPs (McShan et al., 2014). The AgNPs might inhibit the breakdown and digestion of starch and other complex carbohydrates present in diet in gastrointestinal tract by inhibiting the α-amylase enzyme and thereby control the postprandial hyperglycaemia where the blood sugar level rises abnormally after meals. Hyperglycaemic concentration of glucose increases the production of ROS (Fatehi-Hassanabad et al., 2010). The AgNPs could reduce the formation of ROS produced due to presence of high concentrations of glucose either by controlling the postprandial hyperglycaemia by inhibiting the carbohydrate hydrolyzing enzyme or by

showing its antioxidant activity. Besides antidiabetic drugs, antioxidant medications are also used to reduce the long term complications of diabetes mellitus and the biogenic AgNPs could play an important role in the management of diabetes mellitus due to its dual functioning as antioxidant and antidiabetic agent. This indicates the potential usefulness of the biosynthesized AgNPs to treat diabetes mellitus and its associated complications and could be considered as an effective approach for diabetes care.

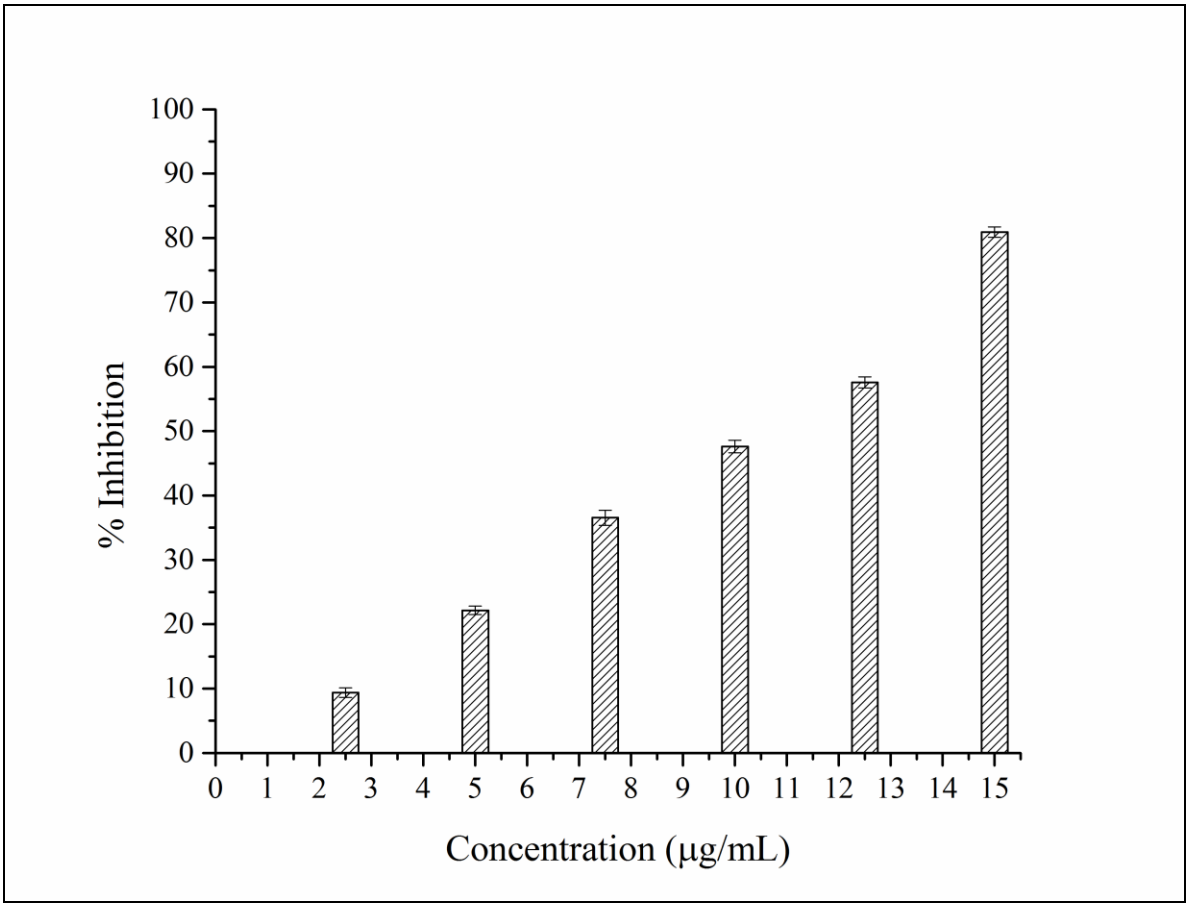


Fig. 11. α-Amylase enzyme Inhibitory activity of AgNPs.

3.6.2. Inhibition of α-glucosidase enzyme

An effective strategy to manage diabetes mellitus, especially the noninsulin- dependent type 2 diabetes, involves control over excessive rise of the blood glucose level by inhibiting the activity of α-glucosidase which is a membrane bound carbohydrate hydrolyzing enzyme present in the epithelium of small intestines (Tan et al., 2017). The enzyme catalyses hydrolysis of complex carbohydrates into absorbable monosaccharides and thereby helps in subsequent influx of glucose from the intestinal tract to blood vessels which is responsible for postprandial hyperglycaemia. In the study, the biogenic AgNPs at different concentrations (1, 2, 4, 6, 8 and 10 μg/ml) exhibited 9.68 ± 0.25 %, 23.59 ± 0.26 %, 36.87 ± 0.09 %, 46.70 ± 0.18 %, 61.03 ± 0.06 % and 76.24 ± 0.25 % inhibition of α-glucosidase, respectively (Fig. 12). The biofabricated AgNPs showed the enzyme inhibitory activity in a concentration dependent manner and the half-inhibitory concentration (IC_{50}) of the AgNPs and acarbose were estimated to be 6.49 ± 0.03 μg/ml and 479.60 ± 4.49 μg/ml, respectively. The result showed that the biogenic AgNPs exhibited remarkable α-glucosidase inhibitory activity. The antidiabetic activity could be due to the interaction of the α-glucosidase enzyme with both of the green synthesized AgNPs and the phenolic compounds present in the AgNPs as capping agent which could form complexes with the enzyme resulting in alteration of their biological structures (Yu et al., 2015). Diabetes is still life-threatening because of limited therapeutic utility of existing synthetic drugs. The number of diabetic patients is expected to be 300 million worldwide by 2025 and thus novel approaches are required to prevent and treat diabetes mellitus (Fatehi-Hassanabad et al., 2010). Synergistic features of plant and metal nanoparticles in the field of phytonanotherapy are unique as they

offer healing properties that may be the clinical bioequivalent to many synthetic drugs with minimal side effects (Anand et al., 2017). The *D. volubilis* flowers mediated green synthesized AgNPs can be regarded as a safe, economical, and beneficial therapeutic form of treatment for diabetes mellitus and its associated complications.

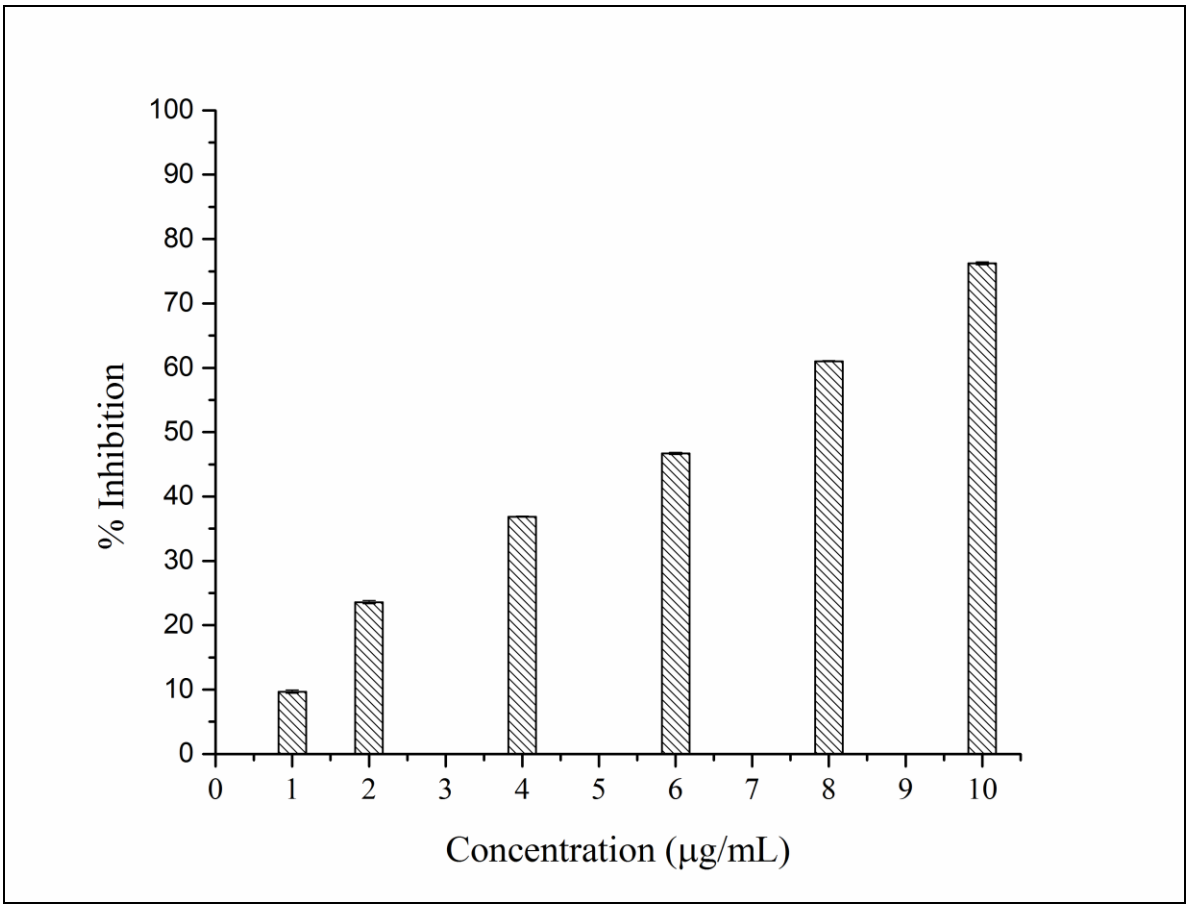


Fig. 12. α-Glucosidase enzyme Inhibitory activity of biogenic AgNPs.

3.7. Antibacterial screening

The green synthesized AgNPs was exposed to both Gram-negative *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* ATCC 25923 and Gram-positive *Bacillus*

subtilis ATCC 6673, *Staphylococcus aureus* ATCC 29737 microorganisms and were found to be efficacious at their respective MIC values as presented in Table 3. The result showed that the biogenic AgNPs exhibited variable degrees of inhibition against the tested microorganisms. The superior antibacterial activity of the biosynthesized AgNPs were due to the high surface area to volume ratio of the nanoparticles resulting in more surface exposure to the bacterial cell surface (Patil, and Kim, 2017). The nanosizes of these particles made it easier to penetrate in the bacterial cell through the cell membrane and thereby the AgNPs interacted with the intracellular materials resulting in cell destruction in the process of multiplication.

Table 3: MIC values of the biogenic AgNPs.

Bacterial strains	**MIC (µg/ml)**	
	AgNPs	Amoxicillin
Pseudomonas aeruginosa	60	65
Escherichia coli	80	10
Bacillus subtilis	40	8
Staphylococcus aureus	30	16

The AgNPs could react with sulfur or phosphorous containing cellular biomolecules of bacteria, such as DNA and thereby inhibit DNA replication resulting in the cellular toxicity leading to cell death (Saratale et al., 2017). The biogenic AgNPs might attack and disrupt the respiratory chain by interacting with thiol groups present in enzymes such as NADH dehydrogenases leading to bacterial cell death. The phytoconstituents present as capping agent in the biogenic AgNPs could play an important role in showing the antibacterial activity of the AgNPs against both types of bacteria. The antibacterial activity of the biosynthesized AgNPs against both types of the test microorganisms was quantitatively determined on the basis of the ZOI (Table 4). The inhibition of bacterial growth around the well was due to the release of diffusible inhibitory compounds from the phytofabricated AgNPs. In the study, higher ZOI were observed for the Gram-positive microorganisms compared to other Gram-negative strains employed in this antibacterial screening. The zeta potential of nanoparticles plays an important role in exhibiting the antibacterial activity and the antibacterial activity is directly proportional to the zeta potential of the nanoparticles (Kora et al., 2010). The zeta potential value of the green synthesized AgNPs used in the present study was found to be -27.43 ± 0.34 mV. The sensitivity of the both types of bacteria towards the biogenic AgNPs was found to be different which might be due to the variation in difference in their cell surface characteristics and their interaction with the negatively charged AgNPs. The cell surfaces of Gram-negative bacteria carry higher negative charge than the Gram-positive bacteria. Consequently, the interaction between Gram-positive bacteria and the green synthesized AgNPs would be definitely stronger than that of Gram-negative bacteria which might be attributed to the higher antibacterial activity of the biogenic AgNPs towards Gram-positive bacteria compared to Gram-negative

bacteria. The composition of bacterial cell wall might play a key role in the difference in the antibacterial activity of the biosynthesized AgNPs towards both types of bacteria because the cell wall of Gram-negative bacteria consists of an outer membrane composed of lipids, proteins and lipopolysaccharides (LPS) which act as a barrier and provide effective protection against antibacterial agents whereas the cell walls of Gram-positive bacteria do not consist of an outer membrane (Kora et al., 2010). Bacteria cannot easily develop resistance against silver because silver targets multiple components in the bacterial cell as compared to the marketed antibiotic drugs (Reddy et al., 2014).

Table 4: Zone of inhibition of the biofabricated AgNPs.

Bacterial strains	**Zone of inhibition (mm)**	
	AgNPs	**Amoxicillin**
Pseudomonas aeruginosa	10.67 ± 0.44	17.83 ± 0.73
Escherichia coli	9.33 ± 0.44	16.83 ± 0.93
Bacillus subtilis	14.67 ± 0.60	15.33 ± 0.44
Staphylococcus aureus	15.67 ± 0.60	16.17 ± 0.60

Antimicrobial resistance is one of the most serious global public health threats in the present age and there is an urgent need to study and develop new antimicrobial agents to take care of multi drug resistant strains. The study confirmed that the *D. volubilis* flowers mediated green synthesized AgNPs could be used as a potent antibacterial agent.

3.8. Mechanism of nanoparticle synthesis

The exact mechanism behind the green synthesis of silver nanoparticles using plant extract still remains speculative. There were large number of research works published in the area of plant extract mediated silver nanoparticle synthesis wherein the role of the plant extract was as reducing and capping agent (Patra et al., 2015). The FTIR study showed that the aqueous extract of *D. volubilis* flowers contained polyphenols, protein and saccharides. The phytoconstituents present in the plant extract might be responsible for the biosynthesis of the AgNPs wherein the biomolecules played their role as reducing agent as well as stabilizing agent that facilitated to no requirement for further addition of capping agents from the outside. The biosynthesis of silver nanoparticles is an example of redox reactions involving electron transfers. The standard reduction potential value of Ag^{+}/Ag^{0} ($E^{0}_{Ag+/Ag0}$) is 0.80 V whereas the value for aldehyde/alcohol, acid/aldehyde, quinone/phenol and proteins systems are below (E_0) 0.80 V (Patra et al., 2015) suggesting that the phytoconstituents present in the flowers extract of *D. volubilis* could act as strong reducing agent in the formation of the biogenic AgNPs. The temperature used in the biosynthesis of the AgNPs could play an important role in the quick formation of the AgNPs. The saccharides and other biomolecules could expand during autoclaving at 121 °C with 15 psi of pressure in the biosynthesis of the AgNPs wherein the functional groups present in the

plant extract would be more accessible for the silver ions to interact (Kora et al., 2010). The reduction of silver ions by the plant extract was also observed in the same reaction condition at room temperature and it was found that the formation of the AgNPs was time consuming resulting in the formation of unstable and aggregated AgNPs due to lack of stabilization. The elevated temperature and pressure accelerates the synthesis of nanoparticles (Kora et al., 2010). It was observed that the autoclaving at 121 ◦C with 15 psi of pressure increased the rate and extent of biosynthesis of the stabilized AgNPs. The autoclaving used in the biosynthesis of the AgNPs could eliminate the microbial contamination such as bacteria, viruses and spores from the flowers of *D. volubilis* which might be acquired during collection and transportation.

4. Conclusion

A facile, quick, economically viable, readily scalable and non-pathogenic production of the AgNPs was depicted in our study, thus this method will be an alternative to whole cell, physical and/or chemical methods of AgNPs synthesis. We have demonstrated a green chemistry approach for the synthesis of AgNPs using *D. volubilis* flowers as a source of plant material. The present study demonstrated the synthesis, characterization and evaluation of antioxidant, antidiabetic and antibacterial activity of the biogenic AgNPs prepared using *D. volubilis* flowers extract. The biofabricated AgNPs possessed the added advantage of incorporation of active biomolecules as capping agent which made it as a potent free radical scavengers, antidiabetic and antibacterial agent. The phytochemical synthesis of the AgNPs using aqueous extract of *D. volubilis* flowers can play an important role in the field of nanotechnology and nanomedicine as it can offer alternative therapeutic

options which are safe, free of side effects and effective. The green synthesized AgNPs are more accepable for medicinal applications due to the toxic chemical-free nature of the biogenic nanoparticles. The study highlights the fact that the AgNPs can be formed and stabilized without the addition of synthetic stabilizing agents from the outside. The surface reactivity of the functionalized AgNPs by the capping agents facilitates it as potential candidate for various pharmaceutical, biomedical and environmental applications. Further future avenues can be directed towards formulating new capping agents for the synthesis of AgNPs with better biological activities.

References

Adams SJ, Kuruvilla GR, Krishnamurthy KV, Nagarajan M, Venkatasubramanian P. Pharmacognostic and phytochemical studies on Ayurvedic drugs *Ativisha* and *Musta*. Revista Brasileira de Farmacognosia. 2013; 23(3): 398-409.

AbdelHamid AA, Al-Ghobashy MA, Fawzy M, Mohamed MB, Abdel-Mottaleb MM. Phytosynthesis of Au, Ag, and Au-Ag bimetallic nanoparticles using aqueous extract of sago pondweed (*Potamogeton pectinatus* L.). ACS Sustainable Chemistry & Engineering. 2013; 1: 1520-9.

Ajitha B, Reddy YA, Reddy PS, Suneetha Y, Jeon HJ, Ahn CW. Instant biosynthesis of silver nanoparticles using *Lawsonia inermis* leaf extract: Innate catalytic, antimicrobial and antioxidant activities. Journal of Molecular Liquids. 2016; 219: 474-81.

Akbar S, Hanif U, Ali J, Ishtiaq S. Pharmacognostic studies of stem, roots and leaves of *Malva parviflora* L. Asian Pacific Journal of Tropical Biomedicine. 2014; 4 (5): 410-5.

Anand K, Tiloke C, Naidoo P, Chuturgoon AA. Phytonanotherapy for management of diabetes using green synthesis nanoparticles. Journal of Photochemistry and Photobiology B: Biology. 2017; 173: 626-39.

Ang LZP, Hashim R, Sulaiman SF, Coulibaly AY, Sulaiman O, Kawamura F, Salleh KM. *In vitro* antioxidant and antidiabetic activites of *Gluta torquata*. Industrial Crops and Products. 2015; 76: 755-60.

Arambewela LSR, Arawwawala LDAM. Standardization of *Alpinia calcarata* Roscoe rhizomes. Pharmacognosy Research. 2010; 2(5): 285-8.

Ashokkumar S, Ravi S, Kathiravan V, Velmurugan S. Synthesis, characterization and catalytic activity of silver nanoparticles using *Tribulus terrestris* leaf extract. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2014; 121: 88-93

Aslam I, Afridi MSK. Pharmacognostic characterization of *Beaumontia grandiflora* (Roxb.) Wall. leaf for taxonomic identification for quality control of a drug. Journal of Applied Research on Medicinal and Aromatic Plants. 2018; 8: 53-9.

babuMaddinedi S, Mandal BK, Maddili SK. Biofabrication of size controllable silver nanoparticles - a green approach. Journal of Photochemistry and Photobiology B: Biology. 2017; 167: 236-41.

Balan K, Qing W, Wang Y, Liu X, Palvannan T, Wang Y, Ma F, Zhang Y. Antidiabetic activity of silver nanoparticles from green synthesis using *Lonicera japonica* leaf extract. Rsc Advances. 2016; 6: 40162-8.

Baloch AB, Xia X, Sheikh SA. Proximate and Mineral Compositions of Dried Cauliflower (*Brassica Oleracea* L.) Grown In Sindh, Pakistan. Journal of Food and Nutrition Research. 2015; 3(3): 213-9.

Bar H, Bhui DK, Sahoo GP, Sarkar P, Pyne S, Misra A. Green synthesis of silver nanoparticles using seed extract of *Jatropha curcas*. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2009; 348: 212-6.

Baret P, Septembre-Malaterre A, Rigoulet M, d'Hellencourt CL, Priault M, Gonthier M, Devin A. Dietary polyphenols preconditioning protects 3T3-L1 preadipocytes from

mitochondrial alterations induced by oxidative stress. The International Journal of Biochemistry & Cell Biology. 2013; 45: 167-74.

Benn TM, Westerhoff P. Nanoparticle silver released into water from commercially available sock fabrics. Environmental science & technology. 2008; 42(11): 4133-9.

Bhutto AA, Kalay Ş, Sherazi ST, Culha M. Quantitative structure-activity relationship between antioxidant capacity of phenolic compounds and the plasmonic properties of silver nanoparticles. Talanta. 2018; 189: 174-81.

Biswas M, Haldar PK, Ghosh AK. Antioxidant and free-radical-scavenging effects of fruits of *Dregea volubilis*. Journal of natural science, biology, and medicine. 2010; 1(1): 29-34.

Bolivar P, Cruz-Paredes C, Hernández LR, Juárez ZN, Sánchez-Arreola E, Av-Gay Y, Bach H. Antimicrobial, anti-inflammatory, antiparasitic, and cytotoxic activities of *Galium mexicanum*. Journal of Ethnopharmacology. 2011; 137: 141-7.

Borah S, Kakoti BB, Mahato K, Chakraborty D, Lahkar S, Gogoi B, Bora NS. Pharmacognostic and preliminary phytochemical studies on shoot of *Calamus leptospadix* Griff.-An ethnomedicinal plant of Assam. Indian Journal of Natural Products and Resources. 2014; 5(4): 320-5.

Carocho M, Morales P, Ferreira IC. Antioxidants: Reviewing the chemistry, food applications, legislation and role as preservatives. Trends in Food Science & Technology. 2018; 71: 107-20.

Chanda S. Importance of pharmacognostic study of medicinal plants: An overview. Journal of Pharmacognosy and Phytochemistry. 2014; 2(5): 69-73.

Cheng D, Zhang Y, Xin X, Gao D. Comparative pharmacognosy of *Pyrrosia petiolosa* and *Pyrrosia davidii*. Revista Brasileira de Farmacognosia. 2014; 24: 368-80.

Choudhury H, Pandey M, Hua CK, Mun CS, Jing JK, Kong L, Ern LY, Ashraf NA, Kit SW, Yee TS, Pichika MR. An update on natural compounds in the remedy of diabetes mellitus: A systematic review. Journal of traditional and complementary medicine. 2018; 8(3): 361-76.

Cumberland SA, Lead JR. Synthesis of NOM-capped silver nanoparticles: size, morphology, stability, and NOM binding characteristics. ACS Sustainable Chemistry & Engineering. 2013; 1: 817-25.

David L, Moldovan B, Vulcu A, Olenic L, Perde-Schrepler M, Fischer-Fodor E, Florea A, Crisan M, Chiorean I, Clichici S, Filip GA. Green synthesis, characterization and anti-inflammatory activity of silver nanoparticles using European black elderberry fruits extract. Colloids and Surfaces B: Biointerfaces. 2014; 122: 767-77.

Dey A, Lakshmanan J. The role of antioxidants and other agents in alleviating hyperglycemia mediated oxidative stress and injury in liver. Food and Function. 2013; 4: 1148-84.

Dias DA, Urban S, Roessner U. A Historical Overview of Natural Products in Drug Discovery. Metabolites. 2012; 2: 303-36.

Dipankar C, Murugan S. The green synthesis, characterization and evaluation of the biological activities of silver nanoparticles synthesized from *Iresine herbstii* leaf aqueous extracts. Colloids and Surfaces B: Biointerfaces. 2012; 98: 112-9.

Farooqui NA, Dey A, Singh GN, Easwari TS, Pandey MK. Analytical techniques in quality evaluation of herbal drugs. Asian Journal of Pharmaceutical research. 2014; 4(3): 112-7.

Fatehi-Hassanabad Z, Chan CB, Furman BL. Reactive oxygen species and endothelial function in diabetes. European journal of pharmacology. 2010; 636: 8-17.

Folashade O, Omoregie H, Ochogu P. Standardization of herbal medicines - A review. International Journal of Biodiversity and Conservation. 2012; 4(3): 101-12.

Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiological reviews. 2013; 93(1): 137-88.

Gad HA, El-Ahmady SH, Abou-Shoer MI, Al-Azizi MM. A Modern Approach to the Authentication and Quality Assessment of Thyme Using UV Spectroscopy and Chemometric Analysis. Phytochemical Analysis. 2013; 24: 520-6.

Gensel J, Borke T, Pérez NP, Fery A, Andreeva DV, Betthausen E, Müller AH, Möhwald H, Skorb EV. Cavitation engineered 3D sponge networks and their application in active surface construction. Advanced Materials. 2012; 24(7): 985-9.

Giovannini P, Howes MJ, Edwards SE. Medicinal plants used in the traditional management of diabetes and its sequelae in Central America: A review. Journal of ethnopharmacology. 2016; 184: 58-71.

Gogoi B, Kakoti BB, Sharma N, Borah S. Pharmacognostic and preliminary phytochemical evaluation of *Cinnamomum bejolghota* (Buch.-Ham.) Sweet bark. Indian Journal of Natural Products and Resources. 2016; 7(1): 59-64.

Hajiaghaalipour F, Kanthimathi MS, Sanusi J, Rajarajeswaran J. White tea (*Camellia sinensis*) inhibits proliferation of the colon cancer cell line, HT-29, activates caspases and protects DNA of normal cells against oxidative damage. Food Chemistry. 2015; 169: 401-10.

Halliwell B. How to characterize a biological antioxidant. Free radical research communications. 1990; 9(1): 1-32.

Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford University Press Inc., New York. 1999.

Hammi KM, Jdey A, Abdelly C, Majdoub H, Ksouri R. Optimization of ultrasound-assisted extraction of antioxidant compounds from Tunisian *Zizyphus lotus* fruits using response surface methodology. Food Chemistry. 2015; 184: 80-9.

Heshmati J, Farsi F, Shokri F, Rezaeinejad M, Almasi-Hashiani A, Vesali S, Sepidarkish M. A systematic review and meta-analysis of the probiotics and synbiotics effects on oxidative stress. Journal of functional foods. 2018; 46: 66-84.

Heleno SA, Barros L, Sousa MJ, Martins A, Ferreira ICFR. Study and characterization of selected nutrients in wild mushrooms from Portugal by gas chromatography and high performance liquid chromatography. Microchemical Journal. 2009; 93: 195-9.

Hossain E, Chandra G, Nandy AP, Gupta JK, Mandal SC. Possible fasciocidal activity of methanol extract of *Dregea volubilis* leaves. Experimental parasitology. 2013; 135(2): 183-7.

Hooshmand S, Kumar A, Zhang JY, Johnson SA, Chai SC, Arjmandi BH. Evidence for anti-inflammatory and antioxidative properties of dried plum polyphenols in macrophage RAW 264.7 cells. Food and Function. 2015; 6: 1719-25.

Hussain J, Khan AL, Rehman NU, Hamayun M, Shinwari ZK, Ullah W, Lee I. Assessment of herbal products and their composite medicinal plants through proximate and micronutrients analyses. Journal of Medicinal Plants Research. 2009; 3(12): 1072-7.

Iravani S. Green synthesis of metal nanoparticles using plants. Green Chemistry. 2011; 13(10): 2638-50.

Iwu MW, Duncan AR, Okunji CO. New antimicrobials of plant origin. Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. 1999; 457-62.

Jadhav RS, Ahmed ML, Swamy P, Sanaullah S. Neuropharmacological studies of drevogenins isolated from *Wattakaka volubilis* (Linn. f.) Stapf. in mice. International Journal of Pharma and Bio Sciences. 2012; 3: 80-8.

Kahrilas GA, Wally LM, Fredrick SJ, Hiskey M, Prieto AL, Owens JE. Microwave-assisted green synthesis of silver nanoparticles using orange peel extract. ACS Sustainable Chemistry & Engineering. 2013; 2: 367-76.

Kala CP, Dhyani PP, Sajwan BS. Developing the medicinal plants sector in northern India: challenges and opportunities. Journal of Ethnobiology and Ethnomedicine. 2006; 2(32): 1-15.

Karaman S, Tütem E, Baskan KS, Apak R. Comparison of total antioxidant capacity and phenolic composition of some apple juices with combined HPLC-CUPRAC assay. Food Chemistry. 2010; 120: 1201-9.

Karthika KS, Sanjaya KS, Hari KR, Jyothi T. A pharmacognostic evaluation of moorvabheda (*Dregea volubilis* (LF) Benth ex. hook F. International Research Journal of Pharmacy. 2012; 3: 127-30.

Khlifi D, Hayouni EA, Valentin A, Cazaux S, Moukarzel B, Hamdi M, Bouajila J. LC–MS analysis, anticancer, antioxidant and antimalarial activities of *Cynodon dactylon* L. extracts. Industrial crops and products. 2013; 45: 240-7.

Kokate CK, Purohit AP, Gokhale SB. Test Book of Pharmacognosy. 42nd ed. Nirali Prakashan, Pune. 2006.

Kora AJ, Sashidhar RB, Arunachalam J. Gum kondagogu (*Cochlospermum gossypium*): a template for the green synthesis and stabilization of silver nanoparticles with antibacterial application. Carbohydrate Polymers. 2010; 82: 670-9.

Kumkrai P, Weeranantanapan O, Chudapongse N. Antioxidant, α-glucosidase inhibitory activity and sub-chronic toxicity of *Derris reticulata* extract: its antidiabetic potential. BMC Complementary and Alternative Medicine. 2015; 15(35): 1-10.

Kunyanga C N, Imungi J K, Okoth M W, Biesalski H K, Vadivel V. Total phenolic content, antioxidant and antidiabetic properties of methanolic extract of raw and traditionally processed Kenyan indigenous food ingredients. LWT - Food Science and Technology. 2012; 45: 269-76.

Kurutas EB. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutrition Journal. 2016; 15(71): 1-22.

Liang YZ, Xie P, Chan K. Quality control of herbal medicines. Journal of chromatography B. 2004; 812: 53-70.

Lu F, Sun D, Huang J, Du M, Yang F, Chen H, Hong Y, Li Q. Plant-mediated synthesis of Ag-Pd alloy nanoparticles and their application as catalyst toward selective hydrogenation. ACS Sustainable Chemistry & Engineering. 2014; 2: 1212-8.

Madhav NVS, Upadhyaya K, Bisht A. Phytochemical screening and standardization of polyherbal formulation for dyslipidemia. International Journal of Pharmacy and Pharmaceutical Sciences. 2011; 3(3): 235-8.

Mandal D, Dash SK, Das B, Chattopadhyay S, Ghosh T, Das D, Roy S. Bio-fabricated silver nanoparticles preferentially targets Gram positive depending on cell surface charge. Biomedicine & Pharmacotherapy. 2016; 83: 548-58.

McShan D, Ray PC, Yu H. Molecular toxicity mechanism of nanosilver. Journal of food and drug analysis. 2014; 22: 116-27.

Meyers MA, Mishra A, Benson DJ. Mechanical properties of nanocrystalline materials. Progress in materials science. 2006; 51(4): 427-556.

Mittal AK, Chisti Y, Banerjee UC. Synthesis of metallic nanoparticles using plant extracts. Biotechnology advances. 2013; 31(2): 346-56.

Mohanty AS, Jena BS. Innate catalytic and free radical scavenging activities of silver nanoparticles synthesized using *Dillenia indica* bark extract. Journal of colloid and interface science. 2017; 496: 513-21.

Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin Journal of Science and Technology. 2004; 26(2): 211-9.

Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Parishcha R, Ajaykumar PV, Alam M, Kumar R, Sastry M. Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: a novel biological approach to nanoparticle synthesis. Nano Letters. 2001; 1(10): 515-9.

Nadagouda MN, Iyanna N, Lalley J, Han C, Dionysiou DD, Varma RS. Synthesis of silver and gold nanoparticles using antioxidants from blackberry, blueberry, pomegranate, and turmeric extracts. ACS Sustainable Chemistry & Engineering. 2014; 2: 1717-23.

Nandi D, Besra SE, Vedasiromoni JR, Giri VS, Rana P, Jaisankar P. Anti-leukemic activity of *Wattakaka volubilis* leaf extract against human myeloid leukemia cell lines. Journal of ethnopharmacology. 2012; 144(3): 466-73.

Nisha SN, Aysha OS, Rahaman JS, Kumar PV, Valli S, Nirmala P, Reena A. Lemon peels mediated synthesis of silver nanoparticles and its antidermatophytic activity. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2014; 124: 194-8.

Olaokun OO, McGaw LJ, Eloff JN, Naidoo V. Evaluation of the inhibition of carbohydrate hydrolysing enzymes, antioxidant activity and polyphenolic content of extracts of ten African *Ficus* species (Moraceae) used traditionally to treat diabetes. BMC Complementary and Alternative Medicine. 2013; 13(94): 1-10.

Ozsoy N, CanA, Yanardag R, Akev N. Antioxidant activity of *Smilax excelsa* L. leaf extracts. Food Chemistry. 2008; 110: 571-83.

Panda N, Mondal NB, Banerjee S, Sahu NP, Koike K, Nikaido T, Weber M, Luger P. Polyhydroxypregnanes from *Dregea volubilis*. Tetrahedron. 2003; 59(42): 8399-403.

Panda N, Mandal D, Mandal NB, Sahu NP, Banerjee S. Flavonoid and flavone C-glycosides from *Dregea volubilis*. Natural Product Communications. 2006; 1(9): 731-3.

Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity. 2009; 2(5): 270-78.

Pandian AM, Karthikeyan C, Rajasimman M. Isotherm and kinetic studies on nano-sorption of malachite green onto *Aspergillus flavus* mediated synthesis of silver nano particles. Environmental Nanotechnology, Monitoring & Management. 2016; 6: 139-51.

Pascu M, Pascu D, Trăistaru GA, Nechifor AC, Bunaciu AA, Aboul-Enein HY. Different spectrophotometric methods for antioxidant activity assay of four Romanian herbs. Journal of the Iranian Chemical Society. 2014; 11: 315-21.

Patil MP, Kim GD. Eco-friendly approach for nanoparticles synthesis and mechanism behind antibacterial activity of silver and anticancer activity of gold nanoparticles. Applied microbiology and biotechnology. 2017; 101: 79-92.

Patra S, Mukherjee S, Barui AK, Ganguly A, Sreedhar B, Patra CR. Green synthesis, characterization of gold and silver nanoparticles and their potential application for cancer therapeutics. Materials Science and Engineering: C. 2015; 53: 298-309.

Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress: A review. European journal of medicinal chemistry. 2015; 97: 55-74.

Poovitha S, Parani M. *In vitro* and *in vivo* α-amylase and α- glucosidase inhibiting activities of the protein extracts from two varieties of bitter gourd (*Momordica charantia* L.). BMC Complementary and Alternative Medicine. 2016; 16(Suppl 1) (185): 1-8.

Pothitirat W, Chomnawang MT, Supabphol R, Gritsanapan W. Comparison of bioactive compounds content, free radical scavenging and anti-acne inducing bacteria activities of extracts from the mangosteen fruit rind at two stages of maturity. Fitoterapia. 2009; 80: 442-47.

Rates SMK. Plants as source of drugs. Toxicon. 2001; 39(5): 603-13.

Ravindran A, Chandran P, Khan SS. Biofunctionalized silver nanoparticles: advances and prospects. Colloids and Surfaces B: Biointerfaces. 2013; 105: 342-52.

Rabinarayan A, Switu J, Harisha CR, Vinay S. Pharmacognostical genetic barcoding and phytochemical analysis on leaves of *Dalbergia volubilis* Roxb.-an extra Pharmacopoeial drug of Ayurveda. International Journal of Pharmacy and Pharmaceutical Sciences. 2018; 10(6): 117-24.

Raj S, Mali SC, Trivedi R. Green synthesis and characterization of silver nanoparticles using *Enicostemm aaxillare* (Lam.) leaf extract. Biochemical and biophysical research communications. 2018; 503: 2814-9.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biology and medicine. 1999; 26(9-10): 1231-7.

Reddy NJ, Vali DN, Rani M, Rani SS. Evaluation of antioxidant, antibacterial and cytotoxic effects of green synthesized silver nanoparticles by *Piper longum* fruit. Materials Science and Engineering: C. 2014; 34: 115-22.

Ren YY, Yang H, Wang T, Wang C. Green synthesis and antimicrobial activity of monodisperse silver nanoparticles synthesized using *Ginkgo Biloba* leaf extract. Physics Letters A. 2016; 380: 3773-7.

Sahoo N, Manchikanti P, Dey S. Herbal drugs: standards and regulation. Fitoterapia. 2010; 81(6): 462-71.

Sahoo N, Manchikanti P. Herbal drug regulation and commercialization: An Indian industry perspective. The Journal of Alternative and Complementary Medicine. 2013; 19(12): 957-63.

Sahu NP, Panda N, Mandal NB, Banerjee S, Koike K, Nikaido T. Polyoxypregnane glycosides from the flowers of *Dregea volubilis*. Phytochemistry. 2002; 61(4): 383-8.

Samanta A, Das G, Das SK. Roles of flavonoids in plants. International Journal of Pharmaceutical Science and Technology. 2011; 6(1): 12-35.

Sanyacharernkul S, Itghiarbha A, Kongtawelert P, Meepowpan P, Nuntasaen N, Pompimon W. A new polyoxypregnane glycoside from the roots of *Dregea volubilis* (Lf) Benth.ex Hook. f and its chondroprotective effect. American Journal of Biochemistry and Biotechnology. 2009; 5(4): 202-9.

Saratale GD, Saratale RG, Benelli G, Kumar G, Pugazhendhi A, Kim DS, Shin HS. Anti-diabetic potential of silver nanoparticles synthesized with *Argyreia nervosa* leaf extract high synergistic antibacterial activity with standard antibiotics against foodborne bacteria. Journal of Cluster Science. 2017; 28: 1709-27.

Savran A, Zengin G, Aktumsek A, Mocan A, Glamoćlija J, Ćirić A, Soković M. Phenolic compounds and biological effects of edible *Rumex scutatus* and *Pseudosempervivum sempervivum*: potential sources of natural agents with health benefits. Food & Function. 2016; 7: 3252-62.

Sharma C, Irshad S, Khatoon S, Arya KR. Pharmacognostical evaluation of Indian folk-traditional plants *Coelogyne cristata* and *Pholidota articulata* used for healing fractures. Indian Journal of Experimental Biology. 2017; 55: 622-7.

Sharma M, Sharma V, Majumdar DK. Entrapment of α -Amylase in Agar Beads for Biocatalysis of Macromolecular Substrate. International Scholarly Research Notices. 2014; 2014: 1-8.

Sharma VK, Yngard RA, Lin Y. Silver nanoparticles: green synthesis and their antimicrobial activities. Advances in colloid and interface science. 2009; 145: 83-96.

Shen Y, Zhang H, Cheng L, Wang L, Qian H, Qi X. *In vitro* and *in vivo* antioxidant activity of polyphenols extracted from black highland barley. Food Chemistry. 2016; 194: 1003-12.

Shukla S, Mehta A, Bajpai VK, Shukla S. *In vitro* antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food and Chemical Toxicology. 2009; 47: 2338-43.

Shukla S, Park J, Kim D, Hong S, Lee JS, Kim M. Total phenolic content, antioxidant, tyrosinase and α-glucosidase inhibitory activities of water soluble extracts of noble starter culture Doenjang, a Korean fermented soybean sauce variety. Food Control. 2016; 59: 854-61.

Siddiqi KS, Husen A, Rao RA. A review on biosynthesis of silver nanoparticles and their biocidal properties. Journal of nanobiotechnology. 2018; 16(14): 1-28.

Silverstein RM, Webster FX, Kiemle DJ. Spectrometric Identification of Organic Compounds.7th ed. John Wiley & Sons, Inc., New Jersey, 2005.

Sreeramulu N, Suthari S, Ragan A, Raju VS. Ethno-botanico-medicine for common human ailments in Nalgonda and Warangal districts of Telangana, Andhra Pradesh, India. Annals of Plant Sciences. 2013; 2(7): 220-9.

Sun Y, Xia Y. Shape-controlled synthesis of gold and silver nanoparticles. Science. 2002; 298: 2176-9.

Taira J, Tsuchida E, Katoh MC, Uehara M, Ogi T. Antioxidant capacity of betacyanins as radical scavengers for peroxyl radical and nitric oxide. Food Chemistry. 2015; 166: 531-36.

Tan Y, Chang SKC, Zhang Y. Comparison of α-amylase, α-glucosidase and lipase inhibitory activity of the phenolic substances in two black legumes of different genera. Food Chemistry. 2017; 214: 259-68.

Tarantilis PA, Troianou VE, Pappas CS, Kotseridis YS, Polissiou MG. Differentiationof Greek red wines on the basis of grape variety using attenuated total reflectanceFourier transform infrared spectroscopy. Food Chemistry. 2008; 111: 192-196.

Thakkar KN, Mhatre SS, Parikh RY. Biological synthesis of metallic nanoparticles. Nanomedicine: nanotechnology, biology and medicine. 2010; 6(2): 257-62.

Ubeyitogullari A, Ciftci ON. Phytosterol nanoparticles with reduced crystallinity generated using nanoporous starch aerogels. RSC Advances. 2016; 6: 108319-27.

Upreti K, Semwal A, Upadhyaya K, Masiwal M. Pharmacognostical and Phytochemical Screening of Leaf Extract of *Zanthoxylum armatum* DC. International Journal of Traditional and Herbal Medicine. 2013; 1(1): 6-11.

Vongsak B, Sithisarn P, Mangmool S, Thongpraditchote S, Wongkrajang Y, Gritsanapan W. Maximizing total phenolics, total flavonoids contents and antioxidant activity of

Moringa oleifera leaf extract by the appropriate extraction method. Industrial Crops and Products. 2013; 44: 566-71.

Wahab S, Hussain A, Ahmad P, Usmani S. Ethanobotanical, pharmacognostical and physico-chemical studies of stem bark of *Bombax ceiba* L., commonly growing in eastern Uttar Pradesh region of India. Pharmacognosy Journal. 2012; 4(32): 55-60.

Wang Z, Fang C, Megharaj M. Characterization of iron-polyphenol nanoparticles synthesized by three plant extracts and their fenton oxidation of azo dye. ACS Sustainable Chemistry & Engineering. 2014; 2: 1022-5.

Wojdyło A, Oszmiański J, Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry. 2007; 105: 940-49.

Wojdylo A, Nowicka P, Carbonell-Barrachina ÁA, Hernández F. Phenolic compounds, antioxidant and antidiabetic activity of different cultivars of *Ficus carica* L. fruits. Journal of Functional Foods. 2016; 25: 421-32.

Yadav RNS, Agarwala M. Phytochemical analysis of some medicinal plants. Journal of Phytology. 2011; 3(12): 10-4.

Yang M, Shen Q, Li L, Huang Y, Cheung H. Phytochemical profiles, antioxidant activities of functional herb *Abrus cantoniensis* and *Abrus mollis.* Food Chemistry. 2015; 177: 304-12.

Yu Z, Gao H, Zhang Z, He Z, He Q, Jia L, Zeng W. Inhibitory effects of *Ligustrum robustum* (Rxob.) Blume extract on α-amylase and α-glucosidase. Journal of Functional Foods. 2015; 19: 204-13.

Zaidun NH, Thent ZC, Latiff AA. Combating oxidative stress disorders with citrus flavonoid: Naringenin. Life sciences. 2018; 208:111-22.

Zayed MF, Eisa WH, Shabaka AA. *Malva parviflora* extract assisted green synthesis of silver nanoparticles. SpectrochimicaActa Part A: Molecular and Biomolecular Spectroscopy. 2012; 98: 423-8.

Zhang H, Tsao R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. Current Opinion in Food Science. 2016; 8: 33-42.

Zhang W, Hu S, Yin JJ, He W, Lu W, Ma M, Gu N, Zhang Y. Prussian blue nanoparticles as multienzyme mimetics and reactive oxygen species scavengers. Journal of the American Chemical Society. 2016; 138: 5860-5.

Zhao J, Ma S, Li S. Advanced strategies for quality control of Chinese medicines. Journal of pharmaceutical and biomedical analysis. 2018; 147: 473-8.

Publications as a first author

South African Journal of Botany 109 (2017) 16–24

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

A new exploration of *Dregea volubilis* flowers: Focusing on antioxidant and antidiabetic properties

B. Das, A. De, M. Das, S. Das, A. Samanta *

Division of Microbiology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India

ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form 30 November 2016
Accepted 13 December 2016
Available online 30 December 2016

Edited by J Van Staden

Keywords:
Dregea volubilis
Antioxidant activity
α-Glucosidase inhibitory activity
α-Amylase inhibitory activity

ABSTRACT

Flowers of *Dregea volubilis* (Family: Apocynaceae) are commonly consumed as seasonal vegetable in India but not yet evaluated for its health beneficiary effects. In the present study, the hydroalcoholic flower extract of *D. volubilis* (DVHA) was evaluated for antioxidant and antidiabetic activities *in vitro*. With high contents of phenolics (39.82 ± 1.22 mg GAE/g) and flavonoids (27.50 ± 0.87 mg QE/g), DVHA showed potential antioxidant activity for scavenging DPPH radical (IC_{50}, 237.86 ± 1.05 μg/mL), hydroxyl radical (IC_{50}, 170.67 ± 0.98 μg/mL), superoxide radical (IC_{50}, 219.07 ± 1.25 μg/mL), nitric oxide radical (IC_{50}, 196.38 ± 1.49 μg/mL) and ferric reducing antioxidant power (176.47 ± 3.18 μmol Fe^{2+}/g), total antioxidant capacity (39.68 ± 1.62 mg GAE/g) along with remarkable inhibitory effects on α-glucosidase (IC_{50}, 3780.09 ± 21.19 μg/mL) and α-amylase (IC_{50}, 360.68 ± 1.26 μg/mL). The characterization of the extract was evaluated by FT-IR and UHPLC analysis. The liquid chromatography study led to the identification and quantification of six compounds viz. gallic acid (412.36 ± 2.29 μg/g), ferulic acid (162.72 ± 0.89 μg/g), rutin (386.25 ± 2.00 μg/g), ellagic acid (208.8 ± 2.00 μg/g), quercetin (306.85 ± 2.24 μg/g) and cinnamic acid (213.71 ± 2.14 μg/g). The results explain the therapeutic potentialities of *D. volubilis* flowers as a potential source of natural antioxidants for use in food and pharmaceutical industries along with their possible applications to control postprandial hyperglycaemia.

1. Introduction

Non-insulin dependent diabetes mellitus, commonly called type 2 diabetes, is a chronic metabolic disease characterized by hyperglycemia which results from insufficient or inefficient insulin secretion. The increasing prevalence of diabetes mellitus has become a major health problem worldwide, reaching epidemic proportions. Controlling post-prandial hyperglycemia through the inhibition of α-amylase and α-glucosidase (carbohydrate hydrolyzing enzymes) present in the gastro-intestinal tract is one of the major management therapies. To maintain biological processes, oxidation is necessary in living organisms for the production of energy (Shukla et al., 2016). Oxygen derived free radicals and other reactive oxygen species (ROS), which are produced uninterruptedly *in vivo*, are responsible for cell death and tissue damage (Ozsoy et al., 2008). Oxygen radicals have shown to be involved in various diseases like ageing, cancer, cardiovascular diseases, diabetes etc. (Halliwell and Gutteridge, 1999). Antioxidants play an important protective role in cell injury promoted by free radical-induced oxidative stress (Kurutas, 2016). Foods containing natural antioxidants, e.g., polyphenols, which might assist to save living body systems against oxidative damage, have taken a great deal of attention to researchers (Baret et al., 2013; Dey and Lakshmanan, 2013; Hooshmand et al., 2015). Polyphenols have been reported to have antioxidant and hypoglycaemic property with ability to inhibit digestive enzymes such as α-amylase and α-glucosidase (Ang et al., 2015). α-Amylase and α-glucosidase inhibitors can retard utilization of dietary carbohydrates into absorbable monosaccharides and suppress postprandial hyperglycemia, making them applicable for treating type 2 diabetes (Wojdylo et al., 2016). Synthetic enzyme inhibitors used to control postprandial hyperglycemia are undesirable for long term usage because of gastrointestinal side effects and are costly too (Poovitha and Parani, 2016). Natural resources enriched with α-amylase and α-glucosidase inhibitors can be utilized as an effective therapy for treating postprandial hyperglycemia with minimal adverse effects.

Dregea volubilis is a large twining shrub growing in India, Sri Lanka, Myanmar, Indonesia, Thailand, and China (Anonymous, 1976). The plant, commonly known as "Jukti" in Bengali (Nandi et al., 2012),

Abbreviations: DVHA, hydroalcoholic flower extract of *D. volubilis*; GAE, gallic acid equivalent; QE, quercetin equivalent; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; IC_{50}, half maximal inhibitory concentration; FT-IR, Fourier transform infrared; UHPLC, ultra high performance liquid chromatography; ROS, reactive oxygen species; PNPG, *p*-nitrophenyl-α-D-glucopyranoside; TPTZ, 2,4,6 tripyridyl-S-triazine; NBT, nitro blue tetrazolium; NED, N-(1-naphthyl)-ethylenediamine dihydrochloride; DNS, 3, 5-dinitrosalicylic acid; FRAP, ferric reducing antioxidant power; HCl, hydrochloric acid; $FeCl_3.6H_2O$, ferric chloride; $FeSO_4 \cdot 7H_2O$, ferrous sulphate; H_2O_2, hydrogen peroxide; AA, ascorbic acid; TBA, thiobarbituric acid; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; TAC, total antioxidant capacity; TPC, total phenolic content; PBS, phosphate buffered saline.

* Corresponding author.
E-mail address: asamanta61@yahoo.co.in (A. Samanta).

http://dx.doi.org/10.1016/j.sajb.2016.12.003
0254-6299/

bears green bisexual sweet scented flowers in drooping umbel with long glabrous branches (Jadhav et al., 2012). The flowers of the plant are consumed as a seasonal vegetable in summer (Hossain et al., 2013). *D. volubilis* is traditionally used to treat inflammation, boil, abscesses, dyspepsia, piles, asthma, tumours, leucoderma, anthelmintic, paralysis, rheumatism, tonsils, neck pain etc. (Kirtikar and Basu, 1935; Chatterjee and Pakrashi, 1995; Sreeramulu et al., 2013). Volubiloside A, volubiloside B, volubiloside C, dregealol, volubilogenone, volubilol, drevogenin D, iso-drevogenin P, 17α-marsdenin, dregeanin, vicenin-2, vitexin, isovitexin, isoorientin, rutin, quercetin, luteolin, apigenin have been isolated from the flower of the plant (Sahu et al., 2002; Panda et al., 2003; Panda et al., 2006).

The present study investigated the total phenolic content, total flavonoid content, antioxidant effects, α-amylase and α-glucosidase inhibitory activities of flowers of *D. volubilis* in order to establish their medicinal and nutritional potentials.

2. Materials and methods

2.1. Chemicals

Gallic acid, ferulic acid, rutin, ellagic acid, quercetin, cinnamic acid, ascorbic acid (AA), acarbose, α-glucosidase, α-amylase, aluminium chloride, PNPG (*p*-nitrophenyl-α-D-glucopyranoside), DPPH (1,1-diphenyl-2-picryl-hydrazyl) and TPTZ (2,4,6 tripyridyl- S-triazine) were purchased from Sigma-Aldrich, St Louis, MO, USA. Folin–Ciocalteu reagent, TBA (thiobarbituric acid), 2- deoxy-D-ribose and NBT (nitro blue tetrazolium) were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Sodium carbonate, phosphoric acid, acetic acid, HCl (hydrochloric acid), sulfuric acid, EDTA (ethylenediaminetetraacetic acid), sodium acetate trihydrate, $FeCl_3 \cdot 6H_2O$ (ferric chloride), $FeSO_4 \cdot 7H_2O$ (ferrous sulphate), H_2O_2 (hydrogen peroxide), sodium hydroxide, TCA (trichloroacetic acid), hydroxylamine hydrochloride, sodium phosphate, ammonium molybdate, sodium nitroprusside, NED (N-(1-naphthyl)-ethylenediamine dihydrochloride), sodium chloride, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, starch, sodium potassium tartrate, DNS (3, 5-dinitrosalicylic acid) and methanol for liquid chromatography were procured from Merck Life Sciences Private Limited, Mumbai, India. The water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used for all tests. All other reagents used were of analytical grade.

2.2. Collection of plant material

Flowers of *D. volubilis* were collected in April, 2015 from Jaynagar Mazilpur, South 24 Parganas, West Bengal, India. Taxonomic identification of the plant was conducted by Dr. V.P. Prasad, Central National Herbarium, Botanical Survey of India, Botanical Garden, Howrah, West Bengal, India. A voucher specimen (voucher no DV/H/141) was deposited in Division of Microbiology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India for future reference.

2.3. Preparation of plant extract

Flowers of *D. volubilis* were shade dried at room temperature and the dried plant material was powdered using mechanical grinder. 100 g of powdered material was extracted with 1000 mL of a hydroalcoholic solvent mixture prepared by mixing 70 volume methanol and 30 volume water using soxhlet apparatus. The crude extract was filtered through Whatman filter paper No. 1 and concentrated by rotary evaporation under vacuum and then the material was evaporated to dryness with a percentage yield of 12.6% (*w*/w). Extract (DVHA) was stored in the dark at a temperature of 10 °C for future use.

2.4. Total phenolic content

The content of soluble phenolics in DVHA was determined using Folin–Ciocalteu assay (Vongsak et al., 2013) with slight modifications and the volumes of the reagents added were scaled down to fit the microtiter plate volume. Folin–Ciocalteu reagent was prepared with ten-fold of dilution in water. Briefly, 40 μL of DVHA (1000 μg/mL) was mixed with 100 μL of Folin–Ciocalteu reagent in microtiter plate and 160 μL sodium carbonate (7.5%, *w*/*v*) was added to the mixture. It was allowed to left for 30 min at room temperature with occasional shaking. The absorbance was read at 765 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA) against reagent blank containing water in place of sample. Gallic acid was used as standard to estimate total phenol content (TPC) and a calibration curve (absorbance vs. μg/mL) was constructed using different concentrations of gallic acid (20–100 μg/mL). The values for TPC are presented in mg gallic acid equivalent (GAE)/g dried extract.

2.5. Total flavonoids content

The aluminium chloride colorimetric method (Pothitirat et al., 2009) was adapted for the determination of total flavonoids with minor changes. An aliquot of 120 μL of DVHA (1000 μg/mL) was mixed with an equal volume of a solution of 2% (*w*/*v*) aluminium chloride solution in a microtiter plate. The absorbance of the mixture was measured at 415 nm after 10 min of incubation against a blank without aluminium chloride solution using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). Result was expressed as mg of quercetin equivalent (QE)/g of dried extract with a calibration curve of quercetin (20–100 μg/mL).

2.6. Fourier transform infrared spectroscopy analysis

Fourier transform infrared (FTIR) spectra of DVHA were recorded in ATR mode using Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA) with a total of 30 scans at a resolution of 4 cm^{-1} in the wave number range between 4000 cm^{-1} and 525 cm^{-1} (Tarantilis et al., 2008). A background spectrum was obtained by collecting the spectrum of the clean ATR crystal immediately before acquiring the spectrum of sample. DVHA was then placed on the ATR accessory and pressed for collecting the FTIR spectrum of the sample. The study was used to acquire information on the nature of the functional groups and chemical bonds present in phytochemicals of DVHA by studying their peak values (cm^{-1}). The spectral acquisitions were done using OMNIC software supplied from the manufacturer of the spectrometer. The analysis represented qualitative information regarding the types of phytoconstituents present in the flowers of *D. volubilis*.

2.7. Identification and quantification of phenolic compounds by UHPLC

The phenolic compounds present in DVHA were identified and quantified by ultra high performance liquid chromatography using a UHPLC+ focused system consisting of a Dionex Ultimate 3000 Pump, a Dionex Ultimate 3000 auto sampler column compartment and a Dionex Ultimate 3000 variable wavelength detector (Karaman et al., 2010). A mixed standard stock solution (1 mg/mL) containing gallic acid, ferulic acid, rutin, ellagic acid, quercetin and cinnamic acid was prepared in HPLC grade methanol and subsequently different concentrations (6, 10, 20, 30, 40 and 50 μg/mL) of standard solutions were prepared by diluting the mixed standard stock solution for calibration curves in order to quantify the phenolic compounds present in DVHA. All solutions were filtered through a 0.45 μm membrane filter. Separations of phenolic compounds were performed using a C18 column (250 mm × 4.6 mm i.d.) with a particle size of 5 μm, Hypersil GOLD (Thermo Fisher Scientific, U.S.A.) and column oven temperature was maintained at 25 °C. The chromatographic separation was carried out

using gradient elution as presented in Table 1 with a flow rate of 1.0 ml min^{-1} using 0.2 (% *v*/v) phosphoric acid as mobile phase A and HPLC grade methanol as mobile phase B, respectively. The injection volume was 5 μL and a wavelength of 280 nm was used for the analysis. The phenolic compounds present were quantified from calibration curves of standards. Data processing of the chromatograms was performed using Chromeleon 7, version 7.2.0.3765 software (Thermo Scientific, U.S.A.).

2.8. DPPH radical scavenging activities

Antioxidant activity of DVHA was evaluated by determining its scavenging ability against DPPH radical (Yang et al., 2015). 50 μL of different concentrations (20–400 μg/mL) of DVHA was mixed with 100 μL of 0.1 mM ethanolic solution of DPPH in a microtiter plate and was recorded as A_s. Absorbance was measured after 30 min of incubation in dark at room temperature at 517 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). A control experiment was performed applying the same procedure using 50 μL water in place of sample and 100 μL of DPPH solution and the absorbance was recorded as A_c. Sample colour contribution was corrected by measuring absorbance of sample colour blank containing 50 μL sample and 100 μL of water. Ascorbic acid was used as standard. The free radical scavenging activity was calculated as percent inhibition according to the following equation:

$$\%\text{inhibition} = ((A_c - A_s)/A_c \times 100).$$

IC_{50} value is the concentration of sample required to produce a 50% decrease in absorbance of initial DPPH concentration. IC_{50} value was calculated for expressing antioxidant activity of DVHA.

2.9. Ferric reducing antioxidant power

The reducing capability of antioxidants can be assessed by ferric reducing antioxidant power (FRAP) assay (Pascu et al., 2014) with slight modifications to accommodate microtiter plate volume which measures the absorbance at 593 nm of a blue colour solution of ferrous form that is generated from a reaction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex with antioxidants. Antioxidant compounds break radical chain reaction by giving hydrogen atom to the ferric complex and thus act as reducing agent. The FRAP reagent was prepared freshly by mixing 5 ml of 300 mM acetate buffer (pH 3.6) with 0.5 ml of 10 mM TPTZ in 40 mM of HCl and 0.5 ml of 20 mM of $FeCl_3 \cdot 6H_2O$. The solution was incubated at 37 °C. Then 10 μL of DVHA (0.5 mg/mL) was mixed with 190 μL of FRAP reagent in a microtiter plate and the mixture was allowed to stand for 30 min in dark. Absorbance readings were measured at 593 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). Sample colour contribution was also corrected. A standard curve of $FeSO_4 \cdot 7H_2O$ (0–1000 μmol/L) was plotted for determining ferric reducing activity. Fe^{2+} content in DVHA is evaluated as mean ± SEM (n = 3) and results are expressed as μmol Fe^{2+}/g dried extract and compared with ascorbic acid.

Table 1
UHPLC gradient programme for the identification and quantification of phenolic compounds in DVHA.

Time (minute)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	1.0	70	30
5.00	1.0	70	30
32.00	1.0	43	57
33.00	1.0	70	30
36.00	1.0	70	30

2.10. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined using 2-deoxy-D-ribose oxidation method (Hajiaghaalipour et al., 2015). Stock solutions of $FeCl_3.6H_2O$ (100 mM), H_2O_2 (1.25 mM), 2- deoxy-D-ribose (2.5 mM), AA (100 mM), 0.5% TBA diluted with sodium hydroxide (0.025 M), 2.8% TCA, DVHA (20–400 μg/mL) were prepared. The volumes of reagents added were minimized to accommodate microtiter plate. The experiment was performed by adding 40 μL of $FeCl_3$, 40 μL of H_2O_2, 40 μL of 2- deoxy-D-ribose and 40 μL of AA to 40 μL of different concentrations of DVHA in a microtiter plate and it was incubated at 37 °C for 1 h. The incubated mixtures were mixed with 20 μL of TBA and 20 μL of TCA and it was maintained at 100 °C for 30 min. It was then cooled down to room temperature and absorbances of the pink coloured mixtures developed were measured at 532 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA) and were recorded as A_s. A control experiment was performed using 40 μL of water in place of DVHA and the absorbance of the solution at 532 nm was recorded as A_c. The samples colour contributions were also corrected by measuring absorbances of solutions containing 40 μL extract (DVHA) and 200 μL water at 532 nm. Ascorbic acid was used as standard. The hydroxyl radical scavenging activity was calculated as percent inhibition according to the following equation:

$$\%\text{inhibition} = ((A_c - A_s)/A_c \times 100).$$

IC_{50} value of DVHA was calculated and compared with ascorbic acid using the formula.

2.11. Superoxide radical scavenging activity

NBT method was used for evaluation of superoxide radical scavenging activity of DVHA (Shukla et al., 2009). Hydroxylamine hydrochloride generates superoxide radical (O_2^-) through auto oxidation in presence of NBT, which gets reduced to nitrile. Nitrile in presence of EDTA shows a colour that is measured at 560 nm. Volumes of the reagents were scaled down to accommodate with the volume of microtiter plate. 50 μL of different concentrations of DVHA (20–400 μg/mL) was separately mixed with a reaction mixture consisting of 50 μL of sodium carbonate (50 mM), 20 μL of NBT (24 mM), 20 μL of EDTA (0.1 mM) in a microtiter plate and absorbance readings were measured immediately at 560 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). 20 μL of hydroxylamine hydrochloride was then added to the reaction mixtures to start the reaction and it was incubated at 25 °C for 15 min and the reduction of NBT was measured at 560 nm. Ascorbic acid was used as standard. The hydroxyl radical scavenging activity was calculated as percent inhibition according to the following equation:

$$\%\text{inhibition} = ((A_c - A_s)/A_c \times 100),$$

where A_c was the absorbance of control reaction mixture (50 μL water was used in place of sample) and A_s was the absorbance of sample reaction mixture at 560 nm. The samples colour contributions were also corrected by measuring absorbances of solutions containing 50 μL extract (DVHA) and 110 μL water at 560 nm. IC_{50} value of DVHA for superoxide radical scavenging activity was determined and compared with ascorbic acid using the formula.

2.12. Total antioxidant capacity

The total antioxidant capacity (TAC) of DVHA was evaluated by phosphomolybdenum method (Hammi et al., 2015) with slight modifications and the volumes of reagents added were adjusted to accommodate microtiter plate volume. In the method Mo(VI) is reduced to Mo(V) by the sample and a green phosphate/Mo(V) complex formed

at acidic pH is measured at 695 nm. 20 μL of DVHA (500 μg/mL) was mixed with 200 μL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the mixture was incubated at 95 °C for 90 min and cooled down to room temperature. The absorbance of the solution was measured at 695 nm against a blank. The antioxidant activity was expressed as mg GAE/g dried extract.

2.13. Nitric oxide radical scavenging activity

Antioxidant activity of DVHA was evaluated by determining its scavenging ability against nitric oxide radical (Hajiaghaalipour et al., 2015). Sodium nitroprusside, at physiological pH, spontaneously produces nitric oxide which reacts with oxygen to produce nitric ions that can be measured by the Greiss reaction. Nitric oxide scavengers compete with oxygen leading to reduced production of nitric oxide radical. 50 μL of various concentrations (20–400 μg/mL) of DVHA was mixed with 50 μL of sodium nitroprusside (5 mM) in a microtiter plate and incubated for 60 min. 100 μL of Greiss reagent was added to the mixtures and incubated for 5 min. Griess reagent was prepared by mixing equal amount of 1% sulphanilamide in 5% phosphoric acid and 0.1% NED. The absorbance of the chromophores formed in the diazotization reaction of nitrile with sulphanilamide and subsequent coupling with NED were measured at 532 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The hydroxyl radical scavenging activity was calculated as percent inhibition according to the following equation:

$$\%\text{inhibition} = ((A_c - A_s)/A_c \times 100),$$

where A_c was the absorbance of control reaction mixture (50 μL water was used in place of sample) and A_s was the absorbance of sample reaction mixture at 532 nm. The samples colour contributions were also corrected by measuring absorbances of solutions containing 50 μL extract (DVHA) and 150 μL water at 532 nm. Ascorbic acid was used as a standard. IC_{50} value of DVHA and ascorbic acid were calculated.

2.14. α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity of DVHA is based on hydrolysis of chromogenic substrate PNPG by α-glucosidase to yellow coloured *p*-nitrophenyl (Shukla et al., 2016). 50 μL of different concentrations (0.5–5 mg/mL) of DVHA was mixed with 100 μL of phosphate buffered saline (PBS, 100 mM, pH 6.9) containing α-glucosidase (1 unit/mL) in a microtiter plate and incubated at 25 °C for 10 min. 50 μL of PNPG (5 mM) prepared in PBS (100 mM, pH 6.9) was mixed to the each reaction mixture wells in the microtiter plate and incubated for 5 min at 25 °C. The absorbances of mixtures were measured at 405 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The α-glucosidase inhibitory activity was presented as percent inhibition according to the following equation:

$$\%\text{inhibition} = ((A_c - A_s)/A_c \times 100),$$

where A_c was the absorbance of control reaction mixture (50 μL PBS was used in place of sample) and A_s was the absorbance of sample reaction mixture at 405 nm. The samples colour contributions were also corrected by measuring absorbances of solutions containing 50 μL extract (DVHA) and 150 μL PBS at 405 nm. Acarbose was used as a standard. IC_{50} values of DVHA and acarbose for enzyme inhibition were calculated.

2.15. α-Amylase inhibitory activity

DVHA was evaluated for α-amylase inhibitory activity (Yu et al., 2015). Stock solutions of starch (1% *w*/*v*) and α-amylase (0.5 mg/mL) were prepared in 20 mM of PBS with pH 6.9, containing 6 mM sodium chloride. 25 μL of different concentrations (0.5–5 mg/mL) of DVHA were mixed with 25 μL of starch solution in a microtiter plate and incubated for 10 min at 25 °C. 25 μL of α-amylase solution was then added to the mixtures and incubated at 25 °C for 10 min. The reaction was terminated by adding 50 μL of dinitrosalicylic acid colour reagent and the reagent mixtures were immediately heated in boiled water for 10 min and cooled down to room temperature. All the solutions in the microtitre plate were diluted by adding 50 μL of distilled water and the absorbences were measured at 540 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The α-amylase inhibitory activity was calculated as percent inhibition according to the following equation:

$$\%\text{inhibition} = ((A_c - A_s)/A_c \times 100),$$

where A_c was the absorbance of control reaction mixture (20 μL water was used in place of sample) and A_s was the absorbance of sample reaction mixture at 540 nm. Acarbose was used as a standard. IC_{50} values of DVHA and acarbose for enzyme inhibition were calculated.

2.16. Statistical analysis

All the experiments were performed in triplicate. The results are presented as mean ± standard error of the mean (SEM). GraphPad PRISM 6 software, USA was used for statistical analysis.

3. Results and discussion

3.1. Total phenolic content

The total amount of phenolic content present in DVHA was quantified as GAE. TPC of *D. volubilis* was 39.82 ± 1.22 mg GAE/g of DVHA (Table 2). The antioxidant and α-amylase, α-glucosidase inhibitory activity of the plant were due to the presence of phenolic phytoconstituents. Plant polyphenols are therapeutically very useful phytoconstituents having hydroxyl groups which exhibit scavenging activity (Shukla et al., 2009). Phenolic compounds are associated with antioxidative action and can inactivate α-amylase, α-glucosidase through non-specific binding to enzymes (Yu et al., 2015). Plant polyphenolic compounds are gaining much attention to food and pharmaceutical industry because they help to improve quality and nutritional aspects of food with the ability to inhibit oxidative degradation of lipids as well as health promoting and disease preventing properties (Wojdyło et al., 2007; Tan et al., 2017).

Table 2
Total phenolic content, total flavonoid content, ferric reducing antioxidant power and total antioxidant capacity of hydroalcoholic flower extract of *D. volubilis*.

Extract	Total phenolic content (mg GAE/g of DVHA)	Total flavonoid content (mg QE/g of DVHA)	Ferric reducing antioxidant power (μmol Fe^{2+}/g of DVHA)	Total antioxidant capacity (mg GAE/g of DVHA)
DVHA	39.82 ± 1.22	27.50 ± 0.87	176.47 ± 3.18	39.68 ± 1.62

DVHA: hydroalcoholic flower extract of *D. volubilis*.
GAE: gallic acid equivalent.
QE: quercetin equivalent.
Results were expressed as mean ± SEM (n = 3).

3.2. Total flavonoids content

The content of total flavonoids present in DHVA was presented as QE. Total flavonoids content of *D. volubilis* was 27.50 ± 0.87 mg QE/g of DVHA (Table 2). Flavonoids are low molecular weight polyphenolic secondary metabolites having C6-C3-C6 carbon frame which are widely distributed in green plant kingdom (Samanta et al., 2011). Flavonoids have antioxidant activity and hydroxyl groups in flavonoids are accountable for free radical scavenging activity (Kumkrai et al., 2015) and they also possess inhibitory activity on carbohydrate hydrolyzing enzymes (Olaokun et al., 2013). Flavonoids are widely present in plants and the flowers of *D. volubilis* can be considered as an important flavonoid-rich food which can protect human beings from diseases related to oxidative stress.

3.3. Fourier transform infrared spectroscopy analysis

A FTIR spectrum of DVHA is presented in Fig. 1. The presence of O—H stretching vibration band at 3266 cm^{-1}, C—O stretching vibration band at 1027 cm^{-1} and O—H bending vibration band at 1393 cm^{-1} are due to the phenolic compounds (Silverstein et al., 2005). The characteristic O—H stretching vibration band at 2927 cm^{-1} and C═ O stretching vibration band at 1700 cm^{-1}, C—O stretching vibration band at 1304 cm^{-1} and O—H bending vibration band at 1430 cm^{-1} are due to presence of phenolic acids. The FTIR spectra (Fig. 1) show the presence of two bands of strong intensity at 2855 cm^{-1} and 2960 cm^{-1}, characteristic of C—H bonds of aliphatic CH2 and CH3 groups, respectively. The spectrum shows the presence of aromatic hydrocarbons due to the presence of low frequency range bands between 900 and 675 cm^{-1} representing out-of-plane bending of ring C—H bonds and in-plane bending bands in the region of 1300–1000 cm^{-1}, C—C stretching band within the ring in the region of 1600–1585 cm^{-1} and 1500–1400 cm^{-1}. The band at 1625 cm^{-1} is due to conjugation of an alkene double bond with an aromatic ring. The FTIR spectrum of extract may serve as a quality control tool for identification of the plant material. The Fourier transform infrared spectroscopy study reveals that the characteristic absorption bands in the spectrum of DVHA confirms the richness of the extract of flower of *D. volubilis* in phenolic compounds.

3.4. Identification and quantification of phenolic compounds by UHPLC

UHPLC analysis was performed to identify and quantify the phenolic phytochemicals present in flower of *D. volubilis*. The phenolic compounds present in the plant can be considered as markers in the quality control of the herbal extract. The flower of the plant is known to contain rutin and quercetin (Panda et al., 2006). The present study shows that the DVHA contains gallic acid, ferulic acid, ellagic acid and cinnamic acid. The phenolic compounds present in DVHA were identified by comparing the retention times of the standards with that of the sample and the retention times of gallic acid, ferulic acid, rutin, ellagic acid, quercetin and cinnamic acid were found to be 3.153, 14.447, 19.750, 21.427, 27.812 and 29.223 min, respectively (Fig. 2). The contents of the phenolic compounds present in DVHA were presented in Table 3. The phenolic compounds can act as antioxidants and OH groups present in phenolic compounds form strong hydrogen linkages with the catalytic residues of the enzymes involved in type 2 diabetes (Ang et al., 2015). It was not possible to analyse all the peaks in the chromatogram of sample due to unavailability of corresponding standards for the HPLC analysis. The flower of *D. volubilis* can be considered as a valuable natural source of polyphenolics with medicinal values.

3.5. DPPH radical scavenging activities

The decrease in absorbance of DPPH solution at 517 nm implies the reduction of DPPH to yellow coloured diphenylpicrylhydrazine which is initiated by antioxidants and the radical scavenging activity of DHVA was compared with ascorbic acid and the IC_{50} values were found to be 237.86 ± 1.05 μg/mL and 27.18 ± 0.15 μg/mL for DVHA and ascorbic acid, respectively (Table 4). DPPH is a stable lipophilic free radical which becomes a diamagnetic molecule by taking an electron or hydrogen radical at room temperature (Shukla et al., 2009). A high level of antioxidants in the extract is associated with greater radical scavenging activity and it is related to lower IC_{50} value. DVHA in different concentrations (20, 50, 100, 200, 300 and 400 μg/mL) range revealed DPPH radical scavenging activity in a dose dependent manner (2.32%, 7.16%, 24.50%, 42.12%, 69.32% and 79.12%) in the study (Fig. 3).

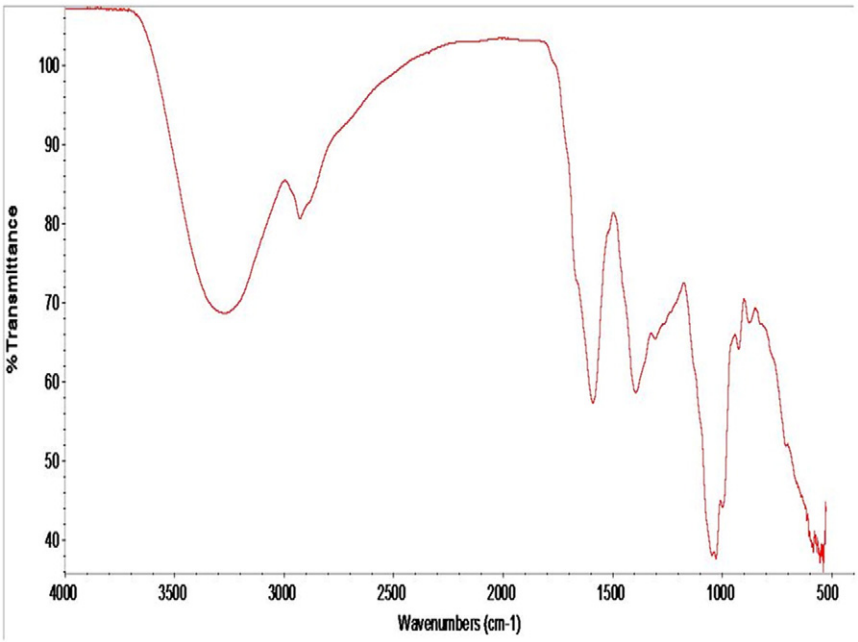


Fig. 1. Fourier transform infrared spectra of the extract of flower of *Dregea volubilis*.

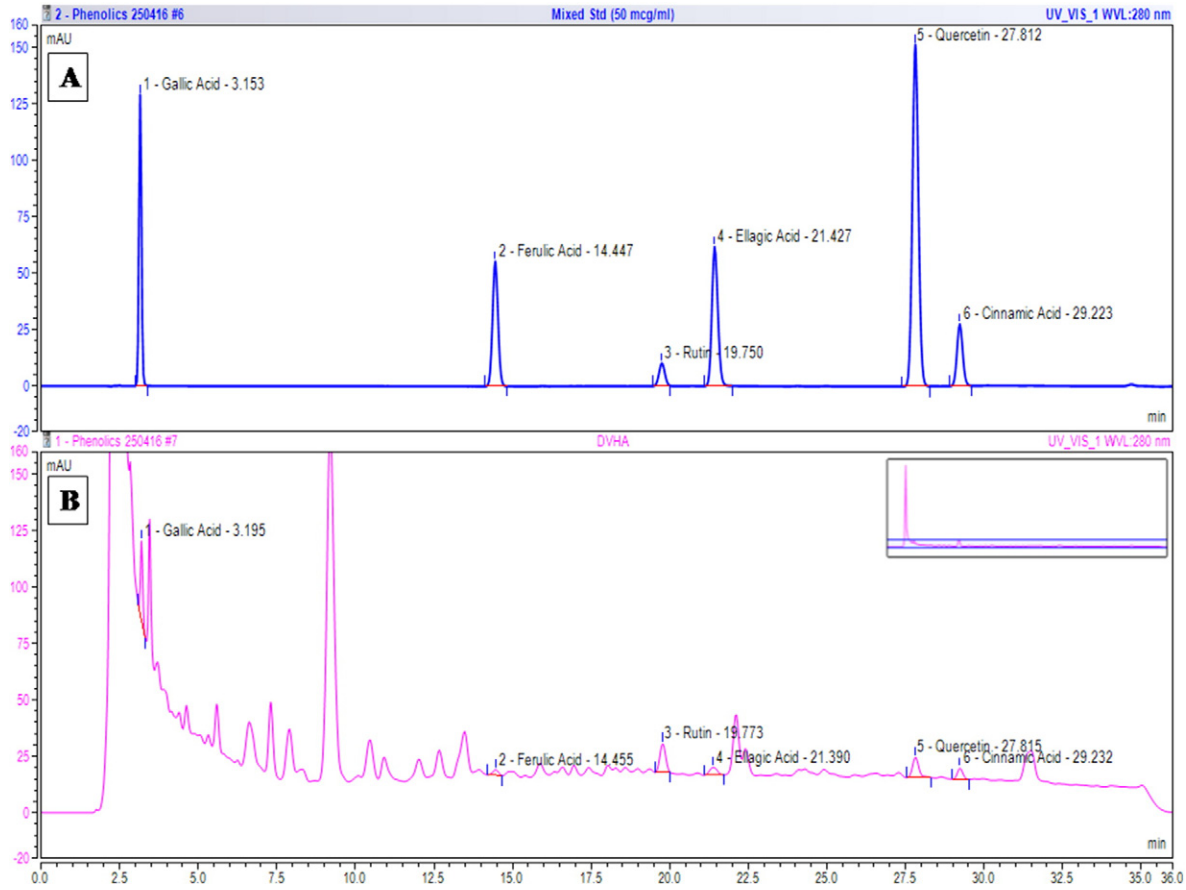


Fig. 2. UHPLC chromatograms of a mixed standard of phenolic compounds (A) and the extract of flower of *Dregea volubilis* (B) as detected at 280 nm (1: gallic acid, 2: ferulic acid, 3: rutin, 4: ellagic acid, 5: quercetin, 6: cinnamic acid).

3.6. Ferric reducing antioxidant power

FRAP assay procedure is a novel method for evaluation of antioxidant activity. Antioxidants form an intense blue coloured ferrous—tripyridyltriazine complex through ferric to ferrous ion reduction of ferric tripyridyltriazine (Fe^{3+}—TPTZ) complex (Shen et al., 2016). Antioxidants contained in various foods act as reductants in the redox linked colorimetric reaction and the antioxidant potential of DVHA and ascorbic acid were found to be 176.47 ± 3.18 μmol Fe^{2+}/g and 2316.63 ± 23.71 μmol Fe^{2+}/g, respectively (Table 2). The high values of total phenolics and flavonoids present in DVHA are responsible for the antioxidant activity of flowers of *D. volubilis*. FRAP assay method is a useful technique for quick estimation of total antioxidant activity of various antioxidants in vegetables.

3.7. Hydroxyl radical scavenging activity

Hydroxyl radical is a highly reactive free radical formed in biological system in a state of oxidative stress. The free radical is responsible for the pathogenesis of various chronic diseases. The radical causes joining of nucleotides in DNA leading to DNA strand breaking which is responsible for carcinogenesis, mutagenesis and cytotoxicity (Shukla et al., 2009). Oxygen radicals have capability to damage DNA by attacking either at sugar or the base of DNA and causes free radical pathology. The inhibitory effect of DVHA on free radical mediated deoxyribose damage was evaluated through iron (II) dependent DNA damage assay. DVHA was capable of scavenging hydroxyl radical at different concentrations (20, 50, 100, 200, 300 and 400 μg/mL) in a dose dependent manner (15.12%, 21.52%, 44.18%, 62.39%, 76.58%, 89.12%) and the IC_{50} values for DVHA and ascorbic acid were found to be 170.67 ± 0.98 μg/mL and 89.64 ± 0.50 μg/mL, respectively (Table 4). The capability of DVHA to scavenge hydroxyl radical is associated with its antioxidant activity and thus DVHA can control process of lipid peroxidation. Phenolic compounds present in DVHA are responsible for free radical scavenging activity due to their high molecular weight, proximity of many aromatic rings and hydroxyl groups.

3.8. Superoxide radical scavenging activity

Superoxide radical is a ROS which is known to be harmful to cellular components and DNA leading to diverse ailments (Shukla et al., 2009). A number of methods are now available for the generation of superoxide

Table 3
The content of phenolic constituents (μg/g of DVHA) detected and quantified in the flower of *D. volubilis* using UHPLC.

Extract	Gallic acid	Ferulic acid	Rutin	Ellagic acid	Quercetin	Cinnamic acid
DVHA	412.36 ± 2.29	162.72 ± 0.89	386.25 ± 2.00	208.8 ± 2.00	306.85 ± 2.24	213.71 ± 2.14

DVHA: hydroalcoholic flower extract of *D. volubilis* (values in μg/g of DVHA).
Results were expressed as mean ± SEM (n = 3) on dry weight basis.

Table 4
Radical scavenging and carbohydrate hydrolyzing enzymes inhibitory activities of hydroalcoholic flower extract of *D. volubilis*.

	IC_{50} value					
	DPPH radical scavenging activity	Hydroxyl radical scavenging activity	Superoxide radical scavenging activity	Nitric oxide radical scavenging activity	α-Glucosidase inhibitory activity	α-Amylase inhibitory activity
DVHA	237.86 ± 1.05	170.67 ± 0.98	219.07 ± 1.25	196.38 ± 1.49	3780.09 ± 21.19	360.68 ± 1.26
Ascorbic acid	27.18 ± 0.15	89.64 ± 0.50	59.33 ± 0.44	48.79 ± 0.26	–	–
Acarbose	–	–	–	–	489.38 ± 1.09	42.62 ± 0.23

DVHA: Hydroalcoholic flower extract of *D. volubilis* (values in μg/mL).
Results were expressed as mean ± SEM (n = 3).

radicals and thus superoxide radical scavenging activity of antioxidants are evaluated. In the study auto oxidation of hydroxylamine produced superoxide radicals in presence of NBT and the decrease of absorbance due to reduction of NBT in presence of antioxidants was measured at 560 nm. DVHA at different concentrations (20, 50, 100, 200, 300 and 400 μg/mL) showed superoxide radical scavenging activity in a dose dependent manner (10.23%, 18.16%, 30.58%, 55.62%, 63.78% and 78.17%) in the experiment (Fig. 3). The IC_{50} values for DVHA and ascorbic acid were found to be 219.07 ± 1.25 μg/mL and 59.33 ± 0.44 μg/mL, respectively (Table 4). The superoxide radical scavenging activity of DVHA may be due to presence of bioactive constituents.

3.9. Total antioxidant capacity

A green phosphate/Mo (V) complex with maximal absorption at 695 nm was formed in the phosphomolybdenum method and the absorbance value was proportional to antioxidant power of DVHA. The reducing capacity or electron donating power of the antioxidants to molybdenum was evaluated in the assay. The reducing ability of a chemical entity may play as a remarkable indicator of its potential antioxidant activity. The total antioxidant capacity of *D. volubilis* was found to be 39.68 ± 1.62 mg GAE/g of DVHA (Table 2). The finding obtained in phosphomolybdenum assay indicates that the phytoconstituents present in DVHA act as antioxidants.

3.10. Nitric oxide radical scavenging activity

Nitric oxide, a ROS, causes oxidative damage to the membrane lipid peroxidation, DNA fragmentation and lipoprotein oxidation which plays an important role in various types of diseases (Taira et al., 2015). Various concentrations of DVHA (20, 50, 100, 200, 300 and 400 μg/mL) showed 12.31%, 21.12%, 38.53%, 58.41%, 69.12% and 81.51% inhibition, respectively (Fig. 3). The results showed that the plant extract exhibited nitric oxide radical scavenging activity in a dose dependent manner. IC_{50} values for DVHA and ascorbic acid were found to be 196.38 ± 1.49 μg/mL and 48.79 ± 0.26 μg/mL, respectively (Table 4). Phenolic compounds are free radical scavengers due to specific structural features and catechol group present in the molecule

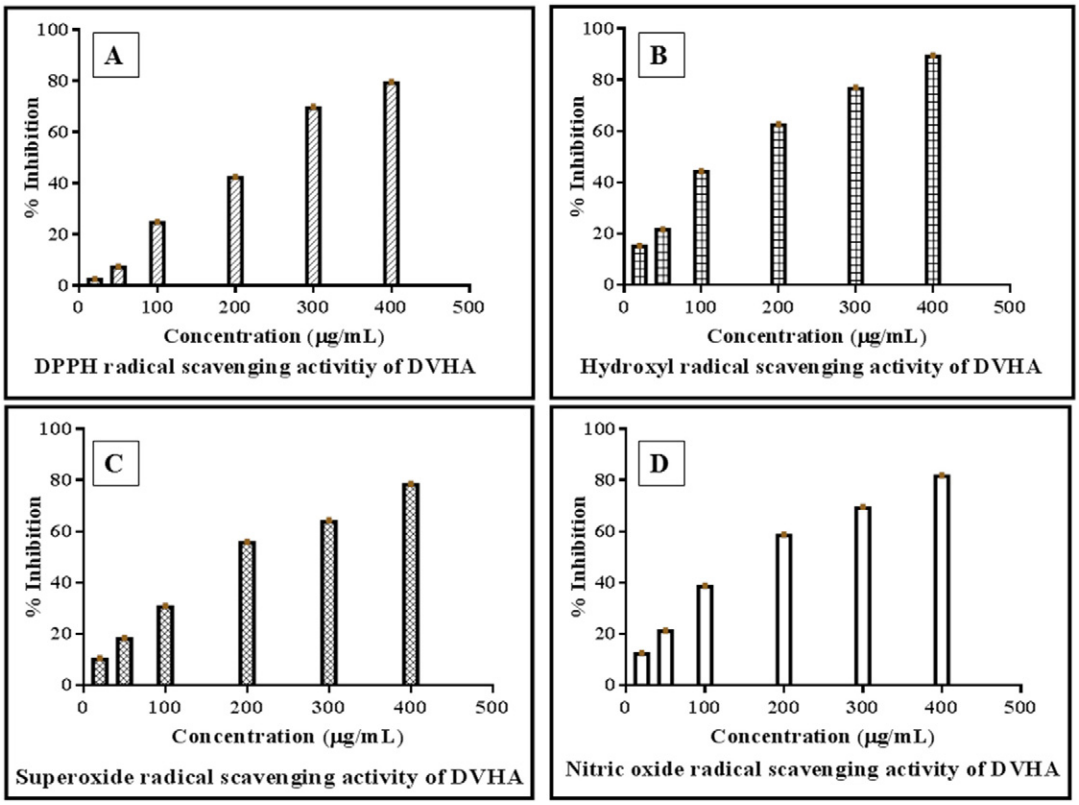


Fig. 3. Free radical scavenging activity (A, DPPH radical; B, hydroxyl radical; C, superoxide radical; D, nitric oxide radical) of the flower extract of *Dregea volubilis*.

is required for excellent nitric oxide radical scavenging activity. Flavonoids exert their bioactivity through scavenging of nitric oxide radical. Polyphenols present in DVHA plays an important role for nitric oxide radical scavenging activity.

3.11. α-Glucosidase inhibitory activity

A fruitful approach to manage diabetes mellitus, especially the non-insulin-dependent Type 2 diabetes, involves control over excessive rise of the blood glucose level by inhibiting the starch digestive enzymes (Tan et al., 2017). α-Glucosidase, a membrane bound carbohydrate hydrolyzing enzyme present in the epithelium of small intestines, catalyses hydrolysis of complex carbohydrates and disaccharides into absorbable monosaccharides and subsequent influx of glucose from the intestinal tract to blood vessels is responsible for postprandial hyperglycaemia (Olaokun et al., 2013). Many scientific studies have reported that vegetables and herbal extracts have the capability to inhibit activity of α-glucosidase leading to control of blood glucose level which suggests that food resources can be utilized as dietary anti-diabetic agents for controlling postprandial hyperglycaemia (Yu et al., 2015; Wojdylo et al., 2016). Natural food resources enriched with phenolic compounds strongly interacts with proteins and inhibits the enzymatic activities by forming complexes with enzymes and changing their biological structures (Yu et al., 2015). In the study, DVHA at different concentrations (500, 1000, 2000, 3000, 4000 and 5000 μg/mL) exhibited 7.21%, 11.85%, 22.93%, 44.70%, 53.15% and 64.65% inhibition of α-glucosidase, respectively (Fig. 4). The extract of flower of *D. volubilis* showed the enzyme inhibitory activity in a concentration dependent manner. The half-inhibitory concentration (IC_{50}) of DVHA and acarbose were estimated to be 3780.09 ± 21.19 μg/mL and 489.38 ± 1.09 μg/mL, respectively (Table 4). The α-glucosidase inhibitory activity of DVHA might be credited to its high content of bioactive phenolic compounds. Therefore, flower of *D. volubilis* can be regarded as a valuable food resource with health-promotion effects.

3.12. α-Amylase inhibitory activity

Natural sources with α-amylase inhibiton activity have taken great attention to researchers due to lower side effects of natural substitutes to synthetic enzyme inhibitors such as acarbose, metformin and orlistat. α-Amylase enzyme catalyses the cleavage of α-D-(1–4) glycosidic linkages of starch, amylose, amylopectin, glycogen and various maltodextrins into shorter oligosaccharides leading to production of absorbable monosaccharides and subsequent intestinal absorption (Yu et al., 2015). Plant phenolics have ability to bind with the reactive sites of α-amylase and can modify the catalytic activity of α-amylase enzyme and hence exhibit hypoglycemic effect (Kunyanga et al., 2012). The inhibitors of α-amylase retard breakdown and digestion of starch and other complex carbohydrates present in diet in gastrointestinal tract and therefore make them useful in dietary management of Type 2 diabetes. The α-amylase inhibitory activity of DVHA at various concentrations (50, 100, 200, 300, 400 and 500 μg/mL) were found to be 5.23%, 12.56%, 20.58%, 45.39%, 55.87% and 69.86% inhibition of α-amylase, respectively (Fig. 4). IC_{50} of DVHA and acarbose were found to be 360.68 ± 1.26 μg/mL and 42.62 ± 0.23 μg/mL, respectively (Table 4). The phenolics present in flower of *D. volubilis* may be attributed to its enzyme inhibition activity. Flower of *D. volubilis* may be consumed as a promising natural source with therapeutic benefits.

4. Conclusion

This study has revealed that DVHA of the flower of *D. volubilis* exerts promising antioxidant potential towards different systems *in vitro* and α-glucosidase and α-amylase inhibiting activities *in vitro*. The phytochemical profiling of the edible vegetable provides data relating to

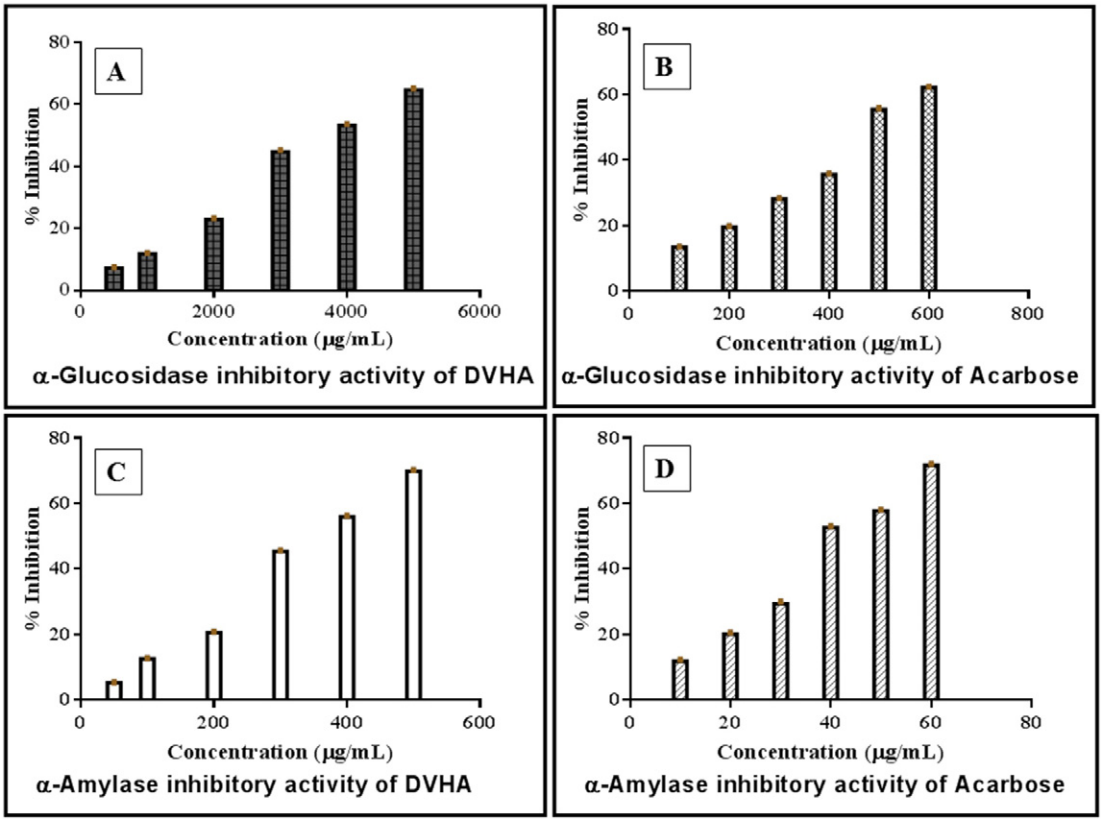


Fig. 4. Enzyme inhibition activity (A, α-glucosidase inhibitory activity of DVHA; B, α-glucosidase inhibitory activity of acarbose; C, α-amylase inhibitory activity of DVHA; D, α-amylase inhibitory activity of acarbose).

phenolic characterization of DVHA from the view of being a source of bioactive compounds with health beneficiary effects. The flower of the plant is therefore recognized as powerful antioxidant as well as carbohydrate hydrolyzing enzymes inhibitor which is helpful in the field of nutrition and medicine. The biological activities might be due to the presence of the phenolic compounds in the flower of the plant which were identified via UHPLC. To the best of our knowledge, this is the first study in evaluating its health promoting effects. In conclusion, flower of *D. volubilis* can be considered as a nutraceutical because of their health beneficial properties which deserve to be further researched and developed in food and pharmaceutical industries.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors acknowledge the University Grants Commission, New Delhi for providing the instrumental facilities under UGC-UPE Phase–II programme at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata.

References

Ang, L.Z.P., Hashim, R., Sulaiman, S.F., Coulibaly, A.Y., Sulaiman, O., Kawamura, F., Salleh, K.M., 2015. *In vitro* antioxidant and antidiabetic activites of *Gluta torquata*. Industrial Crops and Products 76, 755–760.

Anonymous, 1976. Wealth of India vol. X. Publication and Information Directorate, CSIR, New Delhi.

Baret, P., Septembre-Malaterre, A., Rigoulet, M., d'Hellencourt, C.L., Priault, M., Gonthier, M., Devin, A., 2013. Dietary polyphenols preconditioning protects 3T3-L1 preadipocytes from mitochondrial alterations induced by oxidative stress. The International Journal of Biochemistry & Cell Biology 45, 167–174.

Chatterjee, A., Pakrashi, S.C., 1995. The Treatise on Indian Medicinal Plants vol. 4. Publication and Information Directorate, New Delhi.

Dey, A., Lakshmanan, J., 2013. The role of antioxidants and other agents in alleviating hyperglycemia mediated oxidative stress and injury in liver. Food & Function 4, 1148–1184.

Hajiaghaalipour, F., Kanthimathi, M.S., Sanusi, J., Rajarajeswaran, J., 2015. White tea (*Camellia sinensis*) inhibits proliferation of the colon cancer cell line, HT-29, activates caspases and protects DNA of normal cells against oxidative damage. Food Chemistry 169, 401–410.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. third ed. Oxford University Press Inc., New York.

Hammi, K.M., Jdey, A., Abdelly, C., Majdoub, H., Ksouri, R., 2015. Optimization of ultrasound-assisted extraction of antioxidant compounds from Tunisian *Zizyphus lotus* fruits using response surface methodology. Food Chemistry 184, 80–89.

Hooshmand, S., Kumar, A., Zhang, J.Y., Johnson, S.A., Chai, S.C., Arjmandi, B.H., 2015. Evidence for anti-inflammatory and antioxidative properties of dried plum polyphenols in macrophage RAW 264.7 cells. Food & Function 6, 1719–1725.

Hossain, E., Chandra, G., Nandy, A.P., Gupta, J.K., Mandal, S.C., 2013. Possible fasciocidal activity of methanol extract of *Dregea volubilis* leaves. Experimental Parasitology 135, 183–187.

Jadhav, R.S., Ahmed, M.L., Swamy, P., Sanaullah, S., 2012. Neuropharmacological studies of drevogenins isolated from *Wattakaka volubilis* (Linn.f.) Stapf. in mice. International Journal of Pharma and Bio Sciences 3, 80–88.

Karaman, S., Tütem, E., Baskan, K.S., Apak, R., 2010. Comparison of total antioxidant capacity and phenolic composition of some apple juices with combined HPLC–CUPRAC assay. Food Chemistry 120, 1201–1209.

Kirtikar, K.R., Basu, B.D., 1935. Indian Medicinal Plants. vol. 3. International Book Distributors, Dehradun.

Kumkrai, P., Weeranantanapan, O., Chudapongse, N., 2015. Antioxidant, α-glucosidase inhibitory activity and sub-chronic toxicity of *Derris reticulata* extract: its antidiabetic potential. BMC Complementary and Alternative Medicine 15, 1–10.

Kunyanga, C.N., Imungi, J.K., Okoth, M.W., Biesalski, H.K., Vadivel, V., 2012. Total phenolic content, antioxidant and antidiabetic properties of methanolic extract of raw and traditionally processed Kenyan indigenous food ingredients. LWT - Food Science and Technology 45, 269–276.

Kurutas, E.B., 2016. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutrition Journal 15, 1–22.

Nandi, D., Besra, S.E., Vedasiromoni, J.R., Giri, V.S., Rana, P., Jaisankar, P., 2012. Anti-leukemic activity of *Wattakaka volubilis* leaf extract against human myeloid leukemia celllines. Journal of Ethnopharmacology 144, 466–473.

Olaokun, O.O., McGaw, L.J., Eloff, J.N., Naidoo, V., 2013. Evaluation of the inhibition of carbohydrate hydrolysing enzymes, antioxidant activity and polyphenolic content of extracts of ten African *Ficus* species (Moraceae) used traditionally to treat diabetes. BMC Complementary and Alternative Medicine 13, 1–10.

Ozsoy, N., Can, A., Yanardag, R., Akev, N., 2008. Antioxidant activity of *Smilax excelsa* L. leaf extracts. Food Chemistry 110, 571–583.

Panda, N., Mondal, N.B., Banerjee, S., Sahu, N.P., Koike, K., Nikaido, T., Weber, M., Luger, P., 2003. Polyhydroxy pregnanes from *Dregea volubilis*. Tetrahedron 59, 8399–8403.

Panda, N., Mandal, D., Mandal, N.B., Sahu, N.P., Banerjee, S., 2006. Flavonoid and flavone C-glycosides from *Dregea volubilis*. Natural Product Communications 1, 731–733.

Pascu, M., Pascu, D., Trăistaru, G.A., Nechifor, A.C., Bunaciu, A.A., Aboul-Enein, H.Y., 2014. Different spectrophotometric methods for antioxidant activity assay of four Romanian herbs. Journal of the Iranian Chemical Society 11, 315–321.

Poovitha, S., Parani, M., 2016. *In vitro* and *in vivo* α-amylase and α- glucosidase inhibiting activities of the protein extracts from two varieties of bitter gourd (*Momordica charantia* L.). BMC Complementary and Alternative Medicine 16, 185 (1–8).

Pothitirat, W., Chomnawang, M.T., Supabphol, R., Gritsanapan, W., 2009. Comparison of bioactive compounds content, free radical scavenging and anti-acne inducing bacteria activities of extracts from the mangosteen fruit rind at two stages of maturity. Fitoterapia 80, 442–447.

Sahu, N.P., Panda, N., Mandal, N.B., Banerjee, S., Koike, K., Nikaido, T., 2002. Polyoxypregnane glycosides from the flowers of *Dregea volubilis*. Phytochemistry 61, 383–388.

Samanta, A., Das, G., Das, S.K., 2011. Roles of flavonoids in plants. International Journal of Pharmaceutical Science and Technology 6, 12–35.

Shen, Y., Zhang, H., Cheng, L., Wang, L., Qian, H., Qi, X., 2016. *In vitro* and *in vivo* antioxidant activity of polyphenols extracted from black highland barley. Food Chemistry 194, 1003–1012.

Shukla, S., Mehta, A., Bajpai, V.K., Shukla, S., 2009. *In vitro* antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food and Chemical Toxicology 47, 2338–2343.

Shukla, S., Park, J., Kim, D., Hong, S., Lee, J.S., Kim, M., 2016. Total phenolic content, antioxidant, tyrosinase and a-glucosidase inhibitory activities of water soluble extracts of noble starter culture Doenjang, a Korean fermented soybean sauce variety. Food Control 59, 854–861.

Silverstein, R.M., Webster, F.X., Kiemle, D.J., 2005. Spectrometric Identification of Organic Compounds. seventh ed. John Wiley & Sons, Inc., New Jersey.

Sreeramulu, N., Suthari, S., Ragan, A., Raju, V.S., 2013. Ethno-botanico-medicine for common human ailments in Nalgonda and Warangal districts of Telangana, Andhra Pradesh, India. Annals of Plant Sciences 02, 220–229.

Taira, J., Tsuchida, E., Katoh, M.C., Uehara, M., Ogi, T., 2015. Antioxidant capacity of betacyanins as radical scavengers for peroxyl radical and nitric oxide. Food Chemistry 166, 531–536.

Tan, Y., Chang, S.K.C., Zhang, Y., 2017. Comparison of α-amylase, α-glucosidase and lipase inhibitory activity of the phenolic substances in two black legumes of different genera. Food Chemistry 214, 259–268.

Tarantilis, P.A., Troianou, V.E., Pappas, C.S., Kotseridis, Y.S., Polissiou, M.G., 2008. Differentiation of Greek red wines on the basis of grape variety using attenuated total reflectance Fourier transform infrared spectroscopy. Food Chemistry 111, 192–196.

Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y., Gritsanapan, W., 2013. Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. Industrial Crops and Products 44, 566–571.

Wojdyło, A., Oszmiański, J., Czemerys, R., 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry 105, 940–949.

Wojdylo, A., Nowicka, P., Carbonell-Barrachina, Á.A., Hernández, F., 2016. Phenolic compounds, antioxidant and antidiabetic activity of different cultivars of *Ficus carica* L. fruits. Journal of Functional Foods 25, 421–432.

Yang, M., Shen, Q., Li, L., Huang, Y., Cheung, H., 2015. Phytochemical profiles, antioxidant activities of functional herb *Abrus cantoniensis* and *Abrus mollis*. Food Chemistry 177, 304–312.

Yu, Z., Gao, H., Zhang, Z., He, Z., He, Q., Jia, L., Zeng, W., 2015. Inhibitory effects of *Ligustrum robustum* (Rxob.) Blume extract on α-amylase and α-glucosidase. Journal of Functional Foods 19, 204–213.

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 5, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

PHARMACOGNOSTIC STUDIES ON FLOWERS OF *DREGEA VOLUBILIS*: EVALUATION FOR AUTHENTICATION AND STANDARDIZATION

BHASKAR DAS[1], ARNAB DE[1], PIU DAS[1], AMALESH NANDA[2], AMALESH SAMANTA[1]*

[1]Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal, India. [2]Department of Biotechnology, National Institute of Technology, Arunachal Pradesh, India. Email: asamanta61@yahoo.co.in

Received: 01 February 2019, Revised and Accepted: 19 March 2019

ABSTRACT

Objective: The various parts of *Dregea volubilis* (Family: Apocynaceae), locally known as Jukti (Bengali), are commonly used in Indian system of medicine to treat various ailments such as inflammation, piles, leukoderma, asthma, and tumors. Literature review suggested that there has been no detailed work on systemic pharmacognostic and phytochemical studies done on the flowers of the plant. The present study is aimed to lay down quality control parameters for *D. volubilis* flowers to confirm its identity, quality, and purity.

Methods: The present work was designed to study detailed organoleptic, histological, quantitative standards, physicochemical, spectroscopic, and chromatographic characteristics of the flowers of *D. volubilis.*

Results: The total ash, acid insoluble ash, water soluble ash, loss on drying, water, and alcohol soluble extractive values were found to be 11.767±0.130% (w/w), 1.287±0.106% (w/w), 9.140±0.344% (w/w), 14.110±0.061% (w/w), 21.600±0.133% (w/v), and 9.603±0.104% (w/v), respectively. Phytochemical screening of different extracts showed the presence of carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, flavonoids, tannins, and phenolics. The chromatographic study revealed the presence of rhamnose (103.229±4.994 µg/g), fructose (738.670±25.714 µg/g), glucose (285.532±24.465 µg/g), and maltose (49.082±5.206 µg/g).

Conclusion: The characterization parameters of the present study may serve as a reference standard for proper authentication, identification and for distinguishing the plant from its adulterants.

Keywords: *Dregea volubilis,* Organoleptic, Phytochemistry, High-performance liquid chromatography, Fourier transform infrared.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i5.32257

INTRODUCTION

Herbal medicines play an important role in the health-care system to alleviate and treat diseases. There is a great demand for medicinal plants in the herbal industry due to its health beneficiary properties with multi-dimensional chemical structures. Standardization of the medicinal plants is essential to confirm the authenticity and quality to avoid deliberate adulteration and substitution [1].

Dregea, a genus of vines, is a rich source of steroidal pregnanes with potential biological activities [2]. *Dregea volubilis* (L.f.) Benth. ex Hook.f. (Synonym: *Wattakaka volubilis* (L.f.) Stapf., *Marsdenia volubilis* (L.f.) Cooke) belongs to the kingdom of Plantae, subfamily of Apocynoideae, family of Apocynaceae, order of Gentianales, series of Bicarpellatae, subclass of Gamopetalae, class of Dicotyledons and are distributed widely in the tropical zone and South East Asia [3]. *D. volubilis,* a large twining perennial shrub, grows as a woody climber having woody vines and is scattered throughout the India and Car-Nicobar ascending to an altitude of 1500 m [4]. The plant blooms between March and April. The young branches of the plant are green, slender, and smooth; the older branches are gray, very long, and glabrous, often with lenticels or small black dots. Leaves are broadly ovate or somewhat rounded, sub-orbicular, acuminate, 7.5–15 cm long, 5–10 cm wide. Flowers are green or pale green, about 1 cm in a radius, bisexual and sweet-scented in a drooping umbel. Follicles are usually two, slightly tapering to a very blunt point, glabrous, and striated. The seeds are elliptic, concave, flattened, smooth, and shining. Different parts of the plant have been traditionally used in Ayurveda in India for the treatment of various ailments such as asthma, inflammation, tumors, piles, leukoderma, application to boils, rat bite, and urinary discharge [5]. The vernacular names of the medicinally important plant are Jukti (Bengali), Akadbel (Hindi), Harandodi (Marathi), Velipparuthi (Malayalam), Dudhipaala (Telugu), Koti-p-palai (Tamil), Dugdhive (Kannada), Dudghika (Oriya), Khamal lata (Assamese), Kadvo kharkhodo (Gujarati), and Hemajivanti (Sanskrit). The flowers of *D. volubilis* are eaten as a seasonal vegetable in early summer with bitter esthetic principles. Flowers of the plant are a rich source of biologically active phytochemicals and were reported to contain volubiloside A, volubiloside B, volubiloside C, dregealol, volubilogenone, volubilol, drevogenin D, iso-drevogenin P, 17α-marsdenin, dregeanin, vicenin-2, vitexin, isovitexin, isoorientin, rutin, quercetin, luteolin, apigenin, gallic acid, ferulic acid, ellagic acid, and cinnamic acid [6-9]. Antioxidant and antidiabetic activities of hydroalcoholic extract of the flowers of the plant were reported earlier by the present authors [9].

In spite of a lot of attention to health beneficiary effects, flowers of *D. volubilis* have been less explored pharmacognostically. A detailed study on the structural morphology and other physicochemical parameters of the flowers of *D. volubilis* are required. The present study is aimed to evaluate the flowers to fix the pharmacognostical parameters for proper identification, authentication, and quality standardization of the plant.

METHODS

Plant material

The fresh flowers of *D. volubilis* were collected in the month of April 2017 from Jaynagar Mazilpur, South 24 Parganas, West Bengal, India. It was taxonomically identified and authenticated by Dr. V.P. Prasad, Central National Herbarium, Botanical Survey of India, Botanical Garden, Howrah, West Bengal, India. A voucher specimen of the plant was kept at Division of Microbiology and Biotechnology, Department

of Pharmaceutical Technology, Jadavpur University, Kolkata, India. The flowers were dried under shade, powdered using a mechanical grinder and preserved at 25±2°C in airtight container at a dry place.

Chemicals and instruments

Rhamnose, xylose, fructose, glucose, trehalose, and maltose were purchased from Sigma-Aldrich, St Louis, MO, USA. Ethanol was purchased from EMD Millipore, Bedford, MA, USA. Chloroform, acetone, 2-propanol, ethyl acetate, diethyl ether, petroleum benzine (40–60°C), methanol for liquid chromatography, acetonitrile for liquid chromatography, sodium carbonate, anthrone, glacial acetic acid, hydrochloric acid, nitric acid, acetic acid, sulfuric acid, phosphoric acid, ammonia solution 25%, Coomassie Brilliant Blue G250, sodium hydroxide, potassium hydroxide, chloral hydrate, and glycerin were procured from Merck Life Sciences Private Limited, Mumbai, India. The water was purified by a Milli-Q water purification system (EMD Millipore, Bedford, MA, USA) and used for all experiments. All other reagents used were of analytical grade.

Macroscopic evaluation

The macroscopic study of the crude drug includes evaluation of its morphological characteristics which are examined by the naked eye and magnifying lens. The method is the simplest and quickest mean to check the authenticity of a crude drug [10].

Microscopic evaluation

Fresh flowers were collected and washed with water for carrying out the microscopical study. Different parts of the flower were cut into very thin transverse sections (T. S) and boiled in 10% potassium hydroxide solution to remove fatty materials and coloring substances. The sections were stained and observed under Magnus microscope (Olympus [India] Pvt. Ltd., Noida, India). Photomicrographs were captured with Magnus photomicrography units (MIPS USB 2.0Capture and Display Software) at ×40 [11].

Powder characteristics

The mechanically grinded dried powdered material was sieved through mesh number 80 to get uniform powder. It was cleared with chloral hydrate, stained, and mounted in glycerin to observe under Magnus microscope (Olympus [India] Pvt. Ltd., Noida, India) [12].

Quantitative standards

The shade dried powdered material of flowers of *D. volubilis* was evaluated for the determination of ash values, extractive values, and loss on drying (LOD).

Ash values

The total ash, water-soluble ash, and acid insoluble ash of the plant material were performed [13].

Total ash

About 1 g of the material was taken in a previously ignited and tarred silica crucible. The material was spread in even layer and ignited at 450°C by gradually increasing the temperature until it was white indicating the absence of carbon. It was then allowed to cool in a desiccator. The total ash content (% w/w) of the material was calculated according to the following equation:

Total ash (% w/w)=(weight of ash/weight of sample)×100

Acid insoluble ash

The acid insoluble ash was determined by boiling the total ash with 25 ml of 2 (N) hydrochloric acid (HCl) into a China dish. It was covered with a watch glass and gently boiled for 5 min. The watch glass was rinsed with 5 ml of boiled water and the rinsed contents were transferred to the contents of China dish. The insoluble matter of the contents of the china dish was collected on tarred gooch crucible, washed with boiled acidulated water, ignited, cooled in a desiccator and weighed. Acid-insoluble ash content (% w/w) of the material was calculated with reference to the crude drug according to the following equation:

Acid-insoluble ash (% w/w)=(weight of ash/weight of sample)×100

Water-soluble ash

About 25 ml of water was added to the total ash in a China dish and was gently boiled for 5 min. The water-insoluble ash was collected on tarred gooch crucible, washed with boiled acidulated water, ignited, cooled in a desiccator, and weighed. The water-soluble ash was calculated by subtracting the weight of insoluble matter from the weight of total ash. The water-soluble ash content (% w/w) was determined with respect to the air-dried material using the following equation:

Water-soluble ash (% w/w)=(weight of water-soluble ash/weight of sample)×100

Extractive values

The extractive values are indicative weights of the extractable chemical constituents of crude drugs in different solvents. The extractive values of crude drugs were determined in water and alcohol [14]. 5 g each of the crude drugs was taken in a 250 ml stoppered conical flask. 100 ml of the respective solvent was added to the 250 ml stoppered conical flask and was allowed to macerate for 24 h with the aid of mechanical shaker for 6 h. It was then filtered and 25 ml of the filtrate was taken in a tarred Petri dish. It was evaporated to dryness in an oven at 105°C and weighed it again. The extractive value (% w/w) was calculated with respect to the air dried material using the following equation:

Extractive value (% w/w)=(weight of extracted residue/weight of sample)×100

LOD

The LOD was performed [15] by taking 1 g of the crude drug in previously weighed LOD weighing bottle. It was dried in an oven for 1 h, cooled in a desiccator and weighed. The LOD (% w/w) was calculated with respect to the crude drug using the following equation:

LOD (% w/w)=(weight loss/weight of sample)×100

Fluorescence analysis

The fluorescence analysis was performed by treating the dried powdered material with different chemicals and was observed in daylight and ultraviolet (UV) light [16]. Some of the phytochemicals present in plant material show fluorescence in the visible range in daylight. The UV ray produces fluorescence in many crude drugs which do not fluorescence in daylight. A more powerful source of ultraviolet ray is often needed to produce fluorescence in crude drugs. Different types of reagents are often applied to the crude drugs which do not fluoresce to convert them into fluorescent derivatives. The fluorescence analysis is an important parameter for pharmacognostic evaluation for assessing crude drugs qualitatively. The behavior of powdered drugs after treatment with different chemical reagents and their fluorescent characteristics were observed under UV (254 and 366 nm) and visible light using CAMAG UV CABINET 4.

Preliminary phytochemical studies

The shade dried powdered material of flowers of *D. volubilis* weighing about 200 g was soaked with sufficient amount of light petroleum benzine (40–60°C) in a glass beaker for 24 h and then the flowers were extracted with petroleum benzine (40–60°C), chloroform, methanol successively using Soxhlet apparatus and the exhausted material was boiled with water. The petroleum benzine (40–60°C) fraction obtained after extraction using Soxhlet apparatus was combined with the initial fraction of petroleum benzine (40–60°C) obtained after soaking. The extracts of organic solvents were concentrated using rotary evaporator under reduced pressure and evaporated to dryness. The aqueous extract was concentrated using a water bath

and lyophilized. The extracts were preserved in well-closed container and kept in the dark at a temperature of 10°C for future use. The different extracts obtained were tested individually for the presence of different phytoconstituents [17]. Thin-layer chromatography (TLC) fingerprinting of the extracts was performed using Silica Gel G as an adsorbent. TLC plates (Millipore Corporation, USA) were activated in a hot air oven at 110°C for 30 min. The plates were kept in a desiccator for future use. Different combinations of solvents were used for the preparation of the mobile phases.

High-performance liquid chromatography (HPLC) fingerprinting

The chromatographic fingerprinting was performed by ultra HPLC (UHPLC) using a UHPLC+ focused system consisting of a Dionex Ultimate 3000 Pump, a Dionex Ultimate 3000 autosampler column compartment, and a Dionex Ultimate 3000 variable wavelength detector [18]. 1 g of dried and finely powdered (mesh size 85) sample was taken in 10 ml volumetric flask and sufficient Milli-Q water was added and heated in boiling water bath for 20 min and cooled and made up to the volume with Milli-Q water. The solution was filtered through a 0.45 µm syringe filter (Millex, Merck, Germany) which was injected as a test solution and Milli-Q water was injected as blank. Chromatographic separations of phytochemicals of the extract were performed using a C18 column (250 mm×4.6 mm i.d.) with a particle size of 5 µm, Hypersil GOLD (Thermo Fisher Scientific, U.S.A.) and column oven temperature were maintained at 25°C. The chromatographic separation was performed using gradient elution (Table 1) with a flow rate of using 0.2 (% v/v) phosphoric acid as mobile phase A and HPLC grade methanol as mobile phase B, respectively. The UV detector was set at 280 nm and the injection volume was 20 µl. The chromatograms were processed with Chromeleon 7, version 7.2.0.3765 software (Thermo Scientific, U.S.A.).

Fourier transform infrared (FTIR)

FTIR spectra of the different extracts of *D. volubilis* were performed in attenuated total reflection (ATR) mode using Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA) with a total of 30 scans at a resolution of 4 cm^{-1} in the wave number range between 4000 cm^{-1} and 525 cm^{-1}. Background spectrum of a clean ATR crystal was collected immediately before collecting the spectrum of the plant extracts. The extracts were then placed in the ATR accessory and pressed for acquiring the FTIR spectra of the samples. The spectral acquisitions were processed with OMNIC software supplied by the manufacturer. FTIR study provided qualitative information on the types of functional groups and chemical bonds present in the phytochemicals of the extracts by analyzing the peak values (cm^{-1}) of the spectra [9].

Determination of UV-VIS spectra

The different extracts of *D. volubilis* were diluted with respective solvents at a concentration of 0.05% (w/v) in respective solvents and scanned between 200 nm and 800 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA), and the spectra were recorded [19].

Determination of pH

The shade dried powdered material of flowers of *D. volubilis* was mixed with water at a concentration of 1% (w/v), 2% (w/v), and 10% (w/v) and kept in a water bath for 20 min. It was then filtered through Whatman filter paper No. 1 and the pH of the filtrate was measured using a pH meter (Model: 3200P, Agilent Technologies, USA) at 25°C [20].

Determination of protein content

The protein content of the sample was determined according to Bradford method with some modifications [21]. 0.5 g powder of *D. volubilis* flower was mixed with 10 ml of water and the mixture was shaken for 10 min followed by filtration using Whatman filter paper No. 1. 0.2 ml of the sample solution was mixed with 5 ml of Bradford's reagent (0.1 g of Coomassie Brilliant Blue G250 was dissolved in 50 ml of ethanol followed by addition of 100 ml 85% (v/v) phosphoric acid and volume was made up to 1 L). The reaction mixture was kept for 10 min for the development of color completely. The absorbance of the reaction mixtures was measured at 595 nm against a blank using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The protein present in the sample was quantified from calibration curves of absorbance versus concentration in µg/ml of bovine serum albumin which was used as a standard.

Determination of carbohydrate content

The total carbohydrate content of the dried powdered material of flowers of *D. volubilis* was determined by the anthrone method with slight modifications [22]. A standard stock solution (10 mg/ml) containing glucose was prepared in Milli-Q water and different concentrations (20, 40, 60, 80, 90, and 100 µg/ml) of standard solutions were prepared by diluting the stock solution for the calibration curve. 100 mg of dried sample was hydrolyzed by keeping it in a boiling water bath for 3 h with 5 ml of 2.5 (N) HCl and cooled down to room temperature. It was then neutralized with solid sodium carbonate until effervescence ceased and volume of the solution made up to 100 ml with Milli-Q water. It was then centrifuged at 2000 rpm and the supernatant was collected. 1 ml each of standard solutions and sample solution was added to 4 ml of anthrone reagent (0.2% [w/v] anthrone in ice cold concentrated sulfuric acid) and heated for 8 min in boiling water bath and cooled to room temperature. A blank solution was prepared by adding 1 ml Milli-Q water to 4 ml of anthrone reagent. The absorbance of the reaction mixtures was measured at 630 nm against the blank using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, USA).

Determination of free sugar composition

The free sugars were determined by ultra-fast liquid chromatography (UFLC) using a prominence UFLC system (Shimadzu, Japan) equipped with a LC-20AT Solvent Delivery Unit, SIL-20A UFLC version autosampler, and RID-10A refractive index detector [23]. A mixed standard stock solution (10 mg/ml) containing rhamnose, xylose, fructose, glucose, trehalose, and maltose was prepared in Milli-Q water and different concentrations (0.625, 1.25, 2.5, 5, and 7.5 mg/ml) of standard solutions were prepared by diluting the mixed standard stock solution for calibration curves to quantify the sugars present in the sample. 1 g of dried sample was extracted with 40 ml of 80% (v/v) aqueous ethanol at 80°C for 30 min. It was then centrifuged at 15,000 g for 10 min and the supernatant was concentrated at 60°C under reduced pressure. The concentrated sample was defatted 3 times with 10 ml of diethyl ether. The defatted material was concentrated at 40°C and dried. The dried sample was dissolved in Milli-Q water to a final volume of 5 ml. The solution was filtered through a 0.45 µm syringe filter (Millex, Merck, Germany) which was injected as a test solution and Milli-Q water was injected as blank. Chromatographic separations of the sugars were performed using a NH_2 column (250 mm × 4.6 mm i.d.) with a particle size of 5 µm and pore size of 100 Å, Luna NH_2 (Phenomenex, U.S.A.) and column oven temperature was maintained at 40°C. A solvent mixture consisting of seven volumes of acetonitrile and three volumes of Milli-Q water was used as mobile phase. The chromatographic separation was performed with a flow rate of 1.0 ml/min with a run time of 15 min and the injection volume was 10 µl.

Statistical analysis

All experiments were performed in triplicate and the results of the quantitative studies are presented as mean±standard error of mean. The statistical analyses were performed with GraphPad PRISM6 software, USA.

RESULTS AND DISCUSSION

Macroscopic evaluation

The flowers were numerous, green or pale green in color, sweet-scented, and bitter in taste. Inflorescences were in lateral drooping umbellate cymes. 2.5–5 cm long slender peduncles were arising from between the petioles. 1–2.5 cm long slender calyx dividing nearly to

the base was ovate to oblong, obtuse, and ciliolate. Corolla was deeply divided and glabrous outside. Lobes were broadly ovate, obtuse and veined overlapping to the right. Stamina column aroused from the base of the corolla and anther tips were membranous, broadly ovate, oblong, and obtuse. Pollen masses were attached to the pollen carriers by very short caudicles (Fig. 1). The macroscopic evaluation of a crude drug is used for its authentication by comparing the diagnostic characters with the prescribed standards of the standard drug. The macroscopic feature helps to evaluate the basic differentiating characteristics between the various species within a single genus [10]. The parameters of the macroscopic evaluation are mostly subjective and there is a chance of existence of adulterants which are closely resembles the genuine drug. The microscopic and physicochemical analyses are more authentic studies to check whether the parameters of the crude drugs conform to the standard or not. The parameters of the macroscopic evaluation for the flowers of *D. volubilis* can be served as reference diagnostic characters for the authentication of the drug.

Microscopic evaluation

The T. S of the different floral parts of flower (Fig. 2) showed the following observations under the microscope.

Thalamus

The T. S of thalamus showed that epidermal layer was composed of a single layer of compactly arranged tabular cells with cuticularized outer walls. The cortex was composed of 2–3 layers of collenchyma, known as hypodermis. Several layers of thin-walled parenchyma cells, known as parenchymatous zone, were present next to the hypodermis in the cortex. The vascular bundles were collateral and open and each bundle was composed of external phloem and internal xylem. Pith was made of parenchyma cells. Trichomes were uniseriate, multicellular with blunt tip. Cluster crystals of calcium oxalate were present.

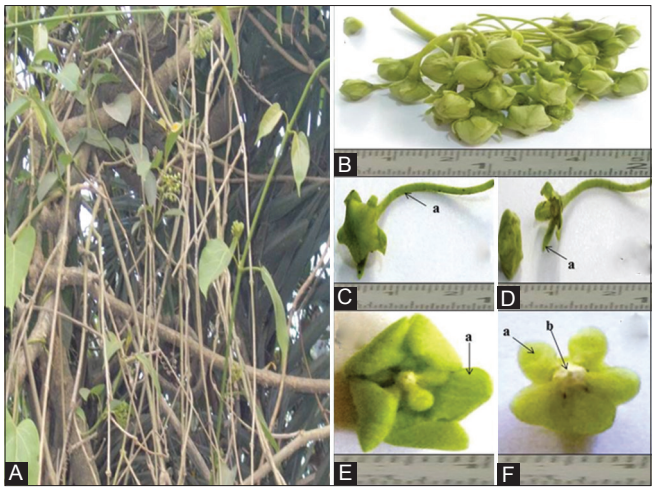


Fig. 1: (A) *Dregea volubilis* in its natural habitat. (B) Pale green flower in dense drooping umbels. (C) Individual flower of *D. volubilis* showing pedicel (a). (D) *D. volubilis* flower part showing sepal (calyx) (a). (E) *D. volubilis* flower part showing petal (corolla) (a). (F) *D. volubilis* flower part showing androecium (a) and gynoecium (b)

Table 1: UHPLC gradient program for the HPLC fingerprinting study

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	1	100	0
10	1	100	0
20	1	90	10
30	1	70	30
40	1	50	50
50	1	30	70
60	1	10	90
70	1	0	100

UHPLC: Ultra-high-performance liquid chromatography, HPLC: High-performance liquid chromatography

Stalk

The T. S of stalk showed that the epidermis was single-layered outermost zone consisting of compactly set tabular living cells, outer walls of which were cuticularized. Cortex was distinctive consisting of hypodermis which was composed of 2–3 layers of collenchyma cells. General cortex was present next to the hypodermis which was composed of several layers of thin-walled parenchyma cells. Vascular bundles were collateral and open consisting of external phloem and internal xylem. Pith was very distinct and large, situated in the center consisting of thin-walled, oval, or polygonal parenchyma cells with abundant intercellular spaces between them. Cluster crystals of calcium oxalate were found to be present. Trichomes were uniseriate, multicellular with blunt tip.

Calyx

The T. S of sepal showed that there were two epidermal layers, for example, upper and lower epidermis. Both the epidermal layers were uniseriate and composed of compactly arranged tabular cells, the outer walls of which were cuticled. The mesophyll was made of parenchyma cells lying between two epidermal layers. The mesophyll was differentiated into (a) upper closely packed, tubular chloroplast containing cells, known as palisade parenchyma, and (b) lower loosely arranged, more or less rounded cells, called spongy parenchyma. Oil globules and cluster crystals of calcium oxalate were also present. Trichomes were uniseriate, multicellular with blunt tip. Stomata were anomocytic.

Corolla

The T. S of petal showed that the epidermal layer was uniseriate and composed of compactly arranged tabular cells, the outer walls of which were cuticled. Trichomes were uniseriate, multicellular with blunt tip. Anomocytic stomata and oil globules were found to be present.

Androecium

The T. S showed that the epidermal layer was uniseriate and composed of compactly arranged tabular cells, the outer walls of which were cuticled. The cluster crystals of calcium oxalate were found to be present.

Gynoecium

The T. S showed the presence of cluster crystals of calcium oxalate.

The detailed microscopic examination of the flowers of *D. volubilis* can be used as a reference to identify the crude drug by comparing the known histological characters. The microscopic examination is an easiest and finest way to set standard parameters depending on the internal anatomy of the plant [15]. The microscopic study alone cannot provide complete evaluation profile for the herbal drugs. The histological characters of the drug along with other analytical parameters can be utilized to set the standardization specifications for the evaluation of the herbal drug.

Powder characteristics

The powder microscopy showed the presence of fibers, cluster crystals of calcium oxalate, and epidermal trichomes. Some fragments consisted of groups and parts of parenchyma cells and epidermal cells. The observations of the study on the powder of *D. volubilis* flowers can serve as useful parameters for the proper identification of the drug. The dried sample in the powdered form gives characteristic features of the drug under a microscope after proper treatments. The microscopical

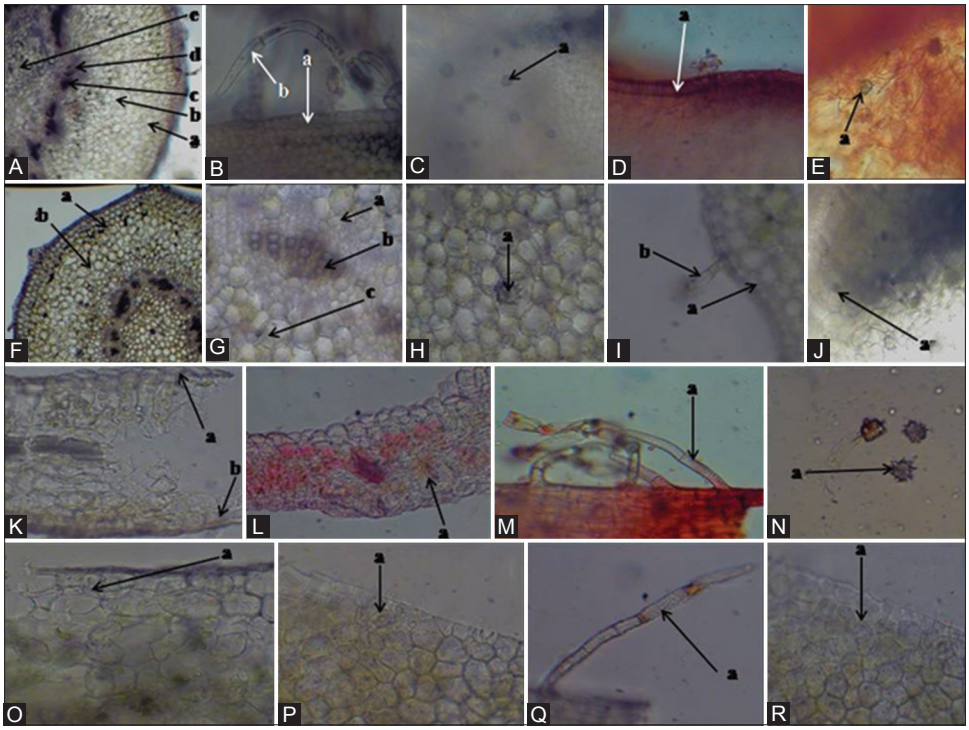


Fig. 2: Transverse section (T. S) of different parts of *Dregea volubilis* flower. (A). T. S of thalamus showing hypodermis (a), parenchymatous zone (b), xylem (c), phloem (d), and pith (e). (B) Epidermis (a) and trichome (b) in thalamus. (C) Cluster crystals of calcium oxalate (a) in thalamus. (D) T. S of androecium showing epidermis (a). (E) Cluster crystals of calcium oxalate (a) in androecium. (F) T. S of stalk showing hypodermis (a) and general cortex (b). (G) Phloem (a), xylem (b), and pith (c) in stalk. (H) Cluster crystals of calcium oxalate (a) in stalk. (I) Epidermis (a) and trichome (b) in stalk. (J) T. S of gynoecium showing cluster crystals of calcium oxalate. (K) T. S of sepal showing upper epidermis (a), lower epidermis (b). (L) Stomata in sepal (a). (M) Trichome in sepal (a). (N) Cluster crystals of calcium oxalate (a) in sepal. (O) T. S of petal showing epidermis (a). (P) Stomata in petal (a). (Q) Trichome in petal (a). (R) Oil globules in petal (a)

examination of epidermal trichomes and calcium oxalate crystals is extremely valuable for authentication of crude drugs [17].

Quantitative standards

The total ash, acid-insoluble ash, and water-soluble ash were found to be 11.767±0.130% (w/w), 1.287±0.106% (w/w), and 9.140±0.344% (w/w), respectively. The water and alcohol soluble extractive values were found to be 21.600±0.133% (w/v) and 9.603±0.104% (w/v), respectively. LOD was 14.110±0.061% (w/w). Therapeutic efficacy of herbal drugs can be ensured by determining the quantitative standards. The ash of crude drugs is consisted of nonvolatile inorganic materials which can be used to set quality control parameter to check the contamination of crude drugs. A high content of ash value beyond the standard limit gives an indication of contamination, substitution, or adulteration [24]. The content of active constituents in a given amount of crude drug is estimated by extractive value in a particular solvent. The extractive values give valuable information regarding the quality of the crude drug whether it is exhausted or not. The high extractive value is an indicative parameter of better extraction of phytoconstituents from crude drugs and it is also helpful for proper selection of solvent that will provide maximum yield [25]. The physical and physicochemical state of the interior of the cell depends on the loss of water. The enzymes present in the cell are responsible for different chemical reactions such as oxidation, hydrolysis, and polymerization of the phytoconstituents present in the plant material when the enzymes come in contact with the active substances during the process of drying. Most of the enzymes present in plant material need sufficient water to act leading to decomposition reactions of the crude drugs. Moisture present in the crude drugs helps in microbial growth leading to degradation of it. It is desirable to keep the water content of the crude drugs at low level to deactivate the enzyme activity as well as to retard microbial degradation to such an extent that the storage stability of the crude drugs is guaranteed [26]. These standardization parameters are essential to ensure the quality of herbal drugs. Quantitative standards can be applied for the evaluation of crude drugs. These parameters can be utilized for maintaining the identity, purity, and quality of crude drugs. Purity depends on the absence of foreign matter in crude drugs. Quality depends on the concentration of the active constituents present in the crude drugs that exert health beneficiary properties.

Fluorescence analysis

The fluorescence analysis of the powdered drug showed various colors after treatment with different chemical reagents and observed visually under daylight, short wavelength ultraviolet light (254 nm), and long wavelength ultraviolet light (366 nm). The results are shown in Table 2.

The fluorescence analysis of the *D. volubilis* flower showed various colors under ordinary light, short wavelength UV light (254 nm), and long wavelength UV light (366 nm) indicating the presence of fluorescent compounds. The analysis is a very important and useful tool for the identification of various phytoconstituents present in the crude drugs which give fluorescence either itself or after derivatization with proper chemical treatment under UV light [16]. The method is very easy and direct method for the identification of fluorescent compounds present in the test sample and the observations can be used as a quality control parameter for the identification of the crude drug.

Preliminary phytochemical studies

The results of preliminary qualitative phytochemical studies of the different extracts of flowers of *D. volubilis* are presented in Table 3. The TLC studies of the different extracts were performed in

Table 2: Fluorescence analysis of powdered flowers of *D. volubilis*

Sl. No.	Treatment	Daylight	UV light	
			254 nm	366 nm
1	Powder as such	Light brown	Dark green	Reddish brown
2	Powder+Acetic acid	Brown	Dark brown	Brownish black
3	Powder+Ferric chloride (5% $FeCl_3$)	Greenish brown	Brownish black	Black
4	Powder+Conc. Hydrochloric acid (HCl, 5N)	Yellowish brown	Brown	Bluish-black
5	Powder+Conc. Nitric acid HNO3	Reddish brown	Brownish black	Black
6	Powder+Conc. Sulfuric acid (H_2SO_4)	Brownish black	Brown	Brownish black
7	Powder+Iodine solution (1%)	Reddish yellow	Brown	Brownish black
8	Powder+Methanol	Light brown	Dark brown	Brownish black
9	Powder+Picric acid	Yellowish brown	Brownish yellow	Brownish black
10	Powder+NaOH Solution (1 N)	Reddish yellow	Dark brown	Brownish black
11	Powder+Distilled water	Yellowish brown	Dark brown	Brownish black
12	Powder+Liquid ammonia ($NH_{3)}$	Deep brown	Dark brown	Brownish black
13	Powder+Conc. HNO_3+NH_3	Reddish brown	Dark brown	Brownish black
14	Powder+Dilute HNO_3	Yellowish brown	Brown	Brownish black
15	Powder+10% potassium dichromate solution	Deep yellow	Dark brown	Black
16	Powder+Acetone	Brown	Yellowish brown	Bluish black

D. volubilis: Dregea volubilis, UV: Ultraviolet

Table 3: Preliminary phytochemical analysis of *D. volubilis* flower extracts

Phytochemicals	Petroleum benzine extract	Chloroform extract	Methanol extract	Aqueous extract
Carbohydrates	–	–	+	+
Proteins	–	–	+	+
Amino acids	–	–	+	+
Steroids	+	+	–	–
Glycosides	–	+	+	+
Alkaloids	–	–	+	+
Tannins and Phenolics	–	–	+	+
Flavonoids	–	–	+	+

D. volubilis: Dregea volubilis

different solvent systems on trial and error method. The retention factor (R_f) values of the different extracts are presented in Table 4. The phytochemical studies of the different extracts of *D. volubilis* flower showed the presence of different types of plant metabolites which are responsible for the medicinal values of the plant. The extraction with different solvents gives rise to the separation of medicinally active portions of the plant according to the polarity of the solvents. The purpose of the standardized extracts is to obtain the therapeutically active compounds and to eliminate unwanted materials by treatment with a selective solvent known as menstruum. The extracts of the crude drugs can be considered a good source of useful drugs [27]. The various types of phytochemicals present contribute medicinal as well as physiological properties to the plants. The TLC analysis of the different extracts was carried out for the development of characteristic fingerprint profile which may be used as a reference for the quality evaluation and standardization of the drug. The bands of the different extracts in the TLC plates were obtained at different R_f values which can be used as identifying markers [11]. The extracts can be utilized as medicinal agents after standardization in different dosage forms of pharmaceutical interest. The preliminary phytochemical studies are of great importance in the field of standardization of crude drugs.

HPLC fingerprinting

The different phytoconstituents present in the sample were separated on C18 column using UHPLC (Fig. 3). The different peaks along with their retention times (min), area (mAU*min), height (mAU), relative area (%), and relative height (%) are presented in Table 5. HPLC plays an important role as an important analytical tool for the quality control of drugs [28]. Natural products have a unique chemical diversity which results in diversity in their biological activity leading to the development of lead compounds which will play an important role in the discovery of drugs for treating various ailments. The modern analytical technique (HPLC) with high power of separation and reproducibility can be used to separate multidimensional chemical structures present in the plant materials. The peak number 7 and 26 with a retention time of 4.912 min and 34.855 min, respectively, are the two more intense peaks among the others in the chromatogram generated after HPLC study. The peak number 7 accounts for 27.54% and the peak number 26 accounts for 23.92% relative area. The chromatograms generated after HPLC study can be used to establish reference HPLC fingerprints of the flower of *D. volubilis* against which raw materials can be evaluated and finished products containing the plant material can be analyzed.

FTIR

The FTIR spectra of the different extracts of *D. volubilis* flowers are presented in Fig. 4. The petroleum benzine and chloroform extracts exhibited characteristic bands for the asymmetrical stretching vibrations of the C–H bonds in CH_2 and CH_3 groups between 2980 and 2810 cm^{-1}, C–H bending vibrations between 1480 and 1400 cm^{-1}, C=O stretching vibrations between 1870 and 1540 cm^{-1}, interactions of O–H bending and C–O stretching in the C–O–H group between 1390 and 1350 cm^{-1}, and the secondary C–O vibrations in the C–O–H group between 1060 and 1025 cm^{-1} which are characteristics of phytosterols [29]. The aqueous, chloroform, and methanol extracts showed characteristic bands for the O–H stretching vibrations between 3550 and 3200 cm^{-1}, C–O stretching vibration band between 1060 and 1000 cm^{-1}, and O–H bending vibration band between 1420 and 1330 cm^{-1} which are due to the presence of phenolic compounds. The extracts also exhibited characteristic O–H stretching vibration band near 3000 cm^{-1} and C=O stretching vibration band near 1700 cm^{-1}, C–O stretching vibration band between 1320 and 1210 cm^{-1}, and O–H bending vibration band between 1440 and 1395 cm^{-1} which

Table 4: Thin-layer chromatography of *D. volubilis* flower extracts

Sl. No.	Solvent system	Extracts	No. of spots (254 nm)	R_f values (254 nm)	No. of spots (366 nm)	R_f values (366 nm)
1	Chloroform: ethyl acetate (6:4)	Aqueous	1	0.48	-	-
		Methanol	4	0.04, 0.09, 0.13, 0.47	1	0.25
		Chloroform	16	0.04, 0.06, 0.10, 0.12, 0.19, 0.30, 0.35, 0.41, 0.48, 0.56, 0.61, 0.72, 0.79, 0.95, 0.96, 0.98	9	0.08, 0.16, 0.30, 0.35, 0.41, 0.93, 0.95, 0.97, 0.98
		Petroleum benzine	7	0.07, 0.23, 0.48, 0.56, 0.72, 0.91, 0.96	3	0.84, 0.92, 0.96
2	Chloroform: ethyl acetate (8:2)	Aqueous	2	0.04, 0.15	-	-
		Methanol	4	0.02, 0.06, 0.10, 0.16	3	0.01, 0.05, 0.14
		Chloroform	9	0.02, 0.05, 0.08, 0.10, 0.15, 0.16, 0.88, 0.91, 0.96	4	0.06, 0.09, 0.90, 0.96
		Petroleum benzine	12	0.10, 0.20, 0.24, 0.29, 0.52, 0.64, 0.67, 0.79, 0.86, 0.89, 0.91, 0.97	3	0.66, 0.89, 0.96
3	Chloroform: methanol: glacial acetic acid (4:5:1)	Aqueous	1	0.69	2	0.10, 0.73
		Methanol	7	0.13, 0.17, 0.24, 0.53, 0.66, 0.76, 0.90	5	0.24, 0.69, 0.78, 0.90, 0.97
		Chloroform	2	0.84, 0.93	2	0.74, 0.87
		Petroleum benzine	3	0.46, 0.48, 0.72	4	0.46, 0.72, 0.78, 0.90
4	Chloroform: ethylacetate: glacial acetic acid (4:5:1)	Aqueous	3	0.06, 0.17, 0.88	1	0.88
		Methanol	8	0.03, 0.04, 0.07, 0.12, 0.19, 0.33, 0.71, 0.87	5	0.04, 0.13, 0.16, 0.70, 0.96
		Chloroform	8	0.02, 0.04, 0.42, 0.55, 0.86, 0.90, 0.94, 0.95	4	0.04, 0.85, 0.94, 0.96
		Petroleum benzine	1	0.94	1	0.94
5	Chloroform: 2propanol: glacial acetic acid (5:4:1)	Aqueous	1	0.05	1	0.05
		Methanol	9	0.03, 0.12, 0.19, 0.48, 0.57, 0.62, 0.72, 0.92, 0.96	7	0.05, 0.13, 0.21, 0.48, 0.62, 0.69, 0.92
		Chloroform	4	0.12, 0.72, 0.88, 0.94	1	0.97
		Petroleum benzine	1	0.94	1	0.97
6	Chloroform: ethanol: triethylamine (6:3:1)	Aqueous	3	0.06, 0.08, 0.11	-	-
		Methanol	12	0.05, 0.13, 0.16, 0.19, 0.31, 0.37, 0.41, 0.47, 0.61, 0.65, 0.90, 0.96	5	0.06, 0.89, 0.92, 0.95, 0.97
		Chloroform	3	0.44, 0.84, 0.95	2	0.91, 0.96
		Petroleum benzine	2	0.81, 0.90	3	0.82, 0.89, 0.96
7	Chloroform: methanol: glacial acetic acid (5:4:1)	Aqueous	6	0.08, 0.12, 0.38, 0.50, 0.69, 0.88	2	0.52, 0.69
		Methanol	11	0.04, 0.09, 0.16, 0.34, 0.44, 0.62, 0.68, 0.78, 0.87, 0.95, 0.98	7	0.16, 0.40, 0.65, 0.79, 0.83, 0.95, 0.98
		Chloroform	2	0.80, 0.92	3	0.79, 0.87, 0.93
		Petroleum benzine	2	0.80, 0.96	2	0.87, 0.96

D. volubilis: Dregea volubilis, Rf: Retention factor

are due to presence of phenolic acids. All the extracts showed characteristic absorption bands for the out-of-plane bending of ring C–H bonds between 900 and 675 cm^{-1}, in-plane bending bands between 1300 and 1000 cm^{-1}, and C–C stretching band within the ring between 1600 and 1585 cm^{-1} and 1500 and 1400 cm^{-1} which are due to the presence of mononuclear and polynuclear aromatic hydrocarbons [30]. The characteristic absorption bands of the different extracts of the plant material confirm the presence of different types of phytoconstituents in *D. volubilis* flowers. FTIR is a useful analytical tool in the field of standardization of drugs. FTIR spectrum is useful in the identification of drugs by comparing the spectrum of the test material with that of the reference material. The FTIR spectra of the different extracts can be served as reference FTIR fingerprints of *D. volubilis* flowers for the quality control of raw materials and finished products containing it [9]. The study also provides qualitative information on the types of chemicals present in the different extracts of the flowers of *D. volubilis.*

Determination of UV-VIS spectra

The UV-VIS spectrum of different extracts of *D. volubilis* flowers is presented in Fig. 5. The aqueous extract showed the peaks at 250 nm, 290 nm (λ_{max}); the methanol extract showed the peaks at 230 nm and 300 nm (λ_{max}); the chloroform extract showed the peaks at 250 nm, 300 nm (λ_{max}), 410 nm, 510 nm, 540 nm, and 670 nm; and the petroleum benzine extract showed the peaks at 300 nm (λ_{max}), 400 nm, 500 nm, 530 nm, and 670 nm. UV-VIS spectrum

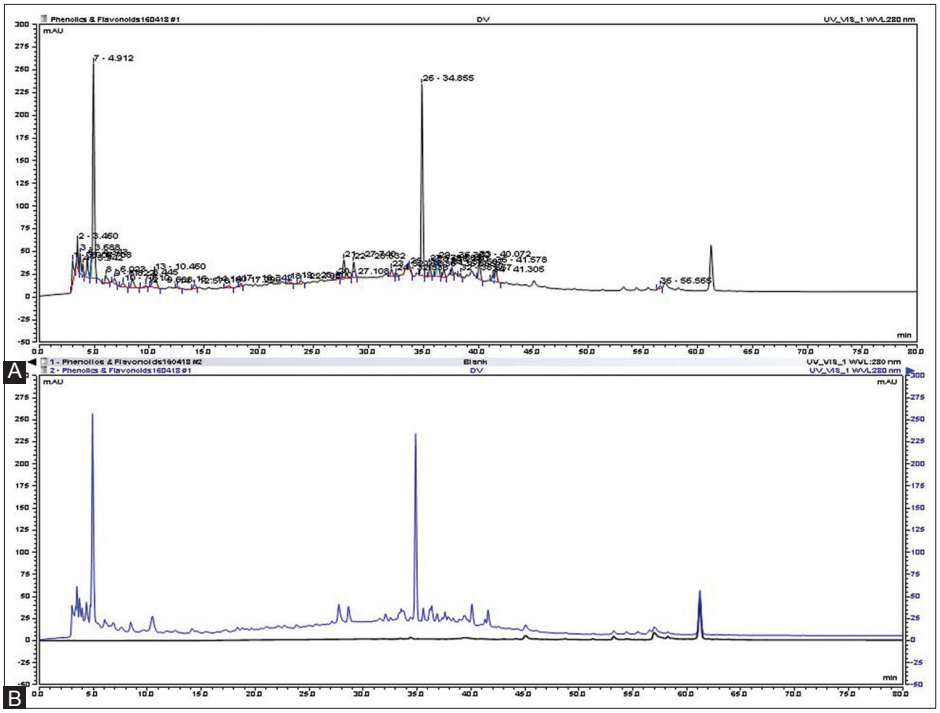


Fig. 3: Ultra-high-performance liquid chromatograms of a sample solution of the flower of *Dregea volubilis* (A) and an overlapped chromatogram (black colored chromatogram represents blank solution and blue colored chromatogram represents sample solution) (B) as detected at 280 nm.

Table 5: HPLC peaks present in the sample solution of the flower of *D. volubilis* as detected at 280 nm

Sl. No. of HPLC Peaks	Retention time (min)	Are(mAU*min)	Height (mAU)	Relative area (%)	Relative height (%)
1	3.007	3.639	26.758	2.52	3.24
2	3.460	2.992	29.535	2.07	3.58
3	3.688	2.887	22.366	2.00	2.71
4	3.942	1.443	13.231	1.00	1.60
5	4.343	3.521	22.164	2.44	2.68
6	4.708	1.774	19.838	1.23	2.40
7	4.912	39.758	236.210	27.54	28.60
8	6.023	2.299	9.147	1.59	1.11
9	6.822	1.329	5.662	0.92	0.69
10	7.610	1.339	4.054	0.93	0.49
11	8.445	2.820	10.939	1.95	1.32
12	9.688	0.501	1.924	0.35	0.23
13	10.460	5.894	17.178	4.08	2.08
14	12.578	0.595	2.365	0.41	0.29
15	14.140	1.131	4.393	0.78	0.53
16	17.290	1.123	2.568	0.78	0.31
17	18.342	0.735	3.604	0.51	0.44
18	22.708	0.772	3.080	0.53	0.37
19	23.800	0.936	3.735	0.65	0.45
20	27.108	0.644	3.177	0.45	0.38
21	27.740	6.010	21.011	4.16	2.54
22	28.632	3.555	17.369	2.46	2.10
23	32.073	1.885	8.404	1.31	1.02
24	32.538	0.623	4.004	0.43	0.48
25	33.720	0.911	4.209	0.63	0.51
26	34.855	34.533	210.94	23.92	25.54
27	35.565	2.167	14.434	1.50	1.75
28	36.180	2.312	14.081	1.60	1.71
29	36.352	3.113	17.537	2.16	2.12
30	36.855	1.667	8.962	1.15	1.09
31	37.582	1.252	8.645	0.87	1.05
32	38.357	0.592	4.046	0.41	0.49
33	40.072	4.243	21.997	2.94	2.66
34	41.305	1.173	6.969	0.81	0.84
35	41.578	3.454	18.030	2.39	2.18
36	56.565	0.749	3.239	0.52	0.39
Total		144.370	825.81	100	100

HPLC: High-performance liquid chromatography, *D. volubilis: Dregea volubilis*

plays an important role in the identification and quantification of many drugs. This analytical tool is very useful for the quality control of drugs [31]. The UV-VIS spectrum of the different extracts of *D. volubilis* flowers can be served as a reference spectrum for quality control of drugs.

Determination of pH

The pH of the 1% (w/v), 2% (w/v), and 10% (w/v) aqueous solutions of the dried powdered material was found to be 5.02±0.02, 4.89±0.02, and 4.74±0.02, respectively. pH can also serve as a quality control tool for the identification of the drugs. A change in the value of pH from the standard value indicates the deterioration of the quality of the product. The aqueous solution of *D. volubilis* flower was found to be acidic in nature. The pH value is of great importance in product development to estimate stability and dissolution of the product. The pH value is also helpful in the development of suitable extraction procedure for the phytoconstituents from the plant [15].

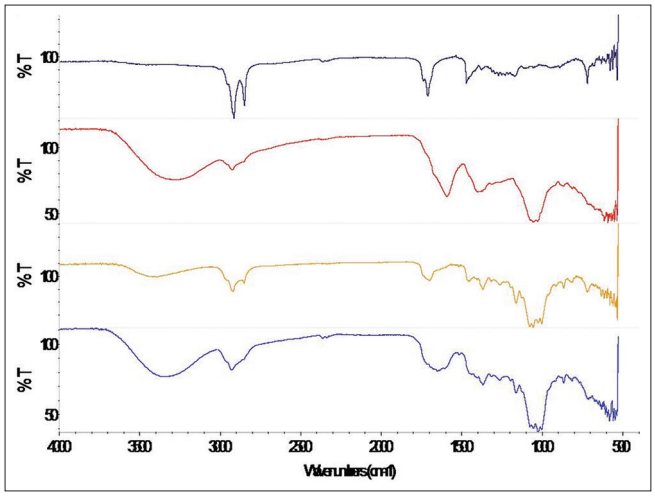


Fig. 4: Fourier transform infrared spectra of the petroleum benzine extract (A), aqueous extract (B), chloroform extract (C), and methanol extract (D) of the flower of *Dregea volubilis*

Determination of protein content

Proteins are important biomolecules with multiple functions within organ and the molecules are differing from one another primarily in their sequence of amino acids. The protein content of the sample was found to be 2.112±0.058 mg/g of the sample. The protein content of the powder of *D. volubilis* flower can be served as a quantitative parameter for standardization of the plant material. Any deviation from the standard value of protein content reflects the changes in the quality of the crude drug. Estimation of protein of herbal drugs plays a crucial role in assessing the nutritional significance and health effects [32]. The parameter can be used as a reference for the quality control of crude drugs.

Determination of carbohydrate content

The carbohydrate content of the dried powdered material of *D. volubilis* flowers was found to be 124.243±3.573 mg/g of the sample. Carbohydrate is one of the most widely used substances in nature and is the main ingredient of food. The quantitative analysis for the estimation of the carbohydrate content of crude drugs can be considered as a quality control parameter for assessing the crude drugs [33].

Determination of free sugar composition

The UFLC analysis (Fig. 6) was performed to identify and quantify the free sugars present in the *D. volubilis* flowers. The retention times of rhamnose, xylose, fructose, glucose, trehalose, and maltose were found to be 4.982, 5.456, 5.734, 6.198, 7.351, and 8.032 min, respectively. The sugars present in the sample were identified by comparing the retention times of the standards with that of the sample. Xylose and trehalose were not detected in the crude drug. The study showed that the flower contained rhamnose (103.229±4.994 µg/g), fructose (738.670±25.714 µg/g), glucose (285.532±24.465 µg/g), and maltose (49.082±5.206 µg/g). Characterization of sugars present in crude drugs is very important for their quality control [33]. The sugars present in the flower can be considered as markers for the standardization of the crude drug.

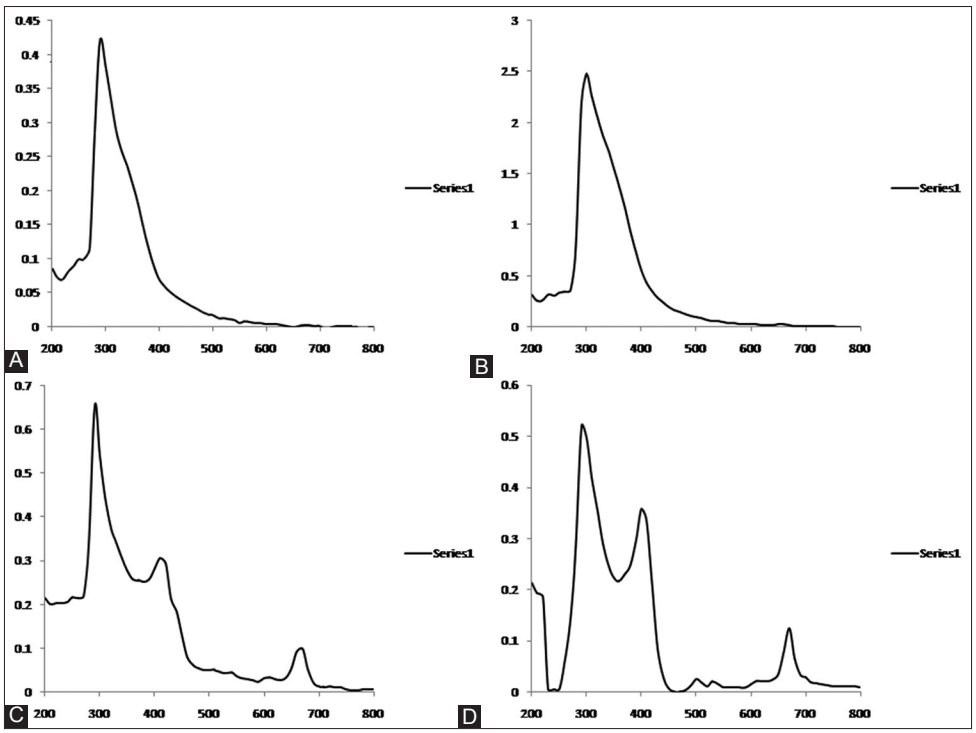


Fig. 5: Ultraviolet-visible spectra of the aqueous extract (A), methanol extract (B), chloroform extract (C), and petroleum benzine extract (D) of the flower of *Dregea volubilis*

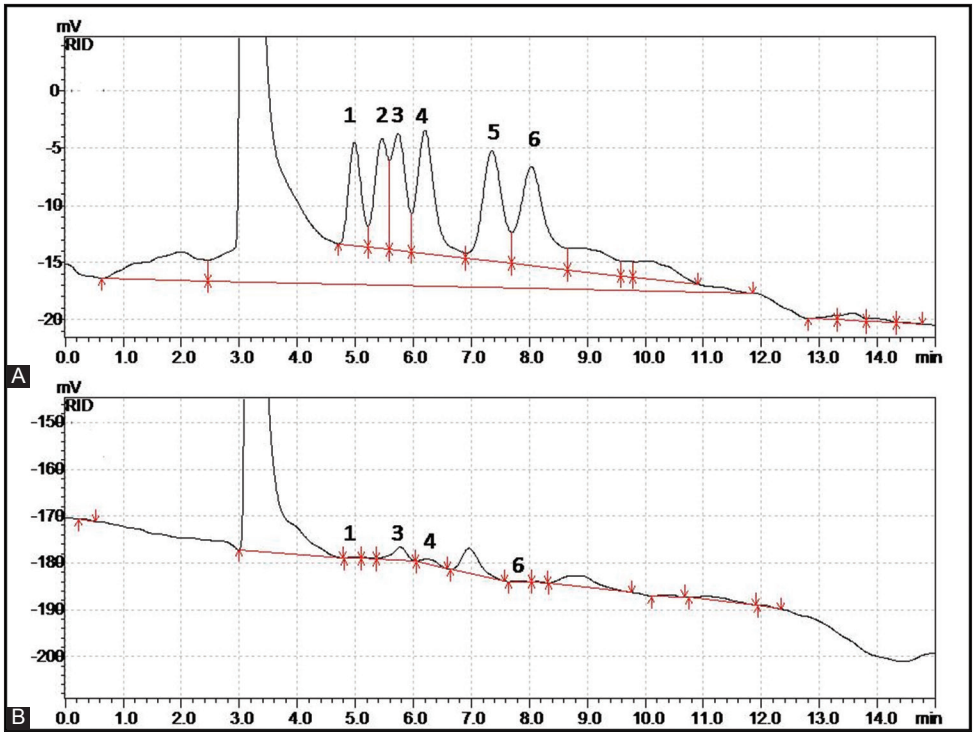


Fig. 6: High-performance liquid chromatography chromatograms of the mixed standard of sugars (A) and the sample solution of the flower of *Dregea volubilis* (B) as detected with refractive index detector (1: Rhamnose, 2: Xylose, 3: Fructose, 4: Glucose, 5: Trehalose, 6: Maltose)

CONCLUSION

Herbal drugs are subjected to variability in quality as they derived from heterogeneous sources. The main concerned area is that the activity of the plant material may vary and even inferior quality material may be produced which may impart a quality impact on the products of the pharmaceutical industry. Standardization of herbal drugs is an important topic of great concern. The present study of pharmacognostical evaluation on the flowers of *D. volubilis* has laid down standard parameters for proper identification, authentication, and for distinguishing the material from its adulterants and substitutes. The detailed study also set the parameters which can be utilized as a pharmacopeial reference for recognition of its distinctiveness, genuineness, and quality. The study also contributes to the documentation of the nutritional composition on the flowers of *D. volubilis* which are consumed as a vegetable.

ACKNOWLEDGMENT

The authors gratefully acknowledge the University Grants Commission, New Delhi, for providing the instrumental facilities under UGC-UPE Phase–II program at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata.

AUTHOR'S CONTRIBUTIONS

Bhaskar Das was involved in performing all the experiments and preparing the manuscript. Arnab De, Piu Das, and Amalesh Nanda were involved in the plant identification, methodology, and interpretation of data. Dr. Amalesh Samanta had revised and finalized the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

REFERENCES

1. Sharma C, Irshad S, Khatoon S, Arya KR. Pharmacognostical evaluation of Indian folk-traditional plants *Coelogyne cristata* and *Pholidota articulata* used for healing fractures. Indian J Exp Biol 2017;55:622-7.
2. Sanyacharernkul S, Itghiarbha A, Kongtawelert P, Meepowpan P, Nuntasaen N, Pompimon W. A new polyoxypregnane glycoside from the roots of *Dregea volubilis* (L.f) Benth. Ex Hook. F and its chondroprotective effect. Am J Biochem Biotechnol 2009;5:202-9.
3. Karthika KS, Sanjaya KS, Hari KR, Jyothi T. A pharmacognostic evaluation on moorva bheda (*Dregea volubilis* (L.f) Benth. Ex Hook.f). Int Res J Pharm 2012;3:127-30.
4. Biswas M, Haldar PK, Ghosh AK. Antioxidant and free-radical-scavenging effects of fruits of *Dregea volubilis*. J Nat Sci Biol Med 2010;1:29-34.
5. Nandi D, Besra SE, Vedasiromoni JR, Giri VS, Rana P, Jaisankar P. Anti-leukemic activity of *Wattakaka volubilis* leaf extract against human myeloid leukemia cell lines. J Ethnopharmacol 2012;144:466-73.
6. Sahu NP, Panda N, Mandal NB, Banerjee S, Koike K, Nikaido T. Polyoxypregnane glycosides from the flowers of *Dregea volubilis*. Phytochemistry 2002;61:383-8.
7. Panda N, Mondal NB, Banerjee S, Sahu NP, Koike K, Nikaido T, *et al*. Polyhydroxypregnanes from *Dregea volubilis*. Tetrahedron 2003;59:8399-403.
8. Panda N, Mandal D, Mandal NB, Sahu NP, Banerjee S. Flavonoid and flavones C-glycosides from *Dregea volubilis*. Nat Prod Commun 2006;1:731-3.
9. Das B, De A, Das M, Das S, Samanta A. A new exploration of *Dregea volubilis* flowers: Focusing on antioxidant and antidiabetic properties. S Afr J Bot 2017;109:16-24.
10. Chanda S. Importance of pharmacognostic study of medicinal plants: An overview. J Pharmacogn Phytochem 2014;2:69-73.
11. Borah S, Kakoti BB, Mahato K, Chakraborty D, Lahkar S, Gogoi B, *et al*. Pharmacognostic and preliminary phytochemical studies on shoot of *Calamus leptospadix* Griff. An ethnomedicinal plant of Assam. Indian J Nat Prod Resour 2014;5:320-5.
12. Hemalatha K, Abirami P. Pharmacognostic studies on *Talinum portulacifolium* (Forssk.) Asch. Ex Schweinf. Asian J Pharm Clin Res 2018;11:470-3.
13. Upreti K, Semwal A, Upadhyaya K, Masiwal M. Pharmacognostical and phytochemical screening of leaf extract of *Zanthoxylum armatum* DC. Int J Tradit Herb Med 2013;1:6-11.
14. Arambewela LS, Arawwawala LD. Standardization of *Alpinia calcarata* roscoe rhizomes. Pharmacogn Res 2010;2:285-8.
15. Aslam I, Afridi MS. Pharmacognostic characterization of *Beaumontia*

grandiflora (Roxb.) Wall. Leaf for taxonomic identification for quality control of a drug. J Appl Res Med Aromat Plants 2018;8:53-9.
16. Mallya R, Bhitre MJ. Pharmacognostic standardisation and chromatographic fingerprinting of leaves and fruits of *Zanthoxylum rhetsa*. Int J Pharm Pharm Sci 2018;10:101-4.
17. Rabinarayan A, Switu J, Harisha CR, Vinay S. Pharmacognostical genetic barcoding and phytochemical analysis on leaves of *Dalbergia volubilis* Roxb. An extra pharmacopoeial drug of Ayurveda. Int J Pharm Pharm Sci 2018;10:117-24.
18. Adams SJ, Kuruvilla GR, Krishnamurthy KV, Nagarajan M, Venkatasubramanian P. Pharmacognostic and phytochemical studies on Ayurvedic drugs ativisha and musta. Rev Bras Farmacogn 2013;23:398-409.
19. Cheng D, Zhang Y, Xin X, Gao D. Comparative pharmacognosy of *Pyrrosia petiolosa* and *Pyrrosia davidii*. Rev Bras Farmacogn 2014;24:368-80.
20. Wahab S, Hussain A, Ahmad P, Usmani S. Ethanobotanical, pharmacognostical and physicochemical studies of stem bark of *Bombax ceiba* L., commonly growing in eastern Uttar Pradesh region of India. Pharmacogn J 2012;4:55-60.
21. Sharma M, Sharma V, Majumdar DK. Entrapment of α amylase in agar beads for biocatalysis of macromolecular substrate. Int Sch Res Not 2014;1:1-8.
22. Baloch AB, Xia X, Sheikh SA. Proximate and mineral compositions of dried cauliflower (*Brassica Oleracea* L.) grown in Sindh, Pakistan. J Food Nutr Res 2015;3:213-9.
23. Heleno SA, Barros L, Sousa MJ, Martins A, Ferreira IC. Study and characterization of selected nutrients in wild mushrooms from Portugal by gas chromatography and high performance liquid chromatography. Microchem J 2009;93:195-9.
24. Kokate CK, Purohit AP, Gokhale SB. Test Book of Pharmacognosy. 42nd ed. Pune: Nirali Prakashan; 2006.
25. Folashade O, Omoregie H, Ochogu P. Standardization of herbal medicines a review. Int J Biodivers Conserv 2012;4:101-12.
26. Madhav NV, Upadhyaya K, Bisht A. Phytochemical screening and standardization of polyherbal formulation for dyslipidemia. Int J Pharm Pharm Sci 2011;3:235-8.
27. Yadav RN, Agarwala M. Phytochemical analysis of some medicinal plants. J Phytol 2011;3:10-4.
28. Farooqui NA, Dey A, Singh GN, Easwari TS, Pandey MK. Analytical techniques in quality evaluation of herbal drugs. Asian J Pharm Res 2014;4:112-7.
29. Ubeyitogullari A, Ciftci ON. Phytosterol nanoparticles with reduced crystallinity generated using nanoporous starch aerogels. RSC Adv 2016;6:108319-27.
30. Silverstein RM, Webster FX, Kiemle DJ. Spectrometric Identification of Organic Compounds. 7th ed. New Jersey: John Wiley and Sons, Inc.; 2005.
31. Gad HA, El-Ahmady SH, Abou-Shoer MI, Al-Azizi MM. A modern approach to the authentication and quality assessment of thyme using UV spectroscopy and chemometric analysis. Phytochem Anal 2013;24:520-6.
32. Hussain J, Khan AL, Rehman NU, Hamayun M, Shinwari ZK, Ullah W, *et al*. Assessment of herbal products and their composite medicinal plants through proximate and micronutrients analyses. J Med Plants Res 2009;3:1072-7.
33. Zhao J, Ma S, Li S. Advanced strategies for quality control of Chinese medicines. J Pharm Biomed Anal 2018;147:473-8.

Publications as a coauthor

Food & Function

PAPER

Cite this: *Food Funct.*, 2016, **7**, 3064

Prebiotic potential of gum odina and its impact on gut ecology: *in vitro* and *in vivo* assessments

Debmalya Mitra, Aditya Kr. Jena, Arnab De, Mousumi Das, Bhaskar Das and Amalesh Samanta*

Received 18th December 2015,
Accepted 11th May 2016

DOI: 10.1039/c5fo01581b

www.rsc.org/foodfunction

The use of prebiotics to escalate certain gut flora is a current aspect of research for effective gut ecology. In the present study we appraise the efficacy of gum odina obtained from the bark of *Odina wodier* (Anacardiaceae), which is not fully degraded (16%) in the upper GI tract and becomes available to the lower region, as a prebiotic. An *in vitro* prebiotic activity assay established a quantitative score to describe the extent to which gum odina supports the selective growth of probiotics with a maximum of 5.60 ± 0.11 for *Lactobacillus plantarum* MTCC 6160. The polysaccharide, upon fermentation, also liberates lactic acid (0.46 ± 0.003 mg ml^{-1}) and acetic acid (1.03 ± 0.003 mg ml^{-1}). *In vivo* studies revealed that natural gum selectively stimulates *Lactobacillus* sp., and eliminates enteric pathogens with a C.F.U. of 384.48 ± 0.11 and 40.56 ± 0.17 respectively on the 8^{th} day. The changes in the level of β-galactosidase signify maturation of macrophages in the gut environment. It also boosts the immune system by increasing sIgA upon infection from the 5^{th} day in the gut, when incorporated into the feed of mice. Moreover an increase in levels of IFNγ on the 5^{th} day also manifest additional protection against various pathogen-induced primary and secondary infections. Thus, gum odina is a potential prebiotic which not only provides nutrition but also improves gut ecology.

1. Introduction

In response to an infection, dehydration or to tackle unfavorable conditions, many trees and shrubs produce an aqueous thick exudation[1] triggered by changes in the existing cell wall by a process known as Gummosis.[2] The solid exudation is referred to as natural gum.[1] Gum odina is extracted from the tree *Odina woodier*, Roxb, Family Anacardiaceae.[3] It is a mixture of various polysaccharides[4] and chemically is a negatively charged polyelectrolyte, belonging to the glycuronogalactan polysaccharides.[5,6] The structure of gum odina was reported earlier[7] which is a polymer of 63.70% D-galactose, 19.50% L-arabinose and 11.50%–17% of two uronic acids (D-galactouronic acid and aldobiuronic acid). The molecular weight of natural gum was reported to be 1.68×10^5 as determined by the static light scattering method.[8] It consists of a 1,6 β-D-galactopyranosyl residue as revealed from compositional analysis with slightly branched galactopyrose units as side chains linked through C_1, C_3, and C_6. Gum odina is chemically similar to inulin *i.e.* the majority of the glycoside linkages are of β-type.[9] Research in the past has revealed the ability of gum to act as a tablet binder[3] and currently the natural gum is used for sustained release drug delivery systems.[10]

Of late the development of prebiotics is another trending area of research.[11] Prebiotics are usually carbohydrates,[12] which are not digested in the stomach by the host, and stimulate growth and activity of specific beneficial strains of bacteria in the gut over others,[13,14] referred to as probiotics[15] in the colon. A large variety of microbial flora is present in human body throughout the gut depending on various physicochemical conditions.[16] Among various resident intestinal bacteria lactobacilli and bifidobacteria are stimulated by prebiotics.[15] Prebiotics upon metabolization[17] liberate lactic, acetic and other short chain organic acids possessing anticancer properties and also provide nutrition to probiotics. Thus synbiotics or a combination of probiotics and prebiotics have a more beneficial effect on the gut than probiotics or prebiotics alone.[15] Synbiotics also eliminate harmful and pathogenic microbes[18] by limiting the space for survival. The association of prebiotics with probiotics has the ability to influence and improve the health of humans.[19] Most of the commercially available prebiotics are generally fructo-oligosaccharide (FOS)[20] like inulin, glacto-oligosaccharides (GOS),[21] arabinoxylans,[22] xylooligosaccharides,[23] pectic-oligosaccharides[24] and galactoglucomannans[25] which are generally plant polysaccharides. Prebiotic research targeted on distal colonic

Division of Microbiology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India. E-mail: asamanta61@yahoo.co.in; Tel: +91-33 24572617 (O) +91 9432315461 (M)

RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2017, **7**, 29129

Gum odina: an emerging gut modulating approach in colorectal cancer prevention

Debmalya Mitra,[a] Abhishek Basu,[b] Bhaskar Das,[a] Aditya Kr. Jena,[a] Arnab De,[a] Mousumi Das,[a] Sudin Bhattacharya[b] and Amalesh Samanta*[a]

It is well established that prebiotics have a profound influence on colonic microbiota which in turn play an essential role in ameliorating the host's health. This study is focused on Gum Odina (GO), a reported prebiotic in our earlier work, and its impact on colorectal cancer (CRC). GO, upon utilization by probiotics, liberates short-chain fatty acids, acetic acid (0.864 ± 0.050 mg ml^{-1}) and butyric acid (2.303 ± 0.083 mg ml^{-1}) predominantly and increases colonization of *Lactobacillus* sp. and *Bifidobacterium* sp. in a gut simulator. The *in vivo* preventive study of CRC was conducted on Swiss albino mice using 1,2-dimethyl hydrazine (DMH) along with inulin and GO as the standard and test prebiotic, respectively. Scanning electron micrographs of the colon depict that the severity of mucosal dysplasia, flat lesions and loss of goblet cells was quite low in the GO group compared to the DMH alone treated group. The same was noticed in the histomicrograph in terms of alteration of the colonic architecture and abnormalities in the submucosa. Administration of DMH also caused oxidative burst as the levels of reactive oxygen species and lipid peroxidation significantly increased ($p < 0.05$) but reduced by 29.35% and 27.65%, respectively, in the GO group. Moreover, the levels of glutathione, glutathione-*S*-transferase, superoxide dismutase and catalase in the colonic tissues significantly increased ($p < 0.05$) by 31.26%, 10.96%, 12.4% and 6.37%, respectively, when compared to IN, a standard prebiotic. Thus, GO possesses CRC-preventing along with antioxidant properties and slows the overall tumor genesis process.

Received 10th April 2017
Accepted 18th May 2017

DOI: 10.1039/c7ra04077f

rsc.li/rsc-advances

1. Introduction

The use of prebiotics for an effective gut ecology is a current trend of research,[1] as the demand in natural products claiming health benefits is increasing day to day. This developing aspect of glycoscience is coming into the limelight due to increased health consciousness and consumer awareness[2] leading to the development of new bioactive compounds working on the principle of "prevention is better than a cure".[3]

Prebiotics are carbohydrates[4] that resist digestion and absorption in the upper parts of the gastrointestinal tract[5] of the host, selectively utilizing specific bacterial strains,[6] and confer health benefits.[7] Considering the profound health benefits of prebiotics, their development from new natural sources like Gum Odina (GO),[1] mushroom polysaccharide,[8] *Aloe vera* mucilage,[9] artichoke fibers (*Cynara cardunculus* L. var. scolymus),[10] almond skin,[11] bamboo shoots[12] (*Phyllostachys praecox*) and *Stevia rebaudiana* (Bertoni) roots[13] is being carried out. Prebiotics not only prevent gastrointestinal diseases[14] by limiting the space of survival for pathogenic bacteria[15] but also improve human health by positive stimulation of the immune system,[16] reduction of intestinal inflammatory diseases and cholesterol levels,[14] regulation of blood glucose,[17] and treatment of pochutis[18] and osteoporosis.[2]

Due to recent occupational hazards and dietary habits like high red meat intake, colorectal cancer (CRC) has become the most deadly cancer in the world at present.[18] CRC is mainly caused by mutation in tumor suppression genes[19] and prebiotics act as a chemopreventive agent by removing food-borne mutagens.[20] Pathogenic bacteria, upon colonization in the intestine, contribute to progression of CRC by inducing gut inflammation,[21] up-regulating inflammatory genes (NF-kB, IL-6, IL-8 and IL-18),[22] epithelial damage and promoting pro-oncogenic responses.[23] Prebiotics, conversely, manipulate gut microbiota[24] *i.e.* selectively stimulate *Lactobacilli* sp. and *Bifidobacterium* sp. immunomodulation[25] by increasing sIgA in the gut environment,[1] enhance apoptosis,[26] and down-regulate the expression levels of COX-2, NF-kB and iNOS.[27] Moreover, upon utilization, prebiotics also liberate SCFA[28] which slows the overall tumerigenesis[29] by providing energy to colonocytes.

GO has been used previously in formulations like tablet binders,[30] emulsifying agents[31] and matrices for sustained drug release,[32] and in designing chitosan–GO complex coacervates for colon targeted drug delivery.[33] The chemical composition[34] and branched structure[35] of this polysaccharide is reported

[a]*Division of Microbiology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India, 700032. E-mail: asamanta61@yahoo.co.in; Tel: +91 33 24572617; +91 9432315461*

[b]*Department of Cancer Chemoprevention, Chittaranjan National Cancer Institute, Kolkata, India*

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 5, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

PREVENTIVE EFFECT OF *CYCAS REVOLUTA* IN 1,2-DIMETHYLHYDRAZINE-INDUCED COLON CANCER IN WISTAR RAT MODEL

SAMIT BERA, BHASKAR DAS, ARNAB DE, AMALESH SAMANTA*

Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal, India. Email: asamanta61@yahoo.co.in

Received: 05 December 2017, Revised and Accepted: 20 January 2018

ABSTRACT

Objective: The aim of this study was to evaluate the colon cancer protective activity of *Cycas revoluta* (Cycadaceae).

Methods: Methanolic extracts of *C. revoluta* (MECR) were assessed for total polyphenols and total flavonoids content. For the *in vivo* study, animals were divided into five groups (n=6). Group I serves as control which received 0.25% carboxymethyl cellulose solution. Groups II-V were treated with 1,2-dimethylhydrazine (DMH) which was given at the dose of 20 mg/kg b.w., s.c. once a week for 4 consecutive weeks. Aqueous suspension of MECR at a dose of 200 mg/kg/day and 400 mg/kg was administered orally to the animals in Groups III-IV every day for 16 weeks. Group V received 5-fluorouracil (5-FU) as a standard drug at a dose of 10 mg/kg b.w., per day s.c. for 16 weeks. After that, animals are sacrificed and colons are taken separately to evaluate biochemical parameters and morphological and histopathological changes.

Results: MECR contains total polyphenols (6.3±0.09 mg of gallic acid equivalent /g) and total flavonoids (4.6±0.06 mg of rutin equivalent/g). The *in vivo* study revealed that superoxide dismutase (SOD), catalase, and reduced glutathione (GSH) activity were decreased in DMH Group. All these parameters were restored significantly ($p<0.05$) toward the near normal value on supplementation with MECR (200 and 400 mg/kg b.w.) to DMH-treated rats (Groups III and IV). In Group V, the synthetic standard drug 5-FU (10 mg/kg b.w.) also increases the activities of SOD, CAT, and GSH significantly ($p<0.05$) more in DMH-treated rats.

Conclusions: It can be concluded that MECR protects rat from DMH-induced colon cancer.

Keywords: *Cycas revoluta*, Methanolic extracts of *C. revoluta*, 1, 2-Dimethylhydrazine, Colon cancer, 5-Fluorouracil.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i5.24126

INTRODUCTION

Colon cancer is the most fatal issues throughout the world and a leading cause of cancer-related mortality. Oxidative stress is one of the main causes of colon cancer [1]. The gastrointestinal tract is more susceptible to toxic chemical exposure which may execute the chronic inflammation, and later, it becomes tumour in the gastrointestinal tract [2]. Over the past few years, the first-line clinical treatments for colon cancer patients are cytoreductive surgery and combined chemotherapy [3]. Drug resistance results in a poor overall survival rate [4]. Therefore, the development of effective and less toxic drugs is urgent for colon cancer patients.

The colon-specific carcinogen 1,2-dimethylhydrazine (DMH) induces the formation of methyl adducts with DNA bases and causes point mutations [5]. This adduct interferes with normal cell growth by altered normal gene transcription. After the Phases I and II metabolism, carcinogenic metabolites are produced from DMH. The activation of carcinogen is done by Phase I enzymes. The pro-carcinogen DMH undergoes hydroxylation in Phase I reactions catalyzed by cellular microsomal monooxygenases to produce strong electrophiles. The produced strong electrophiles are capable of interacting with cellular nucleophiles such as DNA to form adducts causing mutagenesis and neoplastic transformation. By Phase II biotransformation, these electrophilic intermediates are detoxified by enzymes such as glutathione (GSH) S-transferase [6]. Due to binding of DMH metabolite to DNA, gene transcription is modified to create oxidative stress through the production of reactive oxygen species (ROS) inside the cells. The ROS has pathological status by inducing oxidative stress-mediated inflammatory response in several tissues [7]. Increase in ROS level is an important factor to develop colitis-associated colon cancer [8]. There are several endogenous antioxidant such as superoxide dismutase (SOD), CAT, and GSH increase in normal colon cell in response to small increase of ROS, but in DMH-induced rats due to modification gene transcription, excessive ROS is produced crossing the level of endogenous antioxidant production. As a result, DNA damage occurs leading to colon cancer and less production of endogenous antioxidant. After the DNA damage, ROS is produced in fewer amounts for the survival of colon cancer cells.

Cycas revoluta, one of the most primitive living seed plants, contains edible starch in pith, and is used for making sago. Seeds of this plant contain cycasin which is a neurotoxin when consumed orally due to aglycone release from glycoside after acid hydrolysis in the stomach [9]. Although seeds contains toxin, the *C. revoluta* cone is used in the painful urination by the hilly people of Northeast India [10]. It is reported for the seeds to possess profound anticancer activity against colon cancer [11]. The plant contains several important chemicals including dihydroamentoflavone glucosides [12], peptides [11], chitinase [13], mannose-specific lectins [14], and beta-D-glucosidase [15]. *C. revoluta* has been reported to have many pharmacological effects such as antioxidant [16], anti-inflammatory, anticancer, antileishmaniasis [12], and antimicrobial [11]. A recent study [11] has reported the anticancer activity of *C. revoluta* seeds on human colon carcinoma cells (HCT-15). However, till now, no reports are available on the colon cancer protective effect of *C. revoluta* cone (male flower) on the DMH-induced colon cancer. Therefore, the present work was intended to evaluate the efficacy of methanolic extract of *C. revoluta* (MECR) cone against DMH-induced colon cancer in Wistar rat model.

MATERIALS AND METHODS

Materials

DMH, methanol, 5-fluorouracil (5-FU), phenazine methosulphate, NBT, reduced nicotinamide adenine dinucleotide, glacial acetic acid,

Inorganic Chemistry
Cite This: Inorg. Chem. 2018, 57, 12727−12739
Article
pubs.acs.org/IC

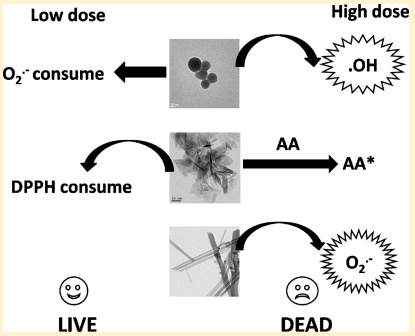
Effect of Morphology and Concentration on Crossover between Antioxidant and Pro-oxidant Activity of MgO Nanostructures

Soumik Podder,[†] Dipak Chanda,[†,‡] Anoop Kumar Mukhopadhyay,[‡] Arnab De,[§] Bhaskar Das,[§] Amalesh Samanta,[§] John George Hardy,[*,∥,⊥] and Chandan Kumar Ghosh[*,†]

[†]School of Materials Science and Nanotechnology, Jadavpur University, Kolkata 700032, India
[‡]Advanced Mechanical and Materials Characterization Division, CSIR-Central Glass and Ceramic Research Institute, Kolkata 700032, India
[§]Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India
[∥]Department of Chemistry, Lancaster University, Lancaster, Lancashire LA1 4YB, U.K.
[⊥]Materials Science Institute, Lancaster University, Lancaster, Lancashire LA1 4YB, U.K.

Supporting Information

ABSTRACT: The toxicity of nanomaterials can sometimes be attributed to photogenerated reactive oxygen species (ROS), but these ROS can also be scavenged by nanomaterials, yielding opportunities for crossover between the properties. The morphology of nanomaterials also influences such features due to defect-induced properties. Here we report morphology-induced crossover between pro-oxidant activity (ROS generation) and antioxidant activity (ROS scavenging) of MgO. To study this process in detail, we prepared three different nanostructures of MgO (nanoparticles, nanoplates, and nanorods) and characterized them by HRTEM. These three nanostructures effectively generate superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$) at higher concentrations (>500 μg/mL) but scavenge $O_2^{\bullet-}$ at lower concentrations (40 μg/mL) with successful crossover at 200 μg/mL. Nanorods of MgO generate the highest levels of $O_2^{\bullet-}$, whereas nanoparticles scavenge $O_2^{\bullet-}$ to the highest extent (60%). Photoluminescence studies reveal that such crossover is based on the suppression of F^{2+} and the evolution of F^{+}, F_2^{+}, and F_2^{3+} defect centers. The evolution of these defect centers reflects the antibacterial activity of MgO nanostructures which is initiated at 200 μg/mL against Gram-positive *S. aureus* ATCC 29737 and among different bacterial strains including Gram-positive *B. subtilis* ATCC 6633 and *M. luteus* ATCC 10240 and Gram-negative *E. coli* ATCC K88 and *K. pneumoniae* ATCC 10031. Nanoparticles exhibited the highest antibacterial (92%) and antibiofilm activity (17%) against *B. subtilis* ATCC 6633 in the dark. Interestingly, the nitrogen-centered free radical DPPH is scavenged (100%) by nanoplates due to its large surface area (342.2 m^2/g) and the presence of the F_2^{+} defect state. The concentration-dependent interaction with an antioxidant defense system (ascorbic acid (AA)) highlights nanoparticles as potent scavengers of $O_2^{\bullet-}$ in the dark. Thus, our findings establish guidelines for the selection of MgO nanostructures for diverse therapeutic applications.



INTRODUCTION

In recent years bacteria have become more resistant against antimicrobials, and if the current trend persists, there are projected to be ca. 10 million lives lost every year by 2050 caused by antimicrobial resistance related issues.[1,2] Importantly, 80% of the antimicrobial-resistant bacterial infections originate from the hospital environment and contact with contaminated surfaces. In this context, metallic and semiconductor nanoparticles have gained the attention of researchers interested in developing potential replacements for antibiotics,[3] as antibiotics induce environmental pressure in which accidentally surviving bacteria (due to degradation of antibiotics) will replicate with genes at a high rate that resist antibiotics by several mechanisms, e.g. further degradation of antibiotics, modification of drug target, expression of efflux pumps, etc., and conventional treatment, e.g. higher doses of drugs, even enhance these strategies to increase an antibiotic resistant bacterial population.[2] The pro-oxidant activity of metallic and semiconductor nanoparticles typically originates from light-induced oxidative stress, caused by reactive oxygen species (ROS), examples of which include: superoxide anions ($O_2^{\bullet-}$),[4] hydroxyl radicals ($^{\bullet}OH$),[4] singlet oxygen ($^1O_2$),[4] dissolution of cations,[2,3,5,6] internalization of nanostructures[5] resulting in disintegration of the cell membrane, inhibition of enzyme activity and DNA synthesis, interruption of energy transduction,[3,7] etc. Generally, ROS damage biomolecules, e.g. proteins, vitamins, and lipids (lipid peroxidation) of microbial cells, due to the strong oxidation potential of ROS.[2,8−12] In addition to antibacterial activity, ROS also introduce anticancer and antitumor activity, leading to the use of these types of materials to have potential therapeutic applica-

Received: July 11, 2018
Published: October 3, 2018

DOI: 10.1021/acs.inorgchem.8b01938
Inorg. Chem. 2018, 57, 12727−12739

NATURAL PRODUCT RESEARCH
https://doi.org/10.1080/14786419.2018.1491039

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

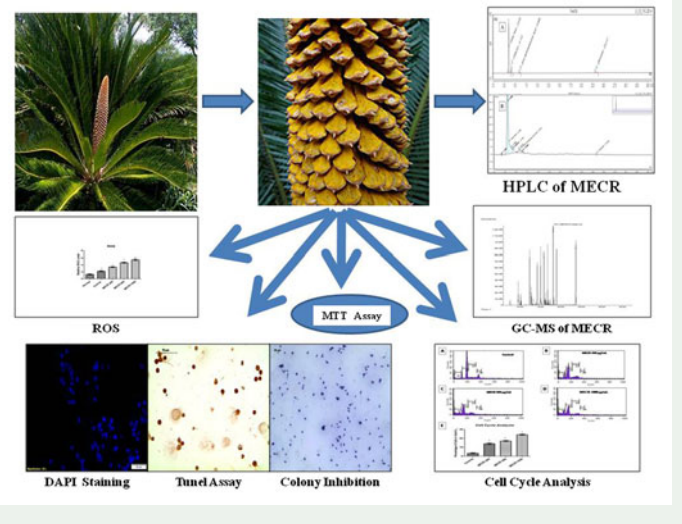
Metabolite profiling and *in-vitro* colon cancer protective activity of *Cycas revoluta* cone extract

Samit Bera[a], Bhaskar Das[a], Arnab De[a], Atish Barua[b], Susmita Das[c], Bratati De[c] and Amalesh Samanta[a]

[a]Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India; [b]Department of Cancer Chemoprevention, Chittaranjan National Cancer Institute, Kolkata, India; [c]Phytochemistry and Pharmacognosy Laboratory, Department of Botany, University of Calcutta, Kolkata, India

ABSTRACT

The methanolic extract of *Cycas revoluta* cone (MECR) was analyzed by GC-MS and UHPLC for metabolite profiling and was evaluated for anti-colon cancer property by using in vitro assays like Cell Viability Assay, Colony Formation Assay, ROS Determination, Flowcytometry, DAPI staining assay, Tunel assay. GC-MS and HPLC analysis confirmed the presence of different phytochemicals in the extract of *Cycas revoluta* cone. *In-vitro* studies showed MECR extract showed significant anti-colon cancer activity by reducing proliferation and inducing apoptosis in colon cancer cell (HCT-8) line, but no such activity was seen in normal colon cell (CCD-18Co) line. The investigation confirms that MECR may be a promising candidate in colon cancer protection.



ARTICLE HISTORY
Received 25 February 2018
Accepted 17 June 2018

KEYWORDS
Cycas revoluta; colon cancer; reactive oxygen species (ROS); GC-MS

List of Abbreviations: MECR: Methanolic extract of *Cycas revoluta*; ROS: Reactive Oxygen Species; DAPI: 4, 6-Diamidino-2-phenylindole; UHPLC: Ultra-high Performance Liquid Chromatography; PI3K/Akt: Phosphoinositide 3-kinase/Akt (Protein Kinase B); AP1:

CONTACT Amalesh Samanta asamanta61@yahoo.co.in

JOURNAL OF THE AMERICAN COLLEGE OF NUTRITION
https://doi.org/10.1080/07315724.2018.1538829

Seasonal Variation of Phyto-Constituents of Tea Leaves Affects Antiproliferative Potential

Sayantan Maitra[a], Arnab De[b], Bhaskar Das[b], Sudipendra Nath Roy[b], Ranadhir Chakraborty[c], Amalesh Samanta[b], and Subhrajit Bhattacharya[d]

[a]Institute of Pharmacy, Jalpaiguri, Government of West Bengal, Jalpaiguri, India; [b]Division of Microbiology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India; [c]Department of Biotechnology, North Bengal University, Siliguri, India; [d]Department of Pharmacology, Emory University School of Medicine, Atlanta, GA, USA

ABSTRACT

Objective: Tea (*Camellia sinensis* Linn.; family: Theaceae) is popular as a stimulant beverage across the globe and is also utilized as a functional antioxidant in alternative medicine. This study has evaluated the impact of seasonal variation on phyto-constituents of tea.

Method: The antiproliferative potential of methanolic extracts of tea leaves collected in the rainy season (MECR) was compared with the extract of tea leaves collected in the autumn season (MECA) of the same mother plant. Evaluation of *in vivo* antitumor activity was carried out in adult female Swiss albino mice groups inoculated with Ehrlich ascites carcinoma (EAC) cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to compare efficacy of MECR with that of MECA in the EAC cell line. Both qualitative and quantitative tests for phyto-chemical constituents present in MECA and MECR were performed. Antitumor efficacy of both the extracts was determined by evaluating different tumor markers showing dose-dependent cytotoxicity.

Results: Statistically significant reduction in EAC-induced tumor was observed in MECR treated mice compared to MECA treated ones. Cell decimation was significantly higher with MECR treatment, where restoration of different parameters including tissue structures returned to normal. Moreover, gas chromatography–mass spectrometry (GC-MS) study revealed the presence of cyclobarbital and benzazulene derivative in MECR, which is thought to be a novel source of these chemicals.

Conclusions: To our knowledge, there is no report that has attempted to reveal nutritional changes in terms of efficacy and variation in anticancer constituents in tea leaves, plucked in two seasons. This study revealed a novel source of barbital and benzazulene derivative. The unique presence of cyclobarbital and benzazulene, as revealed from GC-MS data, in methanolic extract of tea leaves collected during the rainy season (MECR) may have contributed to its enhanced *in vitro* (adopting MTT assay) and *in vivo* (on EAC-infected Swiss albino mice) cytotoxicity vis-à-vis antiproliferative properties compared to methanolic extract of tea leaves collected during the autumn season (MECA). The nature of plucking leaves in the two selected seasons is different.

ARTICLE HISTORY
Received 31 July 2018
Accepted 15 October 2018

KEYWORDS
Camellia sinensis; Ehrlich ascites carcinoma (EAC); MTT; methanolic extracts; seasonal variation; cyclobarbital and benzazulene

Introduction

Tea is one of the most consumed drinks in the world, next to water, with a per-capita worldwide consumption of approximately 0.12 L per day, and the amount of consumption far exceeds other beverages such as coffee, beer, wine, and soft drinks (1, 2). Apart from the unique aroma in tea, several phytoconstituents present in it have shown promising cancer chemopreventive activity (3, 4).

Synthetic anticancer moieties provoke nonspecific decimation of cells, whereas natural products offer protective and therapeutic actions to all cells with low cytotoxicity (5). Developed countries are now inclining toward complementary and alternative therapy due to high mortality risk and long-term morbidity associated with surgical procedures, radiation, and chemotherapy. Potential benefits of functional supplementation with tea as a comestible encompass protective action against carcinogenesis and inhibition of the progress of tumor(s) of differing nature (6, 7). From an earlier study, it is evident that tea elicited a dose-dependent apoptogenic effect on Ehrlich's ascites carcinoma (EAC) cells, grown in the peritoneal cavity of Swiss albino mice (4). Although tea elicits varying results in restricting cancer, considering all reports and by accumulating further evidence, tea may be recommended as a potential dietary supplement for the prevention of cancer (7).

Several studies utilizing spectrometric and chromatographic analyses have been performed to determine the chemical composition of tea leaves and their quantitative changes in relation to factors like genotype and environment (8). The principal constituents of tea leaves encompass a wide range of chemical classes of molecules, such as catechins, flavanols, oxyaromatic

CONTACT Subhrajit Bhattacharya sbhat40@emory.edu Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, USA.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/uacn.

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 3, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

ANTIMICROBIAL INVESTIGATION AND BINDING MODE ANALYSIS OF SOME NEWLY SYNTHESIZED 4-AMINO-5-((ARYL SUBSTITUTED)-4H-1, 2, 4-TRIAZOLE-3-YL)-THIO LINKED HYDROXAMIC ACID DERIVATIVES

MOUSUMI DAS[1], BHASKAR DAS[1], ARNAB DE[1], SUBHASIS BANERJEE[2], AMALESH SAMANTA[1]*

[1]Department of Pharmaceutical Technology, Division of Microbiology and Biotechnology, Jadavpur University, Kolkata, West Bengal, India. [2]Department of Pharmaceutical Chemistry, Gupta College of Technological Sciences, Ashram More, Asansol, Kolkata - 713 301, West Bengal, India. Email: asamanta61@yahoo.co.in

Received: 22 November 2018, Revised and Accepted: 04 January 2018

ABSTRACT

Objective: A series of 5-substituted-4-amino-1, 2, 4-triazole-linked hydroxamic acid derivatives have been synthesized and explored *in vitro* to evaluate antibacterial and antifungal activities.

Methods: Different 5-phenyl group substituted-4-amino-1,2,4-triazole-3-thiol reacted with chlorine substituted hydroxamic acid to produce the desired compounds and characterized spectroscopically. Minimum inhibitory concentration (MIC), zone of inhibition (ZOI), growth kinetic studies, and scanning electron microscopy (SEM) were employed to elicit the antimicrobial efficacy of synthesized compounds against a wide range of bacterial and fungal strains.

Results: Compounds 6a, 6b, 6d, and 6k (MIC of 25 µg/ml) have been found to be more potent against *Klebsiella pneumoniae*, *Bacillus cereus, Bacillus pumilus*, *Micrococcus luteus,* and *Pseudomonas aeruginosa*, compounds 6a-6d, 6k, and 6l (MIC of 25–50 µg/ml) have shown potent antibacterial efficacy against *Klebsiella pneumonia*, *P. aeruginosa,* and *Vibrio cholera* compare to the standard drug amoxicillin (MIC of 60 µg/ml, 65 µg/ml, and 25 µg/ml, respectively). Screening for the antifungal activity revealed that the compounds were found to be most active against *Candida albicans* (6a, 6b, and 6l), *Candida tropicalis* (6b and 6d), and *Aspergillus niger* (6a, 6b, 6d, and 6j) with MIC of 15–25 µg/ml. Bacteriostatic and fungistatic effect of titled compounds was revealed from growth kinetics study.

Conclusion: Electron donating group at the 5-position of the 5-substituted-1,2,4-triazole-linked hydroxamic acid derivatives conferred the biological effectiveness of the synthesized compounds and also offer a therapeutically effective prototypical structure for further development of new chemical entities with superior antimicrobial activity.

Keywords: 1,2,4-triazole, Hydroxamic acid, Antibacterial, Antifungal.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i3.30882

INTRODUCTION

The microbes are getting resistant toward the existing chemotherapeutics in alarming rate which is not only a major concern for public health but also a challenge for the scientific community globally, and the number of cases of multidrug-resistant bacterial infections is increasing nowadays [1]. In clinical practice, the infections caused by the Gram-positive bacteria are very common. However, the infection caused by them is sometime severe. *Bacillus cereus*, *Staphylococcus aureus*, *Bordetella bronchiseptica*, *Micrococcus luteus*, *Bacillus pumilus*, *Bacillus subtilis,* and *Enterococcus faecalis* are some important Gram-positive bacteria causing serious infection in the community, whereas *Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium* are very common Gram-negative bacteria. Among them, *E. coli* is the most common one. The mortality is higher for infections caused by Gram-negative bacteria than the Gram-positive one. To overcome various microbial infections specifically fungal infection, a large number of triazole drugs have been successfully developed [2]. Azole compounds showed the effect by inhibiting lanosterol 14α-demethylase (CYP51) which catalyzes the oxidative removal of the 14α-methyl group of lanosterol to give $\Delta^{14,15}$-desaturated intermediates in ergosterol biosynthesis [3] resulting in accumulation of toxic methyl-sterols in membranes leading to fungistatic or fungicidal effect [4]. Some important azoles derivatives such as voriconazole, itraconazole, posaconazole, and fluconazole (Fluc) are used for the treatment of fast-growing fungal infections, which are the leading cause of mortality and morbidity in immunocompromised patients [5]. However, Fluc is not effective against aspergillosis due to its severe drug resistance [6,7].

These scenarios are highlighting the urgent need for novel, efficacious, less toxic, and safe drug candidates in the pipeline. The heterocyclic compounds containing nitrogen, sulfur, and oxygen have an enormous significance in the field of medicinal chemistry [8]. To address the concern, we develop a series of novel 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives. Assembling the different pharmacophores in a single frame always leads to compounds having fascinating biological profile [9]. Triazole is one of the pharmacophores in the synthesized compounds. Chemical compounds possess triazole as a core; always play a significant role in the field of medicinal chemistry. Like triazole, imidazole moiety has widely used to synthesize compounds to treat diseases causing by different microorganisms. Even though imidazole and triazole have a similar mode of action, triazoles have several advantages over imidazole, like, oral bioavailability, slow metabolic rate and have minimal effect on sterol synthesis in humans. For these, use of triazole moiety increases rather than the imidazole [10]. Triazole is well known for its antimicrobial property and widely used to treat microorganisms associated diseases [11]. Besides, the antibacterial [12-15] and antifungal [16-19] activity; 1,2,4-triazole possess, anti-inflammatory [20], as well as antioxidants properties [21]. Various well-known drug commercialized in the market possesses 1,2,4-triazole moiety such as Fluc, terconazole,

JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Antioxidant and anticancer activity of synthesized 4-amino-5-((aryl substituted)-4H-1,2,4-triazole-3-yl) thio-linked hydroxamic acid derivatives

Mousumi Das, Bhaskar Das and Amalesh Samanta

Division of Microbiology and Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Keywords
1,2,4-triazole; anticancer; antioxidant; hydroxamic acid derivatives; *in vivo* study

Correspondence
Amalesh Samanta, Division of Microbiology and Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, 188, Raja S. C. Mullick Road, Kolkata 700032, India.
E-mail: asamanta61@yahoo.co.in

Received March 23, 2019
Accepted May 21, 2019

doi: 10.1111/jphp.13131

Abstract

Objectives The antioxidant and anticancer activity of twelve 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives were evaluated.

Methods Previously synthesized 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide and 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (**6a–6l**) were evaluated for in vitro antioxidant and in vivo anticancer activity. MDA-MB-231, MCF-7 and HCT 116 cell lines were used to evaluate IC_{50} values, in vitro. Ehrlich ascites carcinoma (EAC)-induced mice model was used to evaluate in vivo anticancer potential. Different biological markers were examined for drug-related toxicities.

Key findings Compound **6b** revealed more potent antioxidant property among all tested compounds, even than the ascorbic acid. The IC_{50} values of compound **6b** were found to be 5.71 ± 2.29 μg/ml (DPPH assay) and 4.12 ± 0.5 μg/ml (ABTS assay). Histopathology of liver sections of drug-treated mice was evaluated. Survival analysis showed that compound **6b** could increase the life span as of the standard drug.

Conclusions After the assessment of all in vivo anticancer study related data, it was found that compound **6b** possess superior anticancer potency in terms of efficacy and toxicity. From this experimental design, it could be concluded that further modification of this prototypical structure will lead to develop more potent antioxidant as well as an anticancer agent in the future.

Introduction

Heterocyclic compounds have been used to be the most researched chemical entity in the area of organic chemistry, having wide verities of application in the field of pharmaceuticals to agro-industries.[1] Among the heterocyclic compounds, nitrogen-containing heterocycles like triazole grab the attention of the scientific community over the decades. 1,2,4-Triazoles is an interesting isomer of triazoles having a diversified pharmacological activities such as anticancer, antidepressant[2], antibacterial[3], antifungal[4] anti-inflammatory, antileishmanial[5] and antiviral activities[6]. It could be found from different experimental design associated with the 1,2,4- triazole that it also possesses superior antioxidant activity. Cetin and Geçibesler[7] demonstrated that phenol and pyridine substituted 1,2,4-triazole attributed exceptional antioxidant property. Pokuri *et al.*[8] have designed and synthesized different derivatives of 1,2,4 triazoles and found significant antioxidant activity over the standard ascorbic acid. Karrouchi *et al.*[9] have synthesized a series of pyrazole containing derivatives of 1,2,4-triazoles and found significant antioxidant activity for all of the synthesized compounds.

Hydroxamic acids (R-CO-NH-OH) are also an important class of chemical compounds which could be derived from hydroxylamine (NH_2OH) and carboxylic acids (R-COOH). This class of compounds has the ability to chelate metal ions and known to have multiple biological activities.[10] They found to have potent antioxidant activities. Like, Koncic *et al.* have synthesized hydroxamic acid derivatives of non-steroidal anti-inflammatory drugs (NSAIDs) and found significant radical scavenging, antioxidant and metal chelating activities. They have found that N-methylhydroxamic acid of diclofenac showed potent

P35

Marketing Survey on Rosuvastatin under Asansol Teritorie

Utsab Chakraborty* and Biswaranjan Chatterjee
Gupta college of technological sciences, asram more G.T Road, Asansol 713301, west Bengal

HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, officially abbreviated HMGCR) is the rate-controlling enzyme (NADH-dependent, NADPH-dependent) of the mevalonate pathway, the metabolic pathway that produces cholesterol and other isoprenoids. Normally in mammalian cells this enzyme is suppressed by cholesterol derived from the internalization and degradation of low density lipoprotein (LDL) via the LDL receptor as well as oxidized species of cholesterol. Competitive inhibitors of the reductase induce the expression of LDL receptors in the liver, which in turn increases the catabolism of plasma LDL and lowers the plasma concentration of cholesterol, an important determinant of atherosclerosis. This enzyme is thus the target of the widely available cholesterol-lowering drugs known collectively as the statins.HMG-CoA reductase is anchored in the membrane of the endoplasmic reticulum, and was long regarded as having seven transmembrane domains, with the active site located in a long carboxyl terminal domain in the cytosol. More recent evidence shows it to contain eight trans membrane domains.In humans, the gene for HMG-CoA reductase is located on the long arm of the fifth chromosome related enzymes having the same function are also present in other animals, plants and bacteria.

P36

Antioxidant Activities of *Cycas revoluta* (Cycadaceae) Extract: Modulatory Effect on Colon Cancer

Samit Bera*, Bhaskar Das, Arnab De and Amalesh Samanta
Department of Pharmaceutical Technology, Jadavpur University, Kolkata-70003

Cycas revoluta is an ornamental shrub of which antioxidant and anti-colon cancer activity of male cone extract is yet to be validated scientifically. The present study investigated anti-colon cancer activity of *Cycas revoluta* cone against 1,2-Dimethyl Hydrazine (DMH) induced colon carcinoma in wistar rats. The animals were divided into five groups taking as a Control, a Carcinogenic, two Tests and a Standard. All groups except Normal Group received DMH once a week for four consecutive weeks. The carcinogen induced group was without any treatment. The third and fourth Test Groups were treated with methanolic extract of *Cycas revolute* (MECR) at two different concentrations. The fifth group was treated with 5-Fluoro Uracil (5-FU). The treatment with MECR and 5-FU were continued for 16 weeks. The study showed MECR significantly ($p<0.05$) increased superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) after treatment. These findings suggest that the methanolic extract of *Cycas revoluta* may play a beneficial role against colon cancer.