Translating the Chemical Vocabulary of Plants by Interpreting the Bioremediation Efficacy of *Cleome rutidosperma* DC.

Submitted by

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To whom it may concern

This is to certify that the thesis entitled "Translating the Chemical Vocabulary of Plants by Interpreting the Bioremediation Efficacy of *Cleome rutidosperma* DC." submitted by Miss Ekta Bhattacharya, who got her name registered on 27th August 2019 (Index No. 103/19/Life Sc./26), for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of Dr. Suparna Mandal Biswas and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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Preface

Phytoremediation is gaining interest in recent years as it is a simple, sustainable and cost-effective strategy for heavy metal decontamination. The first and most straightforward step for remediation of heavy metals is searching for efficient hyperaccumulator species that grow naturally in contaminated sites. Mitigating waste lands by weed species provide some added advantage as they are non-edible they cannot enter into the food chain. Moreover, weed did not need any special care and nutrients. They can tolerate and take-up large amounts of inorganic contaminants to increase the efficiency of phytoremediation. The present thesis work encompasses the first detailed account of hyperaccumulator potentialities of a neglected and underutilized (NUS) species, *Cleome rutidosperma* DC. belonging to the family Cleomaceae, a sister family of Brasssicaceae.

Chapter 1 includes a detailed overview of the sources of pollutants and the health hazards caused by these pollutants especially heavy metals. Rapid urbanization, scientific advancements, lifestyle changes, agricultural revolution and most importantly population outburst has contributed predominantly in polluting the environment and natural resources i.e. water, air or soil. Contamination of natural resources is the major concern of the today's environmental problem. Pollution has, in the long run, resulted in chain of events like global warming, climate change, abrupt changes in weather causing huge agricultural losses, degradation in soil fertility, frequent flooding and lastly increased health risks.

Chapter 2 comprises a comprehensive literature review about phytoremediation. The use of plants to reduce contaminants and restore the soil resource is a cost-effective method of reducing the risk to human and ecosystem health posed by contaminated soil sites. And this phenomenon is termed phytoremediation. Phytoremediation has low installation and maintenance costs compared to other remediation options. The most innovative strategy for

heavy metal clean-up is searching for efficient hyperaccumulator species. A feasible approach for the same is the observation of naturally proliferating species at the contaminated sites, which can help in identifying potential plant species. It has been widely reported that plants that are native to contaminated sites like sewage disposal sites and household dumpyards have shown promise for phytoextraction. Therefore, identification and exploration of novel species inhabiting such areas that yield high biomass and grow naturally were our foremost criteria for the successful phytoremediation of Cd- and Pb-contaminated soils.

Chapter 3 gives a precise overview of the research specifying the problem statement that is addressed with each of the objectives in a tabulated form. The problem statements are mainly based on the aim of validating the role of *Cleome rutidosperma* DC. as a novel potential phytoremediator species. Therefore, we aim a meticulous study of the phytoremediation potential of *C. rutidosperma* against two of the most notorious heavy metals cadmium and lead through hydroponic as well as pot culture experiments. It accounts for the uptake and immobilization of both the tested heavy metals by the plant body and takes into consideration the toxic effects of the high concentrations of metals mainly depicted as the biomass content and total chlorophyll content. We also try to analyze the bioactive compounds present in the root exudates of this plant with the help of GCMS to decrypt the distinctive vocabulary this species uses that enables it to survive in the highly contaminated areas.

Chapter 4 mainly describes the first objective. The methodologies undertaken and the parameters that were taken into consideration for the hydroponic screening experiments. The hydroponic experiments revealed that the plant can tolerate heavy metal stress without showing any significant signs of toxicity. The heavy metal quantification analysis reveal that even at the low concentration of 10 mg/kg, *C. rutidosperma* could accumulate 42.49 mg/kg of Cd in shoots

and 134.71 mg/kg Cd in roots. In case of Pb, the plant could store 27.79 mg/kg in shoots and 491.35 mg/kg in its roots.

In **Chapter 5**, the results of our hydroponic studies were further validated by performing the pot culture experiments. Different concentrations of Cd and Pb were spiked in the soil. After 60 days of incubation, the AES analysis revealed *C. rutidosperma* could efficiently accumulate as high as 639.07 mg/kg of Cd, 8726.03mg/kg of Pb in its roots while 752.83mg/kg Cd and 3732.64mg/kg Pb in its shoots.

Chapter 6 deals with the determination of Bioconcentration factors (BCF) and translocation factors (TF). These indices are used to identify whether *C. rutidopserma* is an excluder or an accumulator species. The TF was 1.18 at 200mg/kg concentration of Cd and 1.38. The highest BCF recorded for Cd was 27 while for Pb 847.23. Therefore, *C. rutidosperma* can be considered as good phytoextractors as the BCF>1 and TF>1 because it can translocate the heavy metals into its shoots efficiently.

The plants mainly communicate through the root with the help of chemical signals released. Therefore, **Chapter 7** includes collection and purification of the root exudates of *C. rutidosperma.* GCMS analysis was done to identify the bioactive compounds present in the root exudates of this plant. Five major compounds, namely, palmitic acid, linoleic acid, oleic acid, campesterol, and stigmasterol, which mainly are metabolic markers for detoxification mechanisms triggered by various stresses were detected.

To conclude, this research work is aimed at validating the species *Cleome rutidosperma* DC as a novel heavy metal accumulator for cadmium and lead. This study may provide a new dimension to exploit this plant as a potential hytoremediator for mitigating the waste lands and the plants can be easily monitored. It would be a safer, cleaner, inexpensive and environment friendly technology which generally have a high public acceptance and can often be carried

out at any site. The possibility of the recovery and re-use of valuable metals (by companies specializing in "phyto mining"). The use of plants reduces erosion and metal leaching in the soil and thus improving the soil health.

It may also have Polyaromatic hydrocarbon degrading abilities as indicated by the root exudate analysis. This aspect of the remediation abilities of this species needs further in depth research that can put forward this species as a potent candidate for successful remediation of soil.

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List of Abbreviations

ABTS	–	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ATDSR	–	Agency for Toxic Substances and Disease Registry
ATP	–	Adenosine triphosphate
CNS	–	Central Nervous System
CPCB	–	Central Pollution Control Board
CTGF	–	Connective tissue growth factor
DPPH	–	2,2-diphenyl-1-picrylhydrazyl
EDTA	–	Ethylenediaminetetraacetic acid
EGFR	–	Epidermal growth factor receptor
GCMS	–	Gas chromatography mass spetrometry
GSH	–	Glutathione S transferase
IARC	–	International Agency for Research on Cancer
MAPK	–	Mitogen-activated protein kinases
MTT	–	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	–	Nicotinamide adenine dinucleotide
NADPH	–	Nicotinamide adenine dinucleotide phosphate
NaS1	–	Nicotianamine synthase 1
NO	–	Nitric Oxide
PCNA	–	Proliferating Cell Nuclear Antigen
PGPR	–	Plant Growth Promoting Rhizobacteria
ROS	–	Reactive Oxygen Species
SLC13A1	–	SLC13A1 solute carrier family 13 member 1 (human)
TGF-β1	–	Transforming growth factor beta 1
USEPA	–	United States Environmental Protection Agency

CONTENTS

Chapter 1

Introduction

The environment, as we all know, is the system where all living beings co-exist. They do so by working together and sharing the resources that are available. Therefore, the Earth is a balanced ecosystem comprising of four spheres; the living beings (biosphere), the land (lithosphere), the air (atmosphere) and the water (hydrosphere).

1.1 Sources of heavy metal pollution

Industrial revolution has drastically change the world scenario in the last few decades. Rapid urbanization, scientific advancements, lifestyle changes, agricultural revolution and most importantly population outburst has contributed immensely in disturbing the natural environment by introducing artificial or man-made components. These contribute immensely in polluting the environment and natural resources be it water, air or soil. Pollution has, in the long run, resulted in chain of events like global warming, climate change, abrupt changes in weather causing huge agricultural losses, degradation in soil fertility, frequent flooding and lastly increased health risks to human as well as all living beings (**Suman et al 2018; Ashraf et al 2019**).

Pollution and heavy metal contamination of the environment is one of the most severe problems in the world (**Liu et al., 2018**). This is posing grave threats to the health of all life this planet uniquely possess by degrading the quality of the main resources which allow life to thrive, that is, water, air, and soil. The main causes behind such decline are anthropogenic activities like irresponsible industrialization, unrestricted agricultural activities, and unrestrained mining practices. These have led to a extreme surge in inorganic pollutants like Arsenic (As), lead (Pb), mercury (Hg), cadmium (Cd), nickel (Ni), chromium (Cr) and aluminium (Al) in the soil (**Alengebawy et al., 2021**). These heavy metals are present in the

soil and water in concentrations that exceed way beyond the permissible limits given by USEPA and CPCB (**Table 1.1**). In addition to these, there may be some natural phenomena like volcanic eruption, geological weathering, metal leaching and soil erosion that contribute to the heavy metal pollution.

Table 1.1: Levels of few heavy metals used to guide clean up and land use decisions (mg/kg)

	US EPA	**CPCB**	
	Soil level requiring clean-up	**Range**	**Typical value**
	Industrial area	**Agricultural area**	
Copper (Cu)	--	2-100	20
Cadmium (Cd)	>70	0.001-7	0.06
Chromium (Cr)	>230	5-3000	100
Nickel (Ni)	>1600	10-1000	40
Lead (Pb)	>400	2- 200	10
Zinc (Zn)	>23,600	10-300	50
Mercury (Hg)	<0.01–1 800	<0.01–1 800	270

Scientists have classified the sources of pollution into two major types; natural and anthropogenic sources. Processes like erosion of sedimentary rocks, volcanic eruptions, soil formation, and rock weathering are the main natural sources, while anthropogenic sources consist of industrial, agricultural, mining, and domestic effluents (**Roozbahani et al 2015**). However, source apportionment may be difficult in many cases, despite sophisticated research technics applied (**Sutkowska 2020**).

1.1.1 Natural Sources of Heavy Metals

Igneous and sedimentary rocks are considered as the most common natural source. **Table 1.2** enlists the concentration ranges (ppm) of heavy metals in these. It has been found that elements that existed in one rock type have varying proportions, as well as quantities of different

elements vary from one rock type to another (**Bradl 2005**). Heavy metals concentration can be estimated according to the type of rocks and the adjacent ecosystem conditions (**Sharma et al 2005**). In addition, soil formation is also considered as one of the main reasons of heavy metals accumulation besides river sediments.

Table 1.2: Range of heavy metal concentrations (ppm) in igneous and sedimentary rocks (Modified from **Cannon et al. 1978**).

Metals	Basaltic Igneous	Granite Igneous	Shale and Clays	Black Shale	Sandstone
Cd	0.006-0.6	0.003-0.18	0.0-11	<0.3-8.4	-
Pb	30-160	4-30	18-120	20-200	-
Cu	48-240	5-140	18-180	20-200	2-41
Zn	2-18	6-30	16-50	7-150	<1-31

1.1.2 Anthropogenic Sources of Heavy Metals

Industries, agriculture, mining, and wastewater are reckoned as anthropogenic sources of heavy metals. These contribute significantly to the increase of heavy metals concentration in the ecosystem, e.g., smelting causes increase in release of Cu, Zn, and As; insecticides contribute to release As; burning of fossil fuels produces Hg, and cars exhaust assists in releasing Pb (**Masindi et al 2018**). In addition, daily human activities, such as farming, industrial processes, and manufacturing, cause imbalance in the biosphere (**He et al 2005**). **Figure 1.1** shows the main anthropogenic sources of heavy metals.

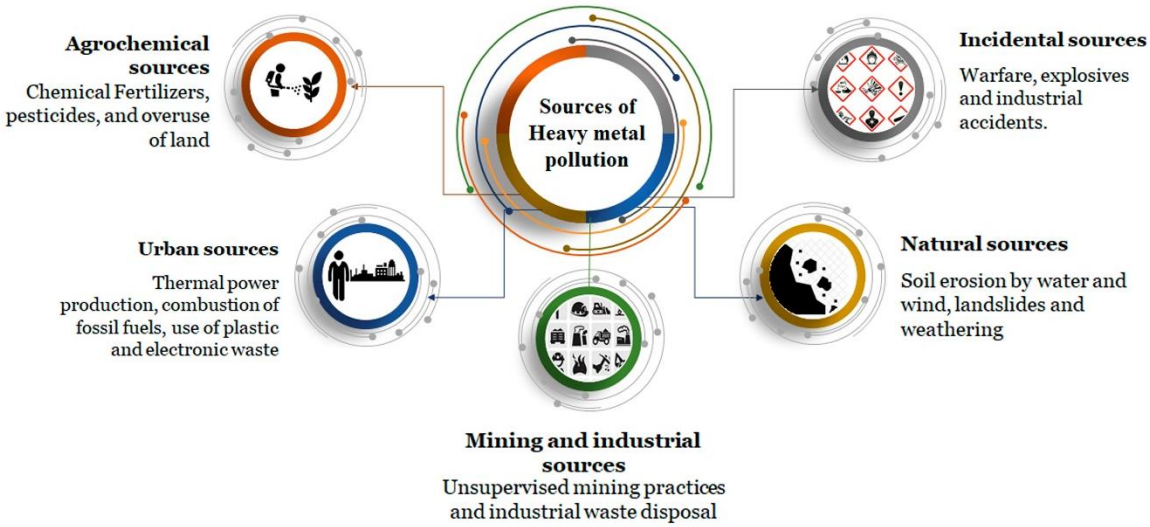


Figure 1.1: Anthropogenic sources of pollution.

1.1.3 Agricultural Sources of Heavy Metals

Agroecosystems are usually are the epicenters of types of agricultural pollutants, which are known as biotic and abiotic byproducts of farming practices. Fertilizers, pesticides, and sewage sludge are the most common among the agricultural sources of heavy metals (**Alloway et al 2013**). These heavy metals differ in nature and the way of accumulation both in soil and in plants. Addition of fertilizers helps in supply necessary nutrients to augment plant growth and productivity and increase the soil health and soil fertility (**Meng et al 2020**). Fertilizers can be of both organic (natural) and inorganic (synthetic) type. Carbon-based or biofertilizers are produced after the anaerobic digestion process in the form of ammonium (**Alengebawy 2021**). Inorganic fertilizers, also known as chemical or synthetic fertilizers, are a combination of inorganic substances and chemical materials (**Cai et al 2019**). Fertilizers, of both organic and inorganic elements, are responsible for producing heavy metals in the soil. According to the region, **Table 1.3** presents a comparative account of the different heavy metals and their amounts in the fertilizers worldwide and European Union (EU). Phosphorus that is very common inorganic fertilizer and also widely used in fertilizer synthesis; plays a significant role in heavy metals accumulation through its application to the soil (**Chen et al 2020**). Phosphate

fertilizers that are water-insoluble, produce phosphate rocks, by precipitation as metal phosphates in the soil which play a major role in the immobilization of metals (**Bolan et al 2003**). Undue and prolonged use of fertilizers result in heavy metals accumulation in agricultural soils which in turn reduces soil fertility, and accordingly decreases plant growth and productivity (**Ai et al 2020**). It is extremely difficult to recover the soil health after it has been contaminated with heavy metals. Cu, Zn, and Cd have a higher accumulation potential in agricultural soil due to the long-term use of fertilizers (**Wang et al 2020**).

Table 1.3: A comparison of the usage of different types of fertilizers and the concentrations of heavy metal input (mg/kg) resulting due to the application of fertilizers along with the EU standards (**Alloway et al 2013**)

Heavy Metals	**Phosphate Fertilizers**		**Nitrogen Fertilizers**		**Lime Fertilizers**		**Manure Fertilizers**	
	Usage	EU	Usage	EU	Usage	EU	Usage	EU
Cd	0.1-170	13	0.05-8.5	0.9	0.04- 0.1	0.2	0.3-0.8	-
Pb	1-300	26	1-15	2	2-125	5.6	2-60	-
Cu	7-225	13	2-1450	1.9	20-1250	8.2	6.6-350	-
Zn	50-1450	236	1-42	5	10-450	22	15-250	-

1.2 Health hazards caused by heavy metals

Heavy metals are defined as metals with a density greater than $5 g/m^3$ (**Megrahi et al 2006**). The main concern behind the accumulation of these metals is that these are nonbiodegradable. Metals also cause toxicological effects by forming covalent bonds with organic compounds. They can bind to cellular macromolecules to form lipophilic compounds which in turn produce toxic effects. Due to their lipophilic nature, the distribution and toxicity of these compounds become more severe as compared to the ionic forms of the same. The heavy metals like lead and mercury bind to the sulphydryl group of the proteins and cause them to either mis-fold or denature (**LoPachin & Gavin 2012**).

Metals can be incorporated in human body by the following ways: a) ingestion-through food and water consumption; b) inhalataion- through the air we breathe; c) contact- through the skin lesions while handling heavy metal rich chemicals in agricultural and industrial sectors. Upon entering our body, these heavy metals tend to bioaccumulate, which in turn, cause the toxic effects. Heavy metals like iron, zinc, silver and gold are actually called essential metals as they are required in our metabolism to perform certain biochemical and physiological roles. But, due to bioaccumulation, these metals cause toxic effects as their concentration increases in the system (**Briffa et al 2020**).

Heavy metals interact with the cellular organelles like mitochondria, chloroplast, lysosomes etc and cause damage as well. Heavy metals can even cause mutation, carcinogenesis and premature apoptosis by directly interacting with the DNA and nuclear proteins. Such damage can be of "direct" or "indirect" in nature. The metals cause conformational changes in the DNA and associated proteins causing damage in the first case. But in the second case, the damage is caused by the Reactive Oxygen Species (ROS) molecules that are produced due to the effects incurred by these metals. Formation of such free radicals are associated with heavy metals like chromium, copper and cobalt which are also considered as carcinogenic (**Engwa et al 2019**) . Metals like iron, copper, vanadium, chromium and cobalt react to form the superoxide and the hydroxyl radical following the Fenton reaction that has been reported to occur in mitochondria, microsomes and peroxisomes (**Valko et al 2005**).

These metal induced ROS generation causes oxidative damage to the DNA like base modifications which result in mutagenic alterations. Some of the main damage on DNA include: (i) base modification caused by chromium and nickel; (ii) crosslinking caused by nickel, copper, iron; (iii) strand excision reported by nickel, cadmium, chromium; and (iv) depurination which is seen by copper, chromium and nickel. Therefore, such oxidative damage of nucleic acids often translate to carcinogenesis. On the other hand, few metals like cadmium

and arsenic are reported to inhibit DNA repair mechanisms. The specific molecular roles and toxic effects of few heavy metals are discussed further.

1.2.1 Vanadium:

Role in metabolism and toxicity: Vanadium has mainly two oxidation states in which it is found in the body, which are vanadyl (V^{4+}) and vanadate (V^{5+}). Vanadium can bind reversibly to the protein transferrin present in the blood, which is then taken up by erythrocytes (**ATSDR 2012**). Pentavalent vanadate is an inhibitor of the plasma membrane Na^+ K^+ ATPase and can also react with a variety of enzymes, making it more toxic than vanadyl. Vanabins (vanadium-binding proteins) that are in the cytoplasm can help the transport and accumulation of vanadium in the vacuoles (**Valko et al 2005**). In vivo studies in mice, showed a decrease in NADPH, NADH and glutathione-SH after an hour of being injected with sodium vanadate. The reason behind can be explained as the metal was seen to act as a phosphate analogue during in vitro studies. It interfered with a variety of ATPases, phosphate-transfer enzymes and phosphatases like (**ATSDR 2012**)

- Na^+ K^+ ATPase
- Ca^{2+}ATPase
- Ca^+ Mg^+ ATPase
- H^+ K^+ ATPase Glucose-6-phosphatase
- Acid and alkaline phosphatase
- Ribonuclease
- Phosphodiesterase
- Phosphotryosyl-phosphatase

Vanadium mainly causes its negative effects due to its interactions with different enzymes. The in vivo mechanism behind the toxicity has not been researched well. It was shown that V_2O_5 induced production of mucin in the epithelial cells in the respiratory tract of mice. Though the mucin production was induced by the independent pathways of EGFR- and MAPK-, and the dependent pathways of RAF1-1KK-NF-κB. An increased production of collagen and/or fibroblasts were seen around the airways due to airway fibrosis caused by V_2O_5. In the presence of vanadium, mRNA levels increased, which encoded pro-fibrogenic growth factors such as PDCF-C, CTGF and TGF-β1 and chemokines such as CXCL9, CXCL10, IFN-α, and IFN-β (**ATSDR 2012**). If there is any kind of injury to cell , Fenton-like reaction due to the presence of vanadium, That produces free radicals and further produce the radical superoxide, which then is reduced by the dismutase, to oxygen and hydrogen peroxide (**Valko et al 2005**).

1.2.2 Chromium:

Role in metabolism and toxicity: In addition to the physical and chemical properties of chromium, the absorption of the metal depends on the activity of macrophages in the alveoli also. Cr^{6+} is absorbed readily than Cr^{3+} in the bloodstream and is more toxic. Chromium which is not absorbed through the lungs, may enter the gastrointestinal tract since it is cleared by the mucociliary clearance. Chromium absorption primarily happens in the jejunum. The extent of absorption mainly depends on oxidation state and formulation of the metal (**ATSDR 2012**). The mechanism behind toxicity and carcinogenicity of chromium is a complex process. DNA lesions such as DNA-protein crosslinks Cr-DNA adducts, DNA-DNA crosslinks, changes in cellular signalling pathways, and DNA strand breaks are caused by complexes made up of Cr^{3+}. Complexation of chromium with peptides, DNA and proteins etc may all be a factor to toxicity and carcinogenicity. Cr^{6+} is more toxic because it has a higher redox potential; and can enter the cells more. Cr^{6+} at physiological pH is found in a tetrahedral chromate anion which has a

similar structure to other natural anions such as phosphate and sulphate, which are permeable through the nonselective membrane channels (**Paiva et al 2009**).Such chromate compounds are observed to induce DNA damage in a variety of ways leading to the production of DNA adducts, chromosomal aberrations, and transcription of DNA as seen in, in vivo and in vitro studies (**O' Brien et al 2003**). Formation of the ROS leads to an oxidative stress which may cause variety of deleterious effects on the cells. This also includes effects like lipid peroxidation, damage to the signalling pathways, modifications in cellular communications and cellular cytoskeleton. Radical scavengers have been noted to block cellular damage by chromium, showing that oxygen radicals are an important key role in the toxicity of chromium (**Jomova & Volka 2011**).

1.2.3 Manganese:

Role in metabolism and toxicity: Manganese is absorbed by the gut by unsaturable simple diffusion via the mucosal layer, or through the high affinity, rapidly storable, low capacity active transport mechanism. Manganese (II) is noted to enter the gastrointestinal tract and bind to albumin or α2-macroglobulin in the plasma. Most manganese is secreted in bile through the liver (**ATSDR 2012**). Manganese is observed to accumulate in the mitochondria present in neurons, oligodendrocytes and astrocytes cells. That, in turn, disrupt ATP synthesis through the inhibition of the F_1/F_0 ATP synthase or the complex 1 (NADH dehydrogenase) in the electron transport chain. Manganese was shown to inhibit ATP synthesis in the brain mitochondria at the complex II (the succinate dehydrogenase) site affecting the mitochondrial function (**Gunter 2017**).Therefore, the disruption of ATP synthesis caused by manganese causes oxidative stress due to the decrease in the intracellular ATP levels together with the generation of free radicles. Such increase in oxidative stress may play a part in the cellular manganese toxicity. Animal studies have shown that the dopaminergic system can be disrupted due to the dopamine being oxidised by manganese and reacting to quinone species. The

dopamine transporter (DAT1) takes up the reactive dopamine species causing dopaminergic neurotoxicity (**Benedetto et al 2010**).

1.2.4 Cobalt:

Role in metabolism and toxicity: Solubility of cobalt plays a vital role in cobalt inhalation and absorption. The more the metal is soluble, the more it is absorbed Cobalt particles gets partially dissolved within the alveolar macrophages. Soluble forms of the metal gets mixed in the bloodstream through bronchial and alveolar walls. Cobalt oral absorption also depends on other factors like iron deficiency and fasting, which increases the absorption of cobalt. Iron and cobalt compete for the absorptive pathway in the intestine. Dermal absorption depends on the skin. If the skin is intact, absorption is less, while if the skin is broken, the absorption of cobalt is higher (**ATSDR 2004**). Cobalt is absorbed firstly by the liver and secondly by the kidney, and also in the lungs when inhaled (**Simonsen et al 2012**). Cobalt has been noted to have a high affinity to the sulphydryl group, thus causing inhibition of essential enzymes. Cobalt has been seen to interfere with the DNA repair process and can cause direct induction DNA damage, and sister-chromatid exchange. Carcinogenic effects of Co^{2+} was observed in animal studies. Formation of the cobalt-mediated free radical has been shown to contribute to toxicity and carcinogenicity caused by the cobalt (**Valko et al 2006**). The Co^{2+}-mediated Fenton reaction was reported to induce DNA cleavage at all the bases. During this reaction, experiments showed the presence of singlet oxygen in the presence of chelators. The site of cytotoxic ROS generation in hepatocytes due to Co^{2+} are the lysosomes (**Zeeshan et al 2017**). Metal-induced activation by cobalt causes hypoxia-inducible factor (HIF) in nearly all the cells, with the transcription of a variety of hypoxia responsive HIF-target genes which probably promotes tumour development and also growth (**Simonsen et al 2012**). During studies, DNA have been seen to be damaged and cell morphology changed due to the presence of $CoCl_2$. Levels of protein were altered, which is indicative of cellular response due to DNA damage

and hypoxia; HIF-1 alpha, p21, p53, and PCNA were modulated (**Zeeshan et al 2017**). The metal and the ions are classified as cytotoxic because these induce apoptosis. At higher concentrations, the metal causes necrosis, including inflammatory response. In vitro tests have shown that cobalt metals and its salts are genotoxic in mammalians, mainly due to the oxidative DNA damage (**Simonsen et al 2012**).

Although it is considered as carcinogenic in animals, according to the IARC, it has not been considered carcinogenic in humans. Cobalt is seen to cause cardiomyopathy and stimulate production of erythropoietin. Studies have shown that cobalt mimics hypoxia through a direct action in the kidney and liver, and on the erythropoietin producing cells, hypothetically by the metal-induced activation of the transcriptional activator HIF. Hyperoxia suppresses the erythropoietin induction effect of cobalt while hypoxia enhances the effect (**Ebert & Jelkmann 2014**).

1.2.5 Nickel:

Role in metabolism and toxicity: Nickel is absorbed through the gastrointestinal tract in the form of low molecular weight lipophilic compounds. Ions and various ligands present in the gut will affect the absorption of nickel in the gut. Studies in animals have shown that nickel is actively transported with facilitated diffusion when it is present in low amounts (**Briffa et al 2020**). In contrast, if nickel is present in high amounts, carriers become saturated, and thus nickel is absorbed through passive diffusion. Nickel binds to albumin together with ultra-filterable ligands, including amino acids and small polypeptides, and is transported in the blood. Nickel competes with copper at the albumin site (**Zhang &Wilcox 2002**). Nickel gets concentrated in the liver through calcium channels found in hepatocytes, as seen during in vitro studies in rats. Nickel carbonyl, is fat-soluble and can thus permeable through the cell membrane (**Duda-Chodak 2008**). Usually, it crosses through calcium channels and diffusion. Nickel storage can hinder calcium mediated cell signalling processes because when nickel

blocks the calcium channels, free calcium is released from the intracellular resulting in less calcium entering the intracellular space damaging the calcium mediated cell signalling processes (**Munaron 2006**).

The metal nickel has a wide extent of carcinogenic mechanisms which comprise of transcription factors, production of free radicals and controlled expression of particular genes. Nickel was noted to be involved in regulating the expression of certain long non-coding RNAs, particular mRNAs and microRNAs.

1.2.6 Copper:

Role in metabolism and toxicity: Copper is essential in functioning of many enzymes normally therefore, classified as an essential element. It can change states from Cu^{2+} to Cu^{3+} by cuproenzymes which are involved in redox reactions. This change in oxidation state may result in formation of superoxide, and hydroxyl radicals causing it to be toxic. A range of homeostatic mechanisms keeps a physiologically essential amount of copper in the limited range. Homeostasis of copper includes the control of absorption, intracellular transport, cellular uptake and efflux, sequestration/storage, and excretion of copper from the body. There is an inversely proportionate relation of copper absorption by the gastrointestinal tract to the dietary intake of the metal. Studies have shown that uptake is saturable, and uptake or efflux are influenced by intracellular amounts of copper (**ATSDR 2004**).

Copper ions contribute in the formation of Reactive oxygen species (ROS). The ROS which can contribute to the redox reactions includes Cu^{2+} (cupric) and Cu^{+} (cuprous). The cupric ROS can be reduced to the cuprous ROS if biological reductants in presence of ascorbic acid and glutathione. Cuprous ROS decomposes the hydrogen peroxide, through the Fenton reaction, which forms $OH^{\bullet}$, which can react with several biomolecules (**Liu et al 2018**). Copper was confirmed of being able to induce DNA strand breaks and cause bases to oxidise by oxygen free radicals and hydroxyl radical. Cupric and cuprous states of copper enhance DNA breakage

though the genotoxic benzene metabolite (1,2,4-benzenetriol), more than iron (**Gaetke & Chow 2003**).

1.2.7 Zinc:

Role in metabolism and toxicity: Zinc is the essential element for metabolic system as it is the active component of various enzymes e.g., alcohol dehydrogenase, alkaline phosphatase, DNA polymerases, RNA transcriptase, Zn-superoxide dismutase etc. In these metalloenzymes, zinc has various functions including catalytic functions, regulatory functions and may also be necessary for the structural stability or integrity of protein. Zinc is mainly carried in the plasma by albumin. Other carriers are amino acids and α2-macroglobulin. Zinc gets concentrated mostly at the liver and then distributed throughout the whole body. Major storage sites for zinc are the liver, bone, pancreas, kidney and the muscles (**ATSDR 2005**). If zinc concentration increases in the extracellular spaces, it increases the intracellular transport too, which in turn, triggers cytotoxicity and apoptosis. At even higher concentration, necrosis of cells occur (**Shuttleworth & Weiss 2011**).

1.2.8 Molybdenum:

Role in metabolism and toxicity: Studies in mice, have revealed that molybdate and sulphate have mutual competitive inhibition in the intestine for absorptive transport, which may be the Na^+ /SO_4^{2-} symporter, known as NaS1 or SLC13A1. NaS1 follows a different absorption transport because it is found in the kidneys instead of the intestine in humans. Molybdenum transporters are expressed in the membranes of bacteria and eukaryotes. MoT_2 is a molybdenum transporter which is also found in humans. Molybdate is transported into cells by MoT_2, and gets incorporated into molybdopterin-cofactor (Moco) which is the biologically prosthetic active group found in the molybdenum-dependent enzymes. This transport is inhibited by sulphate, therefore, indicating a common carrier for both the molybdate and the sulphate (**Tejada et al 2011**). Molybdenum toxicity mechanisms have not been established

properly. Data of molybdenum toxicity between animals and humans is limited. Rabbits were seen to be more sensitive than rats, though studies cannot be compared since there are differences in copper content together with other dietary constituents. Since there is no data to counter, molybdenum toxicity is assumed to be similar in all species, excluding ruminants. In copper-deficient diets, molybdenum induces alterations copper level in the liver, kidneys, and plasms. Such adverse effects gets reversed when high copper doses were administered. High doses of molybdenum caused anaemia and decrease in body weight as recorded in animal studies which are symptoms similar to copper-deficient animals. Copper concentrations were seen to increase in the liver and kidney on high molybdenum exposure (**ATSDR 2017**).

1.2.9 Arsenic:

Role in metabolism and toxicity: Arsenic can be orally taken, inhaled and to a smaller extent can be dermally absorbed also. In humans, the primary mode of entry for arsenic is by oral absorption. It occurs by more than 75% in the forms As^{3+}, As^{5+}, methylarsinic acid (MMA) and dimethylarsinic acid (DMA) (**Buchet et al 1981**). Humans and mice have been reported to absorb arsenic via passive diffusion. However, in mice, it the absorption takes place through a process by the saturable carrier-mediated cellular transport mechanism. Arsenic in soil has a lower bioavailability than the arsenic sodium salts. This is due to the sulphide salts of arsenic that are formed in the soil which are water-insoluble. Arsenic along with its metabolite forms, are distributed throughout the body, without having any preferential distribution (**Naranmandura et al 2006**).

Toxicity and carcinogenicity caused by arsenic have been noted to be associated with different metabolic processes. Pentavalent arsenic (As^{5+}) which is also called arsenate, in the blood is partially reduced, to trivalent arsenic (As^{3+}). As^{3+} is distributed to tissues and is absorbed mostly by the hepatocytes. As^{3+}, which is known as arsenite, is absorbed more than As^{5+}. Therefore, arsenite, is considered more toxic than arsenate. Since arsenite is more toxic

than arsenate, the reduction step can be observed to be a bioactivation instead of a detoxification reaction. Carcinogenic activity was noted in the skin, liver, urinary bladder, and lung, of mice in recent studies, that is similar to arsenic-induced cancer seen in humans. Therefore, it indicates that there must be some common mechanisms of action (**ATSDR 2007**). Cancer through arsenic poisoning is also seen in the prostate and Kupffer cells in humans. Carcinogenic reactions caused by arsenic include epigenetic alterations, generation of ROS and damage to the maintenance system of DNA (**Valko et al 2006**).

1.2.10 Selenium:

Role in metabolism and toxicity: Oral absorption and inhalation of selenium are extensive, however, the rate of absorption depends on the form of the metal. The bioavailability of selenium is usually independent of the levels of exposure, but it may increase in selenium-deficient individuals. Selenium is carried to all the tissues of the body, once it is absorbed through the intestinal tract. When in the blood, selenium forms complexes with protein immediately. Selenite has been reported to accumulate in the erythrocytes through an active transport mechanism. Selenium is found to concentrate in the liver and the kidney in humans. Selenomethionine is not synthesized in humans, though it can get incorporated into proteins instead of methionine (**Rayman et al 2008**).

The main biochemical adverse effect that selenium does have in human the oxidative stress. Reactions between the inorganic selenium forms and the sulphur containing thiol compounds in tissue occurs through redox catalysis, resulting in the formation of the superoxide anion reactive oxygen species. Selenium may form adducts with the proteins that contain regulatory cysteine which may lead to the inactivation of crucial thiol groups resulting in toxicity. Selenol is shown to cause cellular apoptosis while selenium-methylselenocysteine induces cell death in cancer cells by caspases activation (**ATSDR 2003**).

Selenium has protective roles also that works by inhibiting the carcinogen-induced covalent DNA adduct formation, inhibits oxidative damage to lipids, proteins and DNA. It also interferes in alterations of cellular and molecular events that are critical in the inhibition of cell growth and the multistep process of carcinogenesis. Selenium deficiency can affect the intracellular redox function, thus an increase in free radical concentration occurs in animals' deficient of selenium (**Yang et al 1983**). Thyroid hormone function also gets impaired by selenium, through the deiodinase enzymes. The metal is an essential component in iodothyronine 50-deiodinases where the prohormone thyroxine (T_4) is converted to triiodothyronine (T_3) which is the active form. (**Maia et al 2011**).

1.2.11 Cadmium:

Role in metabolism and toxicity: Cadmium salts like chloride, acetate, nitrate and sulphates, ate the most soluble forms, while the sulphide salts are insoluble in water. These sulphides, however, can react with the carbon dioxide in the lungs (**ATSDR 2012**). Cadmium has a high affinity for metallothionein and sulphydryl groups of albumin. Some of the adverse effects of cadmium has been associated with the promotion of apoptosis, oxidative stress, methylation of DNA, and DNA damage (**Valko et al 2005**). Kidneys, lungs and bone are the primary targets of cadmium toxicity. The metal is also known as a potent carcinogenic which affects the kidney, lung, pancreas, and prostate (**Azeh-Engwa et al 2019**). Immediate-early response genes (IEGs) are involved in cell proliferation and cell differentiation. Overexpression of IEGs are crucial for the mitotic growth signals that result in stimulation of cell division that may give rise to carcinogenesis. Cadmium has a stimulatory effect on expression of such IEGs. Cadmium-induced carcinogenicity is also seen by the induction of the expression of a variety of stress response genes like heat shock proteins (HSPs), genes involved in GSH synthesis. A variety of transcription factors, and genes regulating translation, are also affected by cadmium (**Tokumoto et al 2019**). Studies have shown that cadmium induces apoptosis in several organs.

Apoptosis induction in testes have been shown to be negatively correlated with p53, thereby showing that cadmium induces tumorigenesis. Cadmium primarily causes the deactivation of a vital DNA repair activity which is the main effect of its toxicity. Cadmium was noted to disable the mismatch repair (MMR) system. MMR is disordered through the mutations in the proteins MutS homolog (MSH), which causes considerable increase in the genome instability leading to the increase in the frequency of a variety of human cancer (**Jin et al 2003**).

Cadmium does not generate free radicals itself but it can do so indirectly. These include the superoxide radical, nitric oxide, ad hydroxyl radical. Studies have shown that the non-radical hydrogen peroxide may be produced, which can also be a source of radicals through Fenton reactions (**Liu et al 2009**). Cadmium chronic exposure through the oral or inhalation route causes harmful effects in kidney such as damage to the proximal tubule cell, proteinuria, aminoaciduria, glycosuria, polyuria, enzymuria, and a decrease in absorption of phosphate, as observed in laboratory animals. The clinical symptoms include proximal tubule degeneration and atrophy, including interstitial fibrosis in worst-case scenarios. The lipid composition was shown to be disturbed by cadmium, with a higher rate of lipid peroxidation. Antioxidant enzymes, especially the superoxide dismutase and the glutathione peroxidase, are depleted which produces cardiotoxic effects. Metabolism of zinc, copper, iron and selenium are altered by cadmium. These alterations in metabolisms are said to initiate the cadmium-induced toxicity in testes where cadmium interferes with the zinc-protein complexes controlling the DNA transcription and thus leading to apoptosis (**Evcimen et al 2020**). Glomerular membrane polyanions were also recorded to be depleted in presence of cadmium, resulting in an increase in the excretion of proteins with a high molecular weight. When the charge of the glomerular membrane polyanionic is disrupted by cadmium, tubular damage has also been reported along with it. Hepatic glycogen stores were reported to be reduced by cadmium which leads to an increase in the levels of glucose in the blood. Cadmium toxicity is also seen to cause intralobular fibrosis, focal mononuclear infiltrates, cirrhosis, and an explosion of the smooth

endoplasmic reticulum (**Briffa et al 2020**). Decreased bone mineral density is seen due to cadmium toxicity, which increases the chances of bone fractures. Osteoblastic activity is seen to be inhibited by the metal, which then results in bone organic matrix synthesis to decrease together with a decrease in mineralization. Osteoblastic activity reduction may cause an effect on the osteoclastic activity leading to an increase in bone resorption (**ATSDR 2012**). Renal damage caused by cadmium interferes with the 25-hydroxylvitamin D hydroxylation process to form 1,25-dihydroxy-vitamin D. When 1,25-dihydroxy-vitamin D is reduced in serum level, apart from the diminished kidney resorptive function, a deficiency in calcium and phosphate results. Deficiency of these elements causes a release in the parathyroid hormone, which enhances bone resorption (**Il'yasova, & Schwartz, 2005**)

1.2.12 Mercury:

Role in metabolism and toxicity: Metallic mercury is absorbed in the human body mostly through inhalation, followed by ingestion. Like other metals, mercury too has a great affinity for the sulphydryl group, and thus, binds to sulphur-containing amino acids present in the body (**ATSDR 1999**). Mercury in its metallic form passes effortlessly through the blood-brain barrier and placenta by adhering to the red blood cells. Some mercury is taken up by the central nervous system though most of it is oxidized rapidly to mercuric mercury. Metallic mercury is deposited to many tissues in the body including the thyroid, myocardium, breast, muscles, liver, adrenals, kidneys, skin, pancreas, sweat glands, salivary glands, lungs, enterocytes, prostate, testes, and breast milk (**Bernhoft 2012**). The metal also binds with a high affinity onto T cell surfaces and sulphydryl groups of enzymes which influence the T cell function. Mercuric mercury also bonds to the sulphydryl groups on metallothionein, erythrocytes, or glutathione, or it is suspended in plasma. Mercuric mercury is noted to accumulate in the placenta, amniotic fluid and foetal tissues. Ingestion of mercury through diet has been seen to cause an increased risk of heart complications such as myocardial infarction, and death caused by coronary heart

disease or cardiovascular disease. This enhanced risk is probably due to the lipid peroxidation caused by the mercury (**Patrick 2002**).

1.2.13 Lead:

Role in metabolism and toxicity: Lead is taken up by the human body independent of the route, and is distributed throughout the body. It is concentrated mostly in the bones. Pregnancy, menopause, lactation, and osteoporosis are conditions which can increase bone resorption, thus also increasing the lead in the blood. Lead can be found primarily in the red blood cells. Lead can get transferred from mother to foetus via breast feeding process (**ATSDR 2019**). Small particles of inorganic lead can be absorbed through the respiratory tract, while larger particles are removed by the mucociliary cells and transported to the oropharynx and may get swallowed. Absorption of lead by the gastrointestinal tract depends on several factors such as age, nutrition, and diet of the person, including the physiological characteristics of the metal present in the medium ingested. Absorption through the gastrointestinal tract is mostly done in the duodenum through saturable mechanisms. The dermal route is not that efficient when it comes to the absorption of inorganic lead (**Pattee &Pain 2003**).

Lead occurring in chronic exposures contributes to increased blood pressure. Critical hormonal and neuronal systems are affected by lead, which plays a role in the regulation of heart rate, peripheral vascular resistance, and cardiac output. Pb administration was seen to cause hypertension in rats accompanied with depleted nitric oxide, which is vital for the regulation of blood pressure through the peripheral and central mechanisms. Disruption of the NO vasodilatory actions is also caused by Pb through the alteration of the cell-signalling mechanisms present in the endothelial cells. Downregulation of the soluble guanylate cyclase expression is also caused by Pb exposure (**Vaziri 2008**). This enzyme produces cyclic GMP, which facilitates vasodilation caused by NO. Lead interferes with the calcium uptake in renal

cell mitochondria, because lead enters as a substrate for a calcium transporter in the mitochondria.

Oxidative metabolism impairment might contribute to cellular degeneration and transport deficits. Oxidative stress caused by Pb exposure, induces secondary responses to lead include the induction of NO synthase, transketolase, and glutathione S-transferase in the kidney (**Ahamed & Siddiqui 2007**). In vitro studies in rats show that lead depresses the glomerular filtration rate together with the renal blood flow. Lead has also been observed to affect the haematopoietic system in both animals and humans, where an increase in urinary porphyrins are seen. The activity of mitochondrial enzyme ferrochelatase containing zinc is decreased by lead in a non-competitive fashion. The decrease in activity catalyzes the introduction of Fe^{2+} into the protoporphyrin ring, which forms the heam. The binding of lead to the vicinyl sulphydryl group at the active site may inhibit ferrochelatase, which causes an accumulation of the protoporphyrin IX present in the erythrocytes that are circulating, as ZPP. ZPP accumulation is seen only in erythrocytes which are formed in the erythropoietic tissue, in the presence of lead. Haemoglobin concentrations are reduced in the blood, due to the intervention with the heam synthesis. A decrease in the production of haemoglobin, together with an increase in the destruction of erythrocytes leads to hypochromic, normocytic anaemia, linked with reticulocytosis (**Assi et al 2016**).

1.3 Conventional clean-up strategies

Decontamination of soil resource is of utmost importance to protect the human health and environment. The remediation thus, includes mainly, a long term reduction in bioavailability of the metals only if the same results in the reduced risk (**Wuana, & Okieimen 2011**). A range of remediation techniques are applied to soil based on ex-situ or in-situ methodologies. Ex-situ methodology of soil remediation is less expensive, quick, and easier to apply. However, it generates a major amount of waste product that stays to be treated before storing or releasing

it in the landfill sites. On the other hand, in-situ remediation methodology involves reduced land disturbance, provides a broad range of inorganic pollutants to be remediated, cost effective, and reduced risk of spreading contamination (**Liu et al 2018**). Various remediation techniques known for improving the quality of contaminated soil are studied under three categories based on their application:

1.3.1 Physical remediation: Physical processes that are used to remediate the contaminated soil include capping of sediments, washing and excavation.

- **Capping**: This technique is mainly applied in sub-aqueous conditions. Specific proportions of sandy material and apatite are usually stacked, which are placed on the contaminated sediment like a cap. This cap usually comprises of a, (i) stabilizing base layer which supports the added weight of cap; (ii) an isolation base layer, it isolates the contaminants from the sediment; (iii) a filter layer for hydraulic protection for the base layer; (iv) an armor layer, it inhibits erosion for the protection of filter and base layer. Capping can be performed in two ways, Passively (inactive) or Reactively (active). In case of passive method, the cap is composed of clean and neutral material which provides a physical barrier between the environment and contaminated sediment. However, a demerit for such passive methods is that it may cause leaks of toxic metals. However, the active method incorporates the cap with reactive material which can reduce the mobility, toxicity, and bioavailability of contaminants in sediments. This technique is not appropriate for shallow water or marshes or water bodies with large water flows as the capping material can be washed away (**Vandenbossche et al 2015**).

- **Washing of soil:** Sediment washing is an *ex-situ* method that is simple and effective. In this technique, the contaminated sediment is washed with a solution to transfer the pollutants from sediment to an aqueous solution. This is achieved by mixing the soil with an aqueous solution of alkalis, acids, and surfactants (**Wuana, & Okieimen 2011**).

Washing includes (i) excavation of highly contaminated sediment from the bulk soil; (ii) washing of sediment with the help of the aqueous solution; (iii) the solubilized, contaminants are removed from aqueous solution through various chemical processes. Common additives used are inorganic acids (sulfuric acid, nitric acid), organic acid (oxalic acid, ascorbic acids), and surfactants (sophorolipids and rhamnolipids). This technique is suitable for the contaminants which are weakly associated with sediments, and in coarse-grained sediments (**Peng et al 2018**).

- **Excavation of soil:** This technique involves physical removal of majorly contaminated soil from the bulk soil. This can be performed in several ways. The technique can be divided into three methodologies (i) substitution of polluted sediment by removing the soil and replacing it in another soil. This method is more suitable for small land areas being the target contaminated zones; (ii) the deep excavation of contaminated sediment for natural degradation of heavy metals; (iii) importing new soil and mixing with contaminated soil for dilution of heavy metals. This technique is expensive and is efficiently applicable only on land with small areas of contamination (**Dhaliwal et al 2020**).

1.3.2 Chemical remediation: This technique utilises chemical reagents for remediation. Other related techniques are also included in this method. Techniques like solidification, immobilization, vitrification and electrokinetics are some important techniques in this type of remediation.

- **Solidification:** Solidification is the physical encapsulation of contaminants in a solid medium. The encapsulation is formed by cement, bitumen, asphalt, fly ash and thermoplastic binders. During *In-situ* remediation, a binding agent is added to contaminated sediment which is followed by an auger spin mixing to transform the soil into a solid matrix (**Liu et al 2018**). The stabilization technique is also incorporated

where heavy metals undergoes chemical reactions which reduce their mobility in the environment. The entrapped toxic metals are not leachable as the solid block is impermeable to water. A mixture of various salts can be used for the solidification or stabilization of contaminants in soil *ex-situ* or *in-situ*. However, the process does not extract and remove the pollutant. So, over the long term, if the integrity of solid matrix deteriorates due to natural weathering or any uncontrolled physical disaster the contaminants which are trapped can again mobilize into the environment. Therefore, this methodology is applied as a last option for remediation of soil (**Dhaliwal et al 2020**).

- **Immobilization:** As the name suggests, this technique aims primarily to alter the bioavailable phases of metals into more geo-chemically stable phases, resulting in immobilization of chemicals. It is achieved through combined mechanisms of adsorption, complexation, and precipitation. The stabilizing effect of amendments is dependent upon the physical, chemical, and biological characteristics of sediment, heavy metal type, remediation time, remediation method, and evaluation method. The most common inorganic reagents used for immobilization are silico-calcium reagents, phosphates, iron-containing materials, aluminum salts, and mineral-based amendments. Organic reagents for immobilization of heavy metals include manure, biochar, biosolids, bark, wood chips, sawdust, sewage sludge, and turf. A complex formulation of inorganic and organic amendments can also be applied to the contaminated sediments for more efficient stabilization (**Lwin et al 2018**).

- **Vitrification:** This technique of remediation is again a type of stabilization/solidification technique. It involves high thermal energy in contaminated soil, at least 1400°C - 2000°C, for the removal of organic or volatile substances. It is achieved by mixing the contaminated sediments with glass-forming precursors, heating

the mixture till a liquid solution is formed. The steam produced by introducing high thermal energy and the products of pyrolysis are collected from exhaust gas (**Lwin et al 2018**). This reaction occurs successfully, and amorphous homogenous glass is obtained. The contaminants can be stabilized by two ways of interactions with solid glass matrix,that is chemical bonding and encapsulation. For *in-situ* remediation, electrodes can be inserted directly into the contaminated sediments. This technique is efficient but expensive and complex to perform (**Dhaliwal et al 2020**).

- **Electrokinetic method:** In this technique, the electric field is applied to the wet contaminated sediments for the movement of ionized metals towards the cathode or anode. The pollutants are migrated towards electrodes through electro-migration (charged chemical movements), electro-osmotic flow (fluid movements), electrophoresis (charged particle movements), and electrolysis (chemical reaction due to electric field) procedures (**Lwin et al 2018**). On the completion of the remediation process, the contaminant concentrated electrodes can be treated through several techniques for treating the heavy metals (**Peng et al 2018**).

Chapter 2

2.1 Phytoremediation

Plants interact with the environment through signalling compounds that code for their unique language. Chemical signals are widely distributed in plant and animal kingdom and is secreted from one organism and affect other organisms in the neighbourhood and play very important roles in sustainable ecosystems, such as dominance, succession and climax of plant community, biodiversity, crop productivity and pollution abatement and restoration of environment

The use of plants to reduce contaminants and restore the soil resource is a cost-effective method of reducing the risk to human and ecosystem health posed by contaminated soil sites. And this phenomenon is termed phytoremediation. (**Clemens, 2002; Suresh and Ravishankar, 2004; LeDuc and Terry, 2005; Odjegba and Fasidi, 2007; Turan and Esringu, 2007; Saier and Trevors, 2010; Sharma, 2015; Yadav et al 2018; Mahar et al 2016**)

"Phyto" means plant and "remedium" means restoring balance, defined as "the efficient use of plants to remove, detoxify or immobilize environmental contaminants in a growth matrix (soil, water or sediments) through the natural biological, chemical or physical activities and related processes of the plants". The concept of phytoremediation (as phytoextraction) was suggested by **Chaney (1983)**. Remediation techniques that are conventional are not applicable at very large field sites because of the high cost and lower feasibility (**Garbisu and Alkorta, 2003**). On the other hand, phytoremediation has low installation and maintenance costs compared to other remediation options (**Van Aken, 2009**). Regarding cost, phytoremediation can cost as less as 5% of alternative clean-up methods (**Prasad, 2003**). Additionally, the growth of vegetation on polluted soils also helps prevent erosion and metal leaching (**Yan et al 2020**). ''Phytoremediation can be defined as the use of plants and associated soil microbes to reduce

the quantity or toxic effects of contaminants in the environments'' (**Greipsson, 2011**). It can be used for removal of heavy metals and radionuclides as well as for organic pollutants (such as, polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and pesticides).

Plants perform phytoremediation in various ways that can be categorised as follows:

2.1.1 Phytosequestration: Plants can sequester the contaminants and reduce the mobility in the following ways:

- Plants can immobilize the contaminants in the rhizosphere by releasing the phytochemicals leading to precipitation and complexation the target contaminants in the root zone making them less bioavailable.
- Contaminants can irreversibly bind to the transport proteins that are associated with the exterior root membrane and stabilize contaminants on the root surfaces, preventing contaminants from entering the plant
- Plants also have a tendency to translocate and store the contaminants in compartments in the cells called vacuoles that are specialised for compartmentalisation of contaminants (**Shen et al 2021**).

2.1.2 Phytostabilisation: Plants can decrease the likelihood of contaminants especially heavy metals entering into the food chain by decreasing the bioavailability immobilising them belowground (**Wong, 2013; Marques et al., 2009**). Phytostabilization can occur in various ways i) through precipitation of heavy metals ii) reduction in metal valence in the rhizosphere, iii) absorption and sequestration within root tissues, iv) adsorption onto root cell walls (**Ginn et al., 2008; Kumpiene et al., 2012; Gerhardt et al., 2017**). Vegetation growth on contaminated soil not only stabilize the heavy metals in the rhizosphere and reduce their leaching to groundwater but also averts the dispersion of heavy metal containing soil particles by wind (**Vangronsveld et al., 2009; Mench et al.,**

2010). One of the main advantages of phytostabilization over phytoextraction is that disposal of hazardous biomass is not required (**Wuana and Okieimen, 2011**). However, the selection of appropriate plant species is crucial for the process. Plants that are highly tolerant to heavy metal concentration are found to be highly effective in phytostabilization. As plant roots play a central role to immobilize heavy metals, stabilize soil structure, and prevent soil erosion, plants should have dense rooting systems. Plants should be able to grow fast and produce a large amount of biomass so that it can efficiently establish a vegetation cover in the targeted site. Additionally, the vegetation growth should be easy to maintain with minimal care and investment (**Berti and Cunningham, 2000; Marques et al., 2009**). Plant species, which meet the above requirements, have been identified and used for phytostabilization of heavy metal-polluted soils (**Burges et al., 2018**). Phytostabilization efficiency can be improved by adding organic or inorganic amendments to the contaminated soil. These soil amendments can alter properties of the metal contaminants like metal speciation, reduce heavy metal solubility and bioavailability by changing pH value and redox status of the soil (**Alvarenga et al., 2009; Epelde et al., 2009; Burges et al., 2018**). In addition, application of such amendments can result in increase in the organic matter content and essential nutrients of the soil and improve physicochemical and biological properties. This can further benefit plant colonization and improve water-holding capacity of the soil.

2.1.3 Phytoextraction: Phytoextraction can be defined as the use of plants to take up contaminants from soil or water, and translocate and accumulate those contaminants in their aboveground tissues (**Salt et al., 1995; Jacob et al., 2018**). In recent times, phytoextraction is the most significant phytoremediation technique for removal of heavy metals and metalloids from the polluted soil (**Ali et al., 2013; Sarwar et al., 2017**).

Unlike phytostabilization, by which plants only contain heavy metals temporarily, but those heavy metals still remain belowground, phytoextraction is a permanent solution as it physically removes the heavy metals from polluted soil. Therefore, it is more suitable for commercial remediation approaches. The process of phytoextraction of heavy metals includes a few steps: (i) increasing the bioavailibility of heavy metals in rhizosphere, (ii) uptake of heavy metals by plant roots, (iii) translocation of heavy metal from roots to aerial parts of plant, (iv) sequestration and compartmentalization of heavy metal ions in plant tissues (**Ali et al., 2013**). The efficiency of phytoextraction depends on a several factors such as selection of plant species, efficiency of the plant, heavy metal bioavailability, soil, and rhizosphere properties. Appropriate selection of the plant species is key for effective phytoextraction.

The plant species for phytoextraction should possess the following characteristics: (i) resistance to heavy metal toxicity and high tolerance, (ii) high extraction ability with a tendency to accumulate high levels of heavy metals in aboveground parts, (iii) rapid growing with high biomass production, (iv) profuse shoots and extensive root system, (v) having better adaptive fitness with the contaminated environment, strong ability to grow in poor soils, easy and less labour intensive cultivation and harvest, (vi) must be resistant to pathogens and pests, be repulsive to herbivores preferably unpalatable for them to avoid heavy metals entering into the food chain (**Seth, 2012; Ali et al., 2013**). Among these characteristics, metal-accumulating capacities and aboveground biomass are the main factors that determine the phytoextraction potential of a plant species.

Therefore, based on that, two different strategies for plant selection are being employed: (i) using plant species that can hyperaccumulate a good amount of heavy metals in the aboveground tissues and (ii) or by using plants that may have lower metal-accumulating capacities but produce high shoot, thus, having the overall accumulation of

heavy metals comparable to that of hyperaccumulators (**Robinson et al., 1998; Salt et al., 1998; Ali et al., 2013**). Generally, hyperaccumulators are plant species capable of accumulating very high levels of heavy metals in their aboveground parts without phytotoxicity symptoms (**Rascio and Navari-Izzo, 2011; van der Ent et al., 2013**). The efficient heavy metal hyperaccumulator can accumulate metals at levels 100-fold greater than common non-hyperaccumulating species under the same conditions (**Rascio and Navari-Izzo, 2011**). In terms of the bioconcentration indices, the definition of hyperaccumulator should meet the following criteria: (1) the ratio of heavy metal concentration in shoot is to root is greater than 1, which is indicative to the ability to transport metals from roots to shoots (**McGrath and Zhao, 2003; Marques et al., 2009**); (2) the shoot-to-soil ratio of heavy metal concentration is greater than 1, indicating a higher competence to take up heavy metals from soil (**McGrath and Zhao, 2003**); and (3) the concentration of the metal in the shoot is higher than 10 mg/kg for Hg, 100 mg/kg for Cd and Se, 1,000 mg/kg for Co, Cu, Cr, Ni, and Pb, and 10,000 mg/kg for Zn and Mn (**Baker and Brooks, 1989**). A list of some plants, which show high capacity of heavy metal accumulation is given in **Table 2.1**.

However, one thing that must be considered before selecting a plant species for phytoextraction strategies is, using edible crops for phytoremediation should be avoided as heavy metals can bioaccumulate in comestible parts of the plant and thus enter into the food chain by human or animal consumption, raising issues on human health. Hence, selection of the non-edible hyperaccumulators must be a foremost criteria for efficient and safe phytoremediation of heavy metals. Another concern for selection of plant species is the life span of the species. If the life span of the selected species is long, then the growth is also slow, thus leading to limited efficiency of phytoextraction. Alternatively, high biomass producing non-hyperaccumulators can be used for

phytoextraction of heavy metals. Although they usually accumulate lower amounts of heavy metals in their aboveground tissues on a per mass basis, the higher biomass production can recompense for the lower phytoextraction efficiency, and the overall accumulation levels may end up being higher than that of hyperaccumulators (**Ebbs et al., 1997; Vangronsveld et al., 2009; Vamerali et al., 2010**).

2.1.4 Phytovolatilisation: This phenomenon is defined as the process when plants take up toxic pollutants from the soil, transform them into volatile, less toxic elements through their metabolic processes, and release them into the atmosphere via transpiration from their leaves and foliage. This method can be applied for decontamination of organic pollutants and some heavy metals like Se, Hg, and As (**Mahar et al., 2016**). Studies have reported that members of the Brassicaceae family are good volatilizers of Se (**Banuelos& Meek, 1990; Terry et al., 1992; Banuelos et al., 1993**). Inorganic Se is taken up by plntgs and is first assimilated into the organic selenoamino acids like selenocysteine (SeCys) and selenomethionine (SeMet). SeMet is biomethylated to form dimethylselenide (DMSe) which is the volatile form that is dispersed into air and have less toxicity than inorganic Se (**de Souza et al., 2000; Terry et al., 2000**). Another example is of mercury (Hg). Hg exists mainly as a divalent cation Hg^{2+} after release into the environment due to its high reactivity (**Marques et al., 2009**). Such compounds of Hg^{2+} forms, when taken up by plants, gets broken up and Hg^{2+} is converted to elemental Hg and released into air, which relatively much less toxic form of Hg (**Bizily et al., 2000**).

In case of phytovolatilisation, the remediation of the pollutants takes place at the contaminated site itself without any concern of the disposal of the plants, which is an added advantage of this strategy over all other phytoremediation strategies. But there is a limitation too, in this process, the contaminant is not removed completely, i.e., the metal

remains in the gaseous form in the atmosphere. Additionally, these can be again added to the soil due to rains and other precipitations (**Vangronsveld et al., 2009**).

2.1.5 Phytofiltration: As the name suggests, this method involves the use of plants to remove contaminants from the medium by the process of filtration. It can be of three types, (i) rhizofiltration; using the plant roots, (ii) caulofiltration; using the plant shoots and (iii) blastofiltration; using the plant seedlings (**Mesjasz-Przybyłowicz et al., 2004**). Root exudates also help in precipitating the contaminant heavy metals. Therefore, the removal happens either by absorption into the tissues or adsorption onto the plant parts. Ideally, plants used for rhizofiltration should have a dense root system, high biomass production, and be tolerant to heavy metal. Both terrestrial and aquatic plants can be used for rhizofiltration. Plants are grown in a hydroponic environment first. Plants are grown on nutrient media devoid of any contaminant to form dense root system. Then these are transferred to contaminated waters to filter out the heavy metals.

2.2 Role of root exudates in Phytoremediation

The heavy metals present in the soil are not always in an available form for effective bioaccumulation. Only a small fraction of the total heavy metal load in the polluted soil exist as soluble components in the soil and is ready for absorption by plants (**Blaylock and Huang, 2000**). Some heavy metals such as Zn and Cd are found in a more mobile and bioavailable form suitable to be taken up by plant than others (**Lasat, 1999**). According to the bioavailability of heavy metals/metalloids in the soil, heavy metals/metalloids can be classified as readily bioavailable heavy metals (Cd, Ni, Zn, As, Se, Cu), moderately bioavailable heavy metals (Co, Mn, Fe), and least bioavailable (Pb, Cr) (**Prasad, 2003**). Low bioavailability of certain heavy metals such as Pb seriously impedes the uptake of the metals from soil, thus reducing effective phytoextraction. The bioavailability of heavy metals in the soil is determined by their intrinsic

solubility and soil properties, as well as the binding of heavy metals to soil particles. Various soil physicochemical factors, such as the presence of chelating agents, the soil pH, and microbial activity, have been reported to show impacts on bioavailability and solubility of heavy metals in the soil (**Rieuwerts et al., 1998; Wang et al., 2006**).

A plant, itself, can employ various strategies to enhance heavy metal bioavailability. One of these being the secretion of root exudates. Root exudates acidify the rhizosphere by decreasing soil pH, which promotes desorption of heavy metals from insoluble complexes to form free ion, thus increasing the concentration of heavy metals in the soil (**Ma et al 2016**). Plants can also secrete metal-mobilizing compounds through their roots, such as phytosiderophores, carboxylates, and organic acids, which affect physicochemical properties of the soil and facilitate heavy metal chelation, thereby increasing solubility, mobility, and bioavailability of heavy metals in the soil (**Lone et al., 2008; Gerhardt et al., 2009; Robinson et al., 2009; Padmavathiamma and Li, 2012**).

Plants obtain various essential mineral nutrients from the soil through the rhizosphere, that can be explained as the interface of the root and soil. Plants secrete numerous metabolites from roots into the rhizosphere to manage nutrient bio-availability and cope with environmental metal stresses by undertaking measures like to changing the pH or to form metal–metabolite complexes (**Dakora & Philipis 2002, Badri & Vivanco 2009**). Such secretions or exudates contain metabolites which can be a complex mixture of inorganic ions (i.e., H^+, HCO^{3-}), gaseous molecules (i.e., CO_2, H_2) and mainly carbon-based compounds. The chemical composition of these exudates can be broadly divided into two groups: low-molecular-weight compounds including amino acids, organic acids, phenolics and sugar and high-molecular-weight compounds including mucilage and proteins (**Bais et al 2006**). Under low-nutrient conditions, plants release these metabolites to increase nutrient availability by directly binding to mineral nutrients or by changing the rhizosphere pH (**Jones 1998**). An

interesting observation was made regarding heavy-metal-polluted environments, that root exudation can be enhanced by non-essential metal stress to increase external detoxification (**Kochian et al 2004**).

2.3 Plants identified as Hyperaccumulators of heavy metals

A number of plants have been reported till date as hyperaccumulator of various heavy metals. Here is a detailed overview of the same as per the individual heavy metal.

Table 2.1: List of heavy metal hyperaccumulator plants along with the references

Heavy Metal	Plant Species	Concentration(mg/kg)	References
Vanadium	*Medicago sativa*	3440.14	**Yang et al 2011**
	Cynosurus cristatus	1208.3	**Ahemaiti et al 2017**
	Kochia scoparia	1454.7	**Ahemaiti et al 2017**
	Cicer aerietinum	3752.8	**Imitiaz et al 2018a**
	Brassica rapa	39.75	**Liao & Yang 2020**
	Glycine max	788.46	**Yang et al 2017**
	Chenopodium album	384.3	**Ahemaiti et al 2018**
	Brassica napus	73.3	**Gokul et al 2018**
	Artemisia vulgaris	89.4	**Qian et al 2014**
	Scirpoides holoschoenus	218	**Qian et al 2014**
	Reynoutria japonica	225	**Qian et al 2014**
	Betula populifolia	79.4	**Qian et al 2014**
Chromium	*Opuntia cochenilifera*	26000	**Adki et al 2013**
	Prosopis laevigata	8176	**Buendı´a-Gonza´lez et al. 2010**
	Cosmos bipinnatus	4825	**Santiago-Cruz et al. (2014)**
	Leersia hexandra	2978	**Zhang et al 2007**
	Salix matsudana	1794	**Yu et al 2008**
	Gynura pseudochina	1611	**Mongkhonsin et al 2011**
	Salix babylonia	1279	**Yu et al 2008**
	Salix matsudana	1235	**Yu & Gu 2007**

Heavy Metal	Plant Species	Concentration(mg/kg)	References
Manganese	*Antidesma*	46500	**van der Ent et al. 2019**
	Walsura	45200	**van der Ent et al. 2019**
	Casearia	38200	**van der Ent et al. 2019**
	Celosia argentea	32000	**Liu et al 2014**
	Aporosa	26700	**van der Ent et al. 2019**
	Chengiopanax sciadophylloides	23500	**Mizuno et al 2008**
	Baccaurea	21700	**van der Ent et al. 2019**
	Mischocarpus	19400	**van der Ent et al. 2019**
	Scolopia	16600	**van der Ent et al. 2019**
	Xylosma	15400	**van der Ent et al. 2019**
	Phytoalacca americana	13400	**Peng et 2008, Liu et al 2010**
Cobalt	*Haemaniastrum homblei*	4000	**Brooks 1977**
	Alyssum murale	2070	**Tappero et al 2007**
	Acalypha	1971	**Faucon 2007**
	Haemaniastrum robertii	1900	**Faucon et al 2007**
	Glochidion cf. sericeum	1310	**van der Ent et al. 2015**
	Alyssum corsicum	1080	**Malik et al 2000**
Nickel	*Phyllanthus balgooyi*	168500	**Mesjasz-Przybylowicz et al. (2016)**
	Dichapetalum	45600	**van der Ent et al. 2019**
	Antidesma	32700	**van der Ent et al. 2019**
	Rinorea	32200	**van der Ent et al. 2019**
	Planchonella oxyhedra	19600	**Brooks et al1977**
	Rinorea bengalensis	17350	**Wither & Brooks 1977**
	Actephila	14600	**van der Ent et al. 2019**
	Glochidion	11600	**van der Ent et al. 2019**
	Aporosa	10400	**van der Ent et al. 2019**
	Scolopia	9200	**van der Ent et al. 2019**
	Walsura	8600	**van der Ent et al. 2019**
	Baccaurea	8000	**van der Ent et al. 2019**
	Thalaspi caerulescens	6100	**Baker et al 1994**

Heavy Metal	Plant Species	Concentration(mg/kg)	References
	chidion aff acustylum	6060	**Reeves 2003**
	Homalium	5700	**van der Ent et al. 2019**
	Glochidion rubrum	5010	**van der Ent et al. 2015**
	Knema matanensis	5000	**van der Ent et al. 2015**
	Psychotoria grandis	3916	**McAlister et al 2015**
	Hydnocarpus	3200	**van der Ent et al. 2019**
Copper	*Aeollanthus biformifolius*	13700	**Hutchinson 1979**
	Crassula helmsii	9200	**Küpper et al. (2009)**
	Haumaniastrum robertii	6159	**Duvigneaud and Denaeyer-De Smet 1963**
	Coffea arabica	4186	**Lepp and Dickinson (1987)**
	Polypogon fugax	4012	**Ghaderian and Ravandi (2012)**
	Acalypha cupricola	2890	**Faucon et al 2007**
	Crepidorhopalon tenuis	2524	**Faucon et al 2007**
	Geniosporum tenuiflorum	2299	**Rajakaruna and Bohm (2002)**
	Clerodendrum infortunatum	2163	**Rajakaruna and Bohm (2002)**
	Ocimum tenuiflorum	2265	**Rajakaruna and Bohm (2002)**
	Croton bonplandianum	2163	**Rajakaruna and Bohm (2002)**
	Silene cobalticola	1600	**Duvigneaud and Denaeyer-De Smet (1963**
	Waltheria indica	1581	**Ghaderian and Ravandi (2012)**
	Epilobium hirsutum	1504	**Rajakaruna and Bohm (2002)**
	Commelina zigzag	1210	**Duvigneaud and Denaeyer-De Smet (1963**
	Ascolepsis metallorum		**Duvigneaud and Denaeyer-De Smet (1963**

Heavy Metal	Plant Species	Concentration(mg/kg)	References
Zinc	*Brassica juncea*	30550	**Singh & Fulekar 2012**
	Sedum alfredii	9000	**Yang et al 2004**
	Arabis gemmifera	6643	**Kubota and Takenaka (2003), Kashem et al. (2007)**
	Thlaspi caerulescens	6100	**Baker et al 1994**
	Thlaspi praecox	5960	**Likar et al. (2010), Vogel-Mikus et al. (2005)**
	Arabidopsis halleri	5722	**Küpper et al. (2010)**
	Potentilla griffithii	1670	**Qiu et al. (2006); Wang et al. (2009)**
Molybdenum	*Achilla tenuifolia*	1024	**Boojar et al 2011**
	Erodium circonium	725	**Boojar et al 2011**
	Heterocaryum szovitsianum	89.14	**Boojar et al 2011**
	Salsola incanescens	151	**Boojar et al 2011**
	Descuraina sophia	212	**Boojar et al 2011**
	Glysophila pilosa	72.18	**Boojar et al 2011**
	Glaucium elegans	94.5	**Boojar et al 2011**
Arsenic	*Pteris vittata*	4504	**Mandal et al 2018**
	Ptreris cretica	4875	**Eze & Harvey 2018**
	Pongamia pinnata	3662	**Iriel et al 2015b**
	Lemna valdiviana	1190	**deSouza et al 2019**
	Vallisneria gigantean	600	**Iriel et al 2015 a**
	Eichhornia crassipes	498	**deSouza et al 2018**
	Mimosa pudica	29.7	**Sampanpanish and Nanthavong 2019**
Selenium	*Astragalus racemosus*	14920	**White 2016**
	Stanleya pinnata	14900	**Schiavon et al 2015**
	Astragalus bisulcatus	13685	**White 2016**
	Oonopsis wardii	9120	**White 2016**
	Astragalus pattersonii	8512	**White 2016**
	Xylorhiza parryi	5390	**White 2016**
	Stanleya pinnata	4000	**White 2016**

Heavy Metal	Plant Species	Concentration(mg/kg)	References
Cadmium	*Brassica juncea*	25000	**Szczygłowska et al. (2011)**
	Phytoalacca americana	10780	**Peng et al. (2008), Liu et al. (2010)**
	Sedum alfredii	9000	**Yang et al. (2004), Deng et al. (2008)**
	Prosopis laevigata	8176	**Buendı´a-Gonza´lez et al. (2010)**
	Arabis gemmifera	6643	**Kubota and Takenaka (2003), Kashem et al. (2007)**
	Thalaspi caerulescens	6100	**Baker et al 1995**
	Thalaspi praecox	5960	**Liu et al. (2004), Likar et al. (2010), Vogel-Mikus et al. (2005)**
	Arabidopsis halleri	5722	**Kupper et al 2010**
	Viola boashanensis	4825	**Liu et al 2003**
	Salsola kali	2075	**de la Rosa et al. (2004)**
	Potentilla griffithii	1670	**Qiu et al. (2006), Wang et al. (2009)**
	Arabis paniculata	1662	**Qiu et al 2008**
Mercury	*Eichhornia crassipes*	83.2	**Skinner et al 2007**
	Marrubium vulgare	67.2	**Moreno-Jimmenez et al 2006**
	Brassica juncea	116	**Pedron et al 2013**
	Poa annua	236.39	**Pedron et al 2013**
	Helianthus annus	96.3	**Pedron et al 2013**
Lead	*Brassica juncea*	32700	**Singh & Fulekar 2012**
	Potamogeton axphyllus	4210	**Ha et al 2011**
	Arabis paniculata	1662	**Qui et al 2008**
	Ageratum houstonianum	1130	**Ha et al 2011**
	Pteris vittata	1020	**Ha et al 2011**
	Lagerstroemia floribunda	3376	**Meeinkuirt et al. 2012**
	Lolium perenne cv. 'Cadix	2000	**Karami et al 2011**

Heavy Metal	Plant Species	Concentration(mg/kg)	References
	Pelargonium capitatum	1467	**Arshad et al 2008**
	Picea abies	3000	**Grobelak et al 2017**
	Pinus silvestris	4500	**Grobelak et al 2017**
	Senecio sp	4253	**Bech et al 2012**
	Vetiveria zizanoides	934	**Schneider et al. 2016**

2.4 Research Gap Mapped with Problem Statement

Metal toxicity issues do not generally arise in the case of native flora, considering that native plants become adapted over time to the locally elevated metal levels. Native plants may be better phytoremediators for contaminated lands than the known metal hyperaccumulators because these are generally slow growing with shallow root systems and low biomass. Plants tolerant to toxic metals and low nutrient status with a high rate of growth and biomass are the ideal species to remediate degraded soils and habitats like those around mines. The native flora displayed its ability to withstand high concentrations of heavy metals in the soil. Some species also displayed variable accumulation patterns for metals at different soil concentrations. This variation was also observed in different parts of the same plant suggesting that full consideration of plant–soil interactions should be taken into account when choosing plant species for developing and utilizing methods such as phytoremediation. For successful phytoremediation of heavy metals, finding effective hyperaccumulators holds the key, and more than 450 plant species have currently been identified as potential metal hyperaccumulators (**Suman et al 2018**). Another factor that might be taken into consideration is that the selected plant is an undemanding crop plant i.e., the plant should not be an edible crop. Usage of edible crops for phytoremediation should be avoided as heavy metals can accumulate in edible parts of the plant and thus enter into the food chain by human or animal consumption, raising concerns on human health. Hence, selection of the non-edible hyperaccumulators is a key for efficient and safe phytoremediation of heavy metals. For the

same reason, the species must be highly resistant to pathogen and repulsive to herbivores. Such plants usually contain large amounts of glucosinolates. Glucosinolates are a class of plant secondary metabolites that provide defense against herbivores. Along with these roles, glucosinolates are observed to have a role in heavy metal tolerance. Exposition of *Arabidopsis thaliana* a member of brassicaceae to cadmium ions leads to the increased expression of the genes for glutathione synthetase. And they further confirmed the role of glucosinolates in cadmium detoxification (**Sun et al. 2003**). Glucosinolates and phytochellatins are the main compounds of Brassicales family of plants (**van den Bergh et al 2016**).

Hence, based on the mentioned criteria and following the clue of metabolic pathways for glucosinolates and phytochellatins, we came across the closest relative and the less explored family of Brassicaceae, i.e., Cleomaceae which fulfilled all the listed clauses and additionally was found to be a dominant species growing naturally in the local household waste dumpyards. Therefore we decided to explore the potentialities of the species due to which these are able to tolerate and proliferate in such contaminated areas.

2.5 Studies on *Cleome rutidopserma* DC done earlier

Cleome rutidosperma, commonly known as fringed spider flower or purple cleome, is a species of flowering plant in the genus Cleome of the family Cleomaceae, native to tropical Africa. Fringed spider flower is an erect, branched, annual herb, growing up to 15–100 cm tall (**Figure 2.1)**. The plant has angular stems and trifoliolate leaves on stalk. Each lea flet is somewhat diamond-shaped. The flowers are very small (about 15 mm across) with upward pointing purple petals and protruding stamens and pistil.

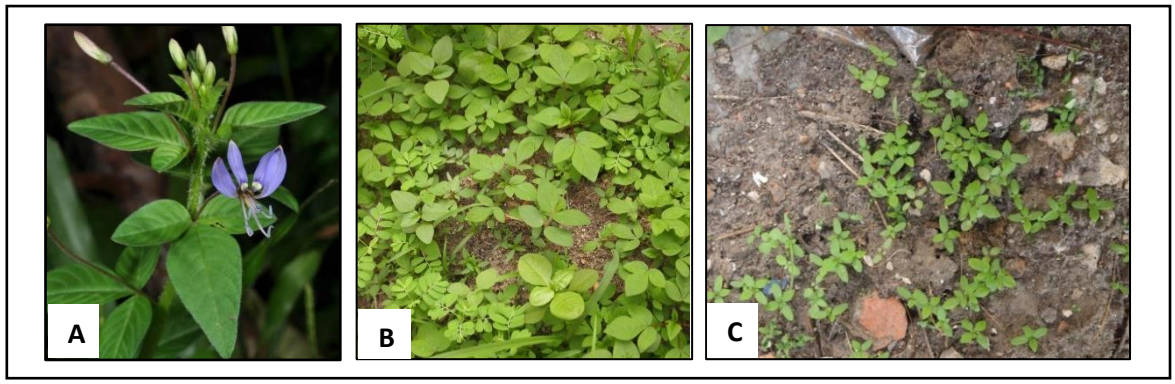


Figure 2.1: *Cleome rutidosperma* DC plant, (**A**) The flowering twig of the plant, (B & C) The habitat where the plant is growing naturally, i.e., contaminated dumpyards and household waste diposal site

In West Bengal, it is known as Nil Hurhure or Beguni Hurhure. *Cleome rutidosperma* belongs to the family of Cleomaceae and native to Tropical Africa. It has been introduced and now naturalized in different regions of Asia, Australia, America and West Indies.

Cleome rutidosperma is a weed in disturbed ground, roadsides, lawn, humid, ruderal and waste places, as well as in natural and semi-natural coastal forest (**Okonwu et al 2017**).

Taxonomy and Nomenclature:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Brassicales

Family: Cleomaceae

Genus: Cleome

Species: *Cleome rutidosperma*

Common Name: Fringed Spider Flower

Bengali Name: Nil Hurhure or Beguni Hurhure

Botanical Name: *Cleome rutidosperma* DC.

Family: Cleomaceae

Phytochemical studies of *Cleome rutidosperma* showed the presence of tannins, lipids, amino acids, flavonoids, cardiac glycosides, alkaloids, steroids, saponins, terpenoids, polyphenols, phlobatannins, pentose and reducing sugars. *Cleome rutidosperma* has been reported to have a wide array of medicinal properties.

- **Anti-pyretic and Anti-inflammatory: Bose et al in 2007** reported *C. rutidosperma* to have anti pyretic and anti-inflammatory effect. Oral administration of the ethanolic extract (200 and 400 mg/kg, p.o) and its fractions (200 mg/kg each) of the aerial parts of *Cleome rutidosperma* to Swiss albino mice produced significant analgesic activity in acetic acid-induced writhing and tail immersion tests, anti-inflammatory effect against carrageenin induced inflammation and adjuvant induced polyarthritis and antipyretic activity against yeast-induced pyrexia. Fractionation of the ethanolic extract potentiated the activities.
- **Analgesic and Locomotory Effect:** Oral administration of crude methanol, chloroform and petroleum ether extracts were also reported to have analgesic and locomotor activity in mice at a dose of 100mg/kg (**Bose et al 2004**).
- **Anti-convulsant Activity:** various extracts like ethanol, petroleum ether, diethyl ether, ethyl acetate and n-butanol extract of aerial parts of this plant. The anticonvulsant activity of above extracts was evaluated by using strychnine induced tonic convulsion in Swiss albino mice (**Jena et al 2009**).

- **Diuretic, Anti-microbial, & Laxative Property:** Crude aqueous extract of *Cleome rutidosperma* was investigated for diuretic and antibacterial activity. The diuretic activity was tested in rats at 400 and 600 mg/kg, orally and compared with furosemide (20 mg/kg, intraperitoneally) as the standard. The antibacterial activity was assessed by disc diffusion method against *Bacillus subtilis, Bacillus laterosporus, Staphylococcus aureus, Micrococcous luteus, Pseudomonas aeruginosa , Vibrio cholerae , Escherichia coli and Salmonella typhi* (**Bose et al 2007**).

- **Wound Healing Property:** The results of wound healing effects of *C. rutidosperma* showed significant promotion of woundhealing activity with both aqueous and methanol extracts in the excision and incision wound models. The methanol extract treated animals showed faster epithelialisation of wound than the animals treated with aqueous roots extract. There was a 100% wound closure for the standard drug nitrofurazone on 13th day of treatment, whereas the methanol extract demonstrated similar effects on 16th day (**Mondal & Suresh 2012**).

- **Anti-diabetic Activity: Okoro et al 2014** found the antidiabetic effects of *C.rutidosperma* root extracts by oral administration to mice. The dosage of administration was ranging from 125mg/kg to 500mg/kg for 28 days, and resulted in significant decrease in blood glucose levels of the mice.

- **Anti-arthritic effect:** The ethanolic extract of *Cleome rutidosperma* exhibited significant anti-arthritic activity. The doses of 200 mg/kg bw of the ethanolic extract of *Cleome rutidosperma*, in chronic model of granuloma pouch in rats produced 48.0% and in arthritis model produced 44.0 % inhibition respectively with that of the standard drug Prednisolone (5 mg/kg) which produced 58.5% and 59% inhibition (**Chakraborty et al 2010**).

- **Anti-plasmodial or Anti-malarial Activity: Bose et al., 2010** studied on the antiplasmodial activity of the *Cleome rutidosperma* on chloroform:methanol (1:1) extract of the leaves and showed significant anti-plasmodial activity in *in-vitro* mode against the strain of *Plasmodium falciparum* (**Bose et al 2010**).

- **Anti- neuro- inflammatory Activity:** Works done by **Ding et al in 2016** show that ethanol extracts of *C.rutidosperma* exhibit anti-neuroinflammatory activities by inhibiting pro-inflammatory mediator expression and production, upregulating HO-1, GCLM and NQO1, blocking NF-κB and modulating JNK signaling pathways. Therefore, it might have therapeutic potential for suppressing overactivated microglia and alleviating neurodegeneration.

- **Antinociceptive Activity:** Methanol extracts of *C.rutidosperma* demonstrated the significantly anti-nociceptive activity in the analgesic and anti-inflammatory tests by reducing nociception in mice models at the dosage concentration of 100 to 200mg/kg when administered orally. The remarkable increase in the latency was observed at 90 and 120 min (**Prawej et al 2016**).

- **Immunity Boosting and Cholesterol degrading activity:** Works done by **Arhogro et al 2014** with combined ethanolic extracts of *C. rutidosperma* and *Costus afer* show that the extract administered significantly decreased white blood cell count and neutrophils. Moreover, the extract significantly reduced triglycerides concentration in the serum when compared with controls. The results of this study suggest that the combined leaf extract could be used in boosting immune system and may have beneficial effect on serum cholesterol concentration.

- **Antidepressant activity:** Using methanol extracts of *C. rutidopserma* at a concentration of 100 and 200 mg/kg, it was seen that the extracts showed significant

($p< 0.01$) dose dependent suppression of motor activity in both open field and hole cross test, $4.67 \pm 0.68^{**}$ and $3.00 \pm 0.45^{**}$, respectively at 200 mg/kg**,** therefore, demonstrating that the extracts used, showed promising CNS depressant effect (**Archi et al 2016**).

- **Antioxidant and anti-cancer activity:** Due to prersence of high amounts of secondary metabolites like tannins, flavonoids, saponins, terpenoids and polyphenols, methanolic extracts of this plant showed high antioxidant activities against in-vitro assays like DPPH and ABTS. MTT assay against HepG2 cancer cells showed significant decrease in cell viability with an IC_{50} value of 50µg/ml (**Prabha et al 2017**).

Chapter 3

Objectives of the study

The main objectives for this work are listed as follows

1. **Hydroponic Screening experiments for selected heavy metals (Cd, Pb) on *Cleome. rutidosperma***
2. **Pot Culture experiments for quantifying the uptake and accumulation of heavy metals by the plant**
3. **Collection, purification and analysis of Root Exudates released in the immediate rhizosphere**
4. **Determination of Bioconcentration Factor and Translocation factor for the tested heavy metals**

3.1 Problem Statements mapped with Objectives

Table 3.1: The Problem statements mapped with the objectives

Problem Statement	Objective
1. Screening of *C. rutidosperma* to check whether the species can tolerate heavy metal stress without showing any signs of phytotoxicity	Hydroponic Screening experiments for selected heavy metals (Cd, Pb)
2. Validation and quantification of the heavy metal uptake using different heavy concentrations	Pot Culture experiments for quantifying the uptake and accumulation of heavy metals by the plant
3. Explaining the nature of remediation performed by the target plant species	Determination of Bioconcentration Factor and Translocation factor for tested heavy metals
4. Chemical characterisation of the signalling compounds that the plant releases in order to remediate the rhizospheric soil of contaminants.	Collection, purification and analysis of Root Exudates released in the immediate rhizosphere

Chapter 4

Hydroponic Screening Experiments

4.1 Introduction

Hydroponics is a widely and frequently used technique for growing plants without soil, providing for a considerable degree of control of the elemental environment surrounding the root (**Jones Jr, J. B. 1982**). It is the best method for growing plants when one needs to minimize the confounding factors interfering in the effects of the treatments administered to the plants. The earliest records of growing plants in water culture without any solid substrate dates back to 1699 in studies done by Woodward. But he failed to figure out the actual mechanism that made such cultures possible. In 1800s, researchers like De Saussure, Sachs, Boussingault and Knop, conducted experiments which helped to determine that certain elements were contributors to plant growth. Knop finally came with the most successful recipe that provided the basic idea of a nutrient solution that helped most of the plants grow in hydroponic culture systems. The main factors to be considered for such nutrient solution were; the osmotic pressure, balance of elements and no precipitate were to be formed of the salts added in the medium which would in turn hinder the availability of the particular nutrient (**Knop, W. 1865**).

There are numerous formulations for preparation of nutrient solution based on different sat combinations and Nitrogen sources. However, most widely used nutrient solution recipe is the formulation given by Hoagland and Arnon (**Hoagland, D. R. & D. I. Arnon. 1950**). Other characteristics of the nutrient solution are equally important, such as the pH, electrical conductivity, form of the elements (particularly for the elements N and Fe), and temperature. But these factors are strictly based on the plant species that is targeted to grow in the specific medium. Therefore, the formulation that provides optimum growth for a particular species of plant is considered to the best nutrient solution for the experiment.

4.2 Plant material and Experimental Design

Cleome rutidosperma DC are a predominant species in the local waste disposal sites. Therefore, plants were acquired from the locations around Kolkata (22.6494° N, 88.3805° E) throughout the months of July - October. The plant samples were collected at matured stage where the plants were bearing flowers and fruits. The samples were identified with the type specimen at Botanical Survey of India, Shibpur, Howrah, West Bengal, India. A voucher specimen (Cr02a) has been preserved in our departmental herbarium for future reference. Seeds of the identified plants were collected after proper ripening of fruits. Approximately 20 gm of seeds were collected from the plant samples. The seeds were first washed with 10ml of distilled water. Then the seeds were surface sterilised with 10 ml of 2.5% NaOCl for 1min (**Sidhu et al 2017**) followed by washing in distilled water upto 5 times to remove any residue of NaOCl. Seeds were dried and immediately stored in a cool dry place to be used further for all the experimental procedures (**Bhattacharya &Biswas 2022**)

The experimental setup included a glass beaker (1litre capacity), and a styrofoam floater with perforations (5mm dia) that could float freely in the beaker and 500ml of nutrient media per beaker (**Niu, et al., 2007**). Hydroponic experiments require plants that are at least 5-6cm in length because the plant needs to have a well differentiated shoot and root system to be able to be placed in the floaters so that the shoots remain above it while the roots remain immersed in the medium (**Table 4.1)**. The surface sterilized seeds were placed on moist filter papers to germinate for 10 days at room temperature. The individual seedlings were selected on the basis of their length and number of leaves i.e., 5-6cm in length and 4-5 leaves per seedling. Hoagland's nutrient media with minor modifications was used in the experiments. The heavy metal salts (reagent grade) used in this study included $CdCl_2 \cdot 2.5H_2O$, $Pb(NO_3)_2 \cdot H_2O$. The salts were separately diluted in deionized water and added into nutrient medium respectively. As this was a screening experiment, treatment were prepared at 10mg/kg concentrations for both

the metals. A control set was maintained containing the nutrient medium devoid of the metal salts. Another set that contained the metal salts but no plants were planted was also maintained to monitor any change in metal concentrations due to environmental factors. All solutions were adjusted to pH 7.0–7.2. The plant were allowed to grow in this hydroponic culture for 30 days in controlled greenhouse conditions. A total of 15 sets were prepared including the control (**Bhattacharya & Biswas 2022**).

Table 4.1: The nutrient media composition for the hydroponic experiments with *Cleome rutidoserpma* DC.

Micronutrient	Amount
KNO_3	6.0mM
$Ca(NO_3)_2$	4.0mM
$NH_4H_2PO_4$	0.1mM
$MgSO_4$	1.0mM
$CaCl_2$	25.0µM
H_3BO_3	12.5µM
$MnSO_4$	1.0 µM
$CuSO_4$	0.5 µM
H_2MoO_4	0.1 µM
$NiSO_4$	0.1 µM

4.3 Phytophysiological Parameters

Plants, being unable to physically move and avoid unfavourable conditions, have developed different mechanisms to cope the kind of stresses. Plants respond to external stimuli including heavy metal toxicity *via* several mechanisms. These include (i) sensing of external stress stimuli, (ii) signal transduction and transmission of a signal into the cell, and (iii) triggering appropriate measures to counter balance the negative effects of stress stimuli by modulating the physiological, biochemical, and molecular status of the cell.

As response to heavy metal stress, plant undergoes various physiological changes too. Plants growing on heavy metal-rich soils suffer from both decreased growth and yield (**Keunen**

et al., 2011), indicating an implication of heavy metal toxicity in hampering the overall growth performance of the stressed plants (**Kikui et al., 2005; Panda et al., 2009; Buendía-González et al., 2010; Gangwar et al., 2010, 2011; Gangwar and Singh, 2011; Eleftheriou et al., 2012; Hayat et al., 2012; Silva, 2012; Anjum et al., 2014**). Studies done by **Zobel et al in 2007,** suggest that heavy metals might cause an inhibition in root growth that alters water balance and nutrient absorption. This in turn, affect their transportation to the aboveground plant parts and thus negatively affecting shoot growth. Overall this might ultimately result in decreasing biomass accumulation. Heavy metal toxicity might also cause stunted stem and root length, and chlorosis in younger leaves that can extend to the older leaves after prolonged exposure (**Israr et al., 2006; Guo et al., 2008a,b; Warne et al., 2008; Gangwar and Singh, 2011; Gangwar et al., 2011; Srivastava et al., 2012**). Heavy metal stress also cause negative impacts on various biochemical processes in the plant. It has been shown that plants that have been given exposures to high concentrations of Cadmium, Manganese, Zinc and Lead, have shown decline in chlorophyll contents along with lower photosynthetic rates (**Dong et al 2005, Maleva et al 2012, Li et al 2012**) . Therefore, the parameters chosen to measure the extent of heavy metal toxicity in *C. rutidosperma* upon exposed to heavy metal treatments were; (A) Total Dry Biomass (g per pot) (B) Total Chlorophyll content (mg/g dry weight).

Total chlorophyll content was measured using the following equation (**Arnon 1949**).

$$\textbf{Chlorophyll (a + b) (mg/g tissue):} \frac{[\mathbf{20.21}\,(\boldsymbol{A645}) + \mathbf{8.02}(\boldsymbol{A663})]*\boldsymbol{V}}{\mathbf{1000}*\boldsymbol{W}}$$

A = Absorbance of specific wavelength; V = Final volume of Chlorophyll extract in 80% Acetone; W = Fresh weight of Tissue extract

4.4 Quantification of heavy metal uptake

Plants were harvested from the treatment sets after a period of 30 days. The plant tissues were then washed with deionised water followed by 0.01% EDTA solution and finally again with water three times to get rid of any heavy metal residue in them. The tissue samples were dried

in a hot air oven at 70^{o}C for two weeks. After proper drying, the plant samples were then weighed in the analytical balance (Winsor Corp.). Then the dried tissues were digested using the 'Tri-Acid mixture' (**Hseu 2004**). The tissues were placed in 25ml glass conical and Nitric acid, Perchloric acid and Sulphuric acid were added in the ratio 10:4:1 respectively (for approx. 500mg dry biomass). The acid solution was then evaporated on hot plate at 70^{o}C until the total acid solution had decreased to 2-3ml. Then the conicals were kept to cool at room temperature for an hour. Upon cooling, deionised water was added to the conicals for dissolution of the heavy metals. The residue was then filtered with Whatman 42 filter paper. The filtrate is collected in 50ml volumetric flask and the volume was made up with deionised water. On the contrary, the heavy metal concentration of the nutrient medium was recorded by digesting with aqua regia (HNO_3 67%: HCl 37% = 3:1) (**Hseu et al 2002**). After digestion, however, the remaining process is kept the same and 50ml of digestion product was collected. The heavy metal concentration was determined by Microwave plasma - atomic emission spectrometry (MP-AES). The detailed operating conditions are given in **Table 4.2**

Table 4.2: The operating conditions for the MPAES analysis for quantification of heavy metals from plant tissue as well as medium.

Instrument model	**4210 MPAES (Aegilant Technologies Software 1.16.10384**
Magnetron Voltage (V)	5
Magnetron Temp.(^{o}C)	23.1
Wavelength Temp. Front (^{o}C)	50.8
Wavelength Temp. Back (^{o}C)	48.3
Nitrogen generator pressure (psi)	140
Uptake time (s)	20
Rinse time(s)	20
Stabilization time	25 s/ sample delay
Pump speed (rpm)	15

Instrument model	4210 MPAES (Aegilant Technologies Software 1.16.10384	
Read time (s)	3	
Resolution pixel	4	
Replication	3	
Background correction	Auto	
Wavelength (nm)	**Cd**	**Pb**
	228.8	405.8
Viewing position	-10	-20
Nebulizer flow (L/min)	0.5	0.75

4.5 Results and Discussion

During evolution of angiosperms, only 19 elements such as C, O, H, Mg, S, N, Ca, P, and K (macronutrients) and Cu, Zn, Mn, Fe, Mo, B, Ni, Co, Cl, and B (micronutrients) were selected for basic metabolism (**Ernst, 2006**). Si is also considered as a beneficial element, as it has been reported to be involved in the maintenance of plant structures in some plants (**Epstein, 1999**). Macro and micronutrients play an important role in physiological and biochemical processes of plants such as chlorophyll biosynthesis, photosynthesis, DNA synthesis, protein modifications, redox reactions in the chloroplast and the mitochondrion, sugar metabolism, and nitrogen fixation. For example, Zn is a cofactor for more than 300 enzymes and 200 transcription factors associated with the maintenance of membrane integrity, auxin metabolism, and reproduction (**Marschner, 1995; Barker and Pilbeam, 2007; Briat et al., 2007; Williams and Pittman, 2010; Prasad, 2012; Ricachenevsky et al., 2013**). Although many heavy metals occur naturally in the earth's crust at various levels, the problem arises when they are released in excess into the environment due to natural and/or anthropogenic activities. The 53 elements belonging to the d-block have been categorized as “heavy metals” based on their density (>5 g/cm3) (**Jarup, 2003, Megrahi et al 2006**).

At higher concentrations, heavy metals produce severe toxicity symptoms in plants, and therefore, their uptake and utilization are closely controlled by the plant cells (**Janicka-Russak et al., 2008; Saito et al., 2010; Singh et al., 2012; Srivastava et al., 2012; DalCorso et al., 2013a; Farias et al., 2013; Fidalgo et al., 2013**). Some heavy metals, such as Cd, Cr, Pb, Al, Hg, etc., although being non-essential and do not have any physiological function, are very toxic even at very low concentrations (**Ernst et al., 2008; Janicka-Russak et al., 2008; Garzón et al., 2011; Hayat et al., 2012; Shahid et al., 2012; Chong-qing et al., 2013; Gill et al., 2013**). Heavy metals even if they are essential or non-essential, generally produce common toxic effects on plants, such as low biomass accumulation, chlorosis, inhibition of growth and photosynthesis, altered water balance and nutrient assimilation, and senescence, which ultimately leads to the death of the plant. Heavy metals employ toxicities in plants through four proposed mechanisms. These include (i) structural similarities with the nutrient cations, which causes these to compete for absorption at root surface; for example, As and Cd compete with P and Zn, respectively, for their absorption; (ii) heavy metals binds directly with sulfhydryl group (-SH) of functional proteins, which disrupts their structure and function, and thus, renders them inactive; (iii) displacement of essential cations from specific binding sites that lead to a collapse of function; and (iv) generation of reactive oxygen species (ROS), which consequently damages the macromolecules (**Sharma and Dietz, 2009; Dal Corso et al., 2013a**).

The screening experiment was performed to reconfirm whether *C. rutidosperma* could tolerate heavy metal stress without showing any signs of toxicity. Toxicity was measured in terms of total biomass per pot (g) and Total chlorophyll content (mg/g). After the period of 30 days, the plants were harvested and dried. The dry biomass was measured with all the individual plants pooled per replicate. While the total chlorophyll was measured as a mean of

chlorophyll content per individual plant in an experimental set. **Figure 4.1** shows the data for the parameters.

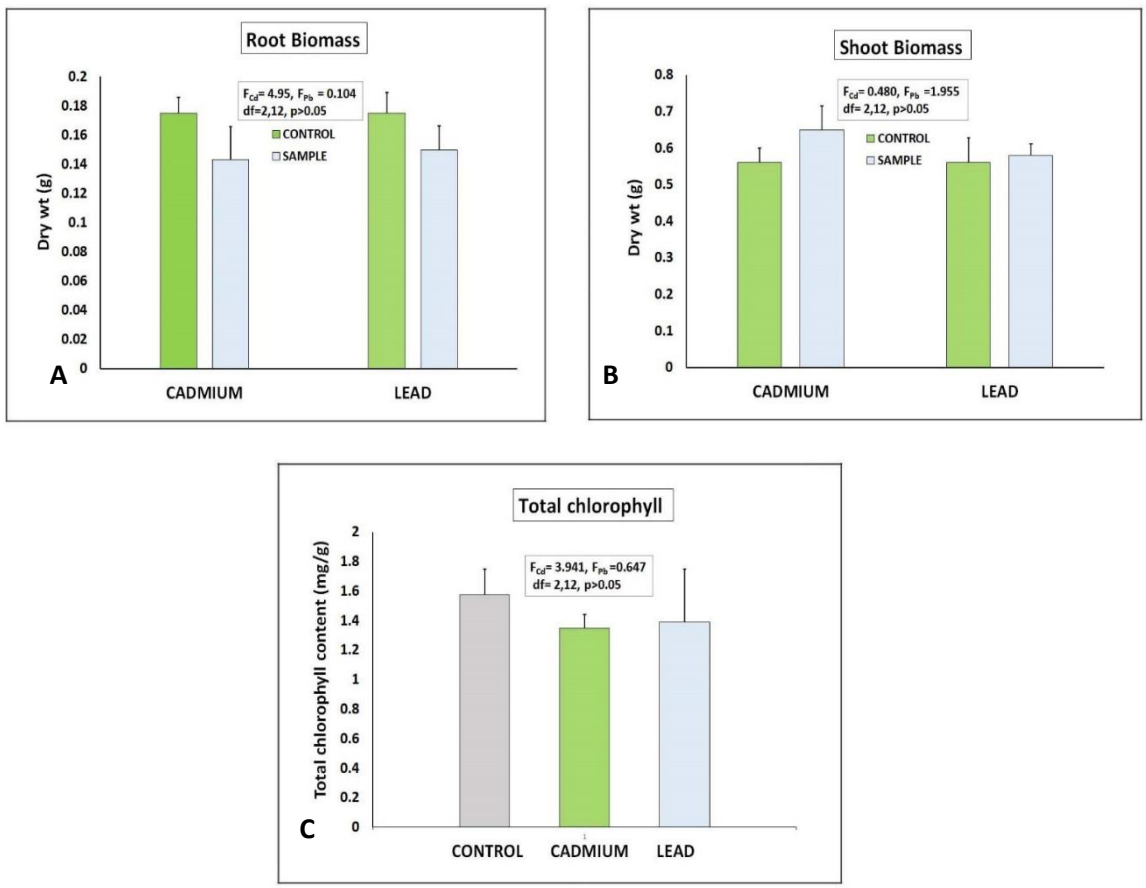


Figure. 4.1: Phytophysiological effects of heavy metal stress on *Cleome rutidosperma* DC based on hydroponic experiments; (**A**) & (**B**): Biomass of shoot and root respectively; (**C**): Total chlorophyll content. The results did not differ significantly w.r.t control (F values and 'Degrees of freedom (df)' are given in the respective graphs).

Hydroponic screening experiment revealed that there were no significant differences in the total biomass and total chlorophyll content compared to the control (**Figure.4.1**) which indicates that the plant can tolerate heavy metal stress without showing any significant signs of toxicity. But, the AAS analysis reveal that even at the low concentration of 10 mg/kg, *C. rutidosperma* could accumulate 42.49 mg/kg of Cd in shoots and 134.71 mg/kg Cd in roots. In

case of Pb, the plant could store 27.79 mg/kg in shoots and 491.35 mg/kg in its roots (**Figure 4.2**). These values were significantly higher than the control plants.

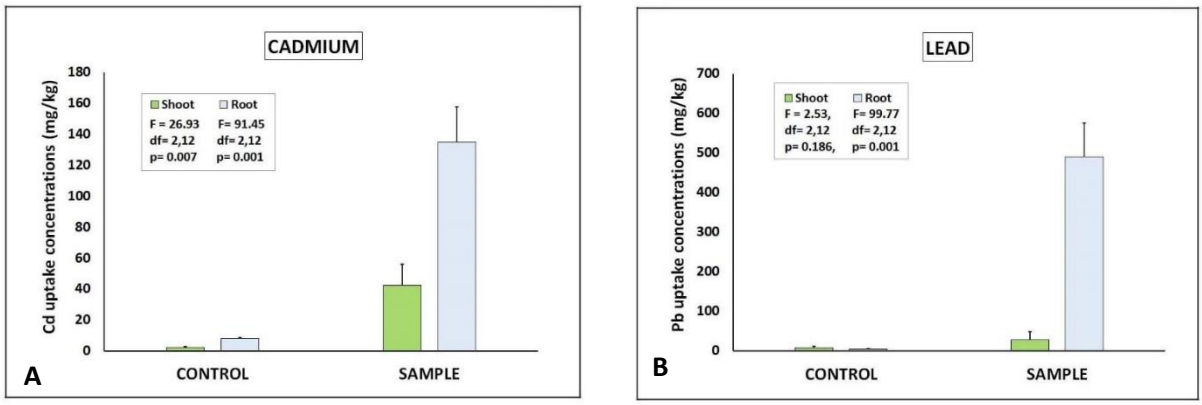


Figure 4.2: Heavy metal uptake by roots and shoots of *Cleome rutidosperma* recorded in hydroponic experiments at 10mg/kg tested concentration of; (**A**): Cadmium & (**B**): Lead. There was a significant difference in the metal concentrations in the sample w.r.t control as well as the roots w.r.t to the shoots (F values and 'df' are given in the respective graphs)

The studies, therefore, suggest that *C. rutidosperma* is a candidate plant that can be explored for its heavy metal accumulating abilities as the plants do not exhibit any significant signs of heavy metal toxicities. However, it is a matter of further evaluation and research to see whether this plant can tolerate higher metal concentrations. Another fact is to observe if the plant can only immobilize the metal pollutants or can translocate these metals to its aboveground tissues. These are the statements being addressed in the following chapters.

Chapter 5

Pot culture experiments

5.1 Introduction

Accumulation of heavy metals by plants also depend on several factors and mechanisms. Detailed study of these help in optimizing the potential of the plant to actively mediate a clean-up strategy. According to studies by **Sinha et al in 2009**, the plants which can grow and survive in naturally contaminated soils can be classified as two types;

(i) **Excluders**: That can survive on contaminated medium by avoiding the stress and restricting the uptake of contaminants into the plant tissues.

(ii) **Accumulators**: These plants can survive by taking up the contaminants, bioconcentrating and biodegrading them into inert forms inside the plant system.

In this study, therefore, we were concerned whether the species in question is an excluder or an accumulator. Hydroponic experiments only, did not suffice for determining the nature of *C. rutidosperma*. This was due to the fact that uptake by the accumulators depend on several factors like;

(a) **The Plant species**: The plant species needs to be screened for its potential to hyperaccumulate the heavy metals in its biomass. This potential depends on the mechanisms that are innate to the species. Therefore, the main factor for a successful phytoextraction strategy is a proper exploration and identification of a potential candidate species (**Prasad & Freitas 2003; Burken & Schnoor 1996**).

(b) **The bioavailability of metal**: The efficiency of plants to take up the heavy metals also depend on the characteristics of the soil. Heavy metal mostly exists as insoluble form in soil, which is not bioavailable to plants. Plants can increase their bioavailability by

releasing a variety of root exudates, which can change rhizosphere pH and increase heavy metal solubility (**Dalvi and Bhalerao, 2013**). The bioavailable metal is sorbed at the root surface and moves across the cellular membrane into the root cells. Some heavy metals such as Zn and Cd are more mobile and bioavailable for plant than others (**Lasat, 1999**). According to the bioavailability of heavy metals/metalloids in the soil, heavy metals/metalloids can be classified as readily bioavailable heavy metals (Cd, Ni, Zn, As, Se, Cu), moderately bioavailable heavy metals (Co, Mn, Fe), and least bioavailable (Pb, Cr) (**Prasad, 2003**). Various soil physicochemical factors, such as the presence of chelating agents, the soil pH, and microbial activity, have shown impacts on bioavailability and solubility of heavy metals in the soil (**Rieuwerts et al., 1998; Wang et al., 2006**). For example, the amount of lead absorbed by plants is affected by the pH, organic matter, and the phosphorus content of the soil. To reduce lead uptake by plants, the pH of the soil is adjusted with lime to a level of 6.5 to 7.0 (**Traunfeld and Clement 2001**).

(c) **Rhizospheric microbiota**: The microbial population thriving in the immediate rhizospheric environment also contribute largely in the extent of heavy metal uptake by the plant species. The microbial community of the rhizosphere may directly stimulate root proliferation and, thus, promote plant growth, increase heavy metal tolerance and plant fitness (**Gupta et al., 2013a; Fasani et al., 2018**). These microbes can also help in increasing the heavy metal availability by secreting enzymes that form metal-chelate complexes and increase the rate of uptake and translocation within the plant tissues (**Vamerali et al., 2010; Sheoran et al., 2011**). For example, PGPR and PGPE (plant growth-promoting endophytes) can increase solubility of water insoluble Zn, Ni, and Cu through the secretion of protons or organic anions (**Becerra-Castro et al., 2011**). PGPR also secrete biosurfactants and siderophores to mobilize heavy metals in the soil.

Siderophores are Fe chelators with strong affinity for ferric iron and variable affinity for other heavy metals, such as Cd, Ni, As, and Pb (**Schalk et al., 2011**).

Therefore, to ensure the potentiality of *C. rutidosperma* of heavy metal uptake we performed pot culture experiments with artificial spiking of heavy metal in different concentrations and monitored the toxic effects of the stress along with quantifying the uptake in the plant biomass.

5.2 Experimental design

In order to perform the pot experiments with soil, we referred the protocol detailed by S**idhu et al 2018**. The completely randomized design of experiment was put to work. The natural garden soil was tested and all the quality parameters were quantified. The natural soil was spiked with heavy metals salts namely $CdCl_2 \cdot 2.5H_2O$ and $Pb(NO_3)_2 \cdot H_2O$. Three replicates were set up for each of the 10 different concentration levels of the metal treatments including control. Altogether 30 individual pots were maintained in the study. There were nine different Pb and Cd concentrations, equivalent to 10, 20, 30, 40, 50, 75, 100, 150 and 200 mg/kg soil respectively. The spiked soils (1kg each) were filled in polythene bags and placed in plastic pots to avoid metal leaching from soils. Altogether the plants were maintained for 60 days under the same conditions before the harvest. Triplicates of the control plants of all species were maintained in soil devoid of metal salts. The moisture content was maintained at 60% throughout the experimental period using distilled water. After spiking prior to seedling plantation, the soils were equilibrated for 7days.

5.3 Soil parameters

The soil parameters that were tested before and after the experiment along with the methods for quantification are given below.

(a) **Soil pH**: The soil pH is an important criteria that needs to be measured beforehand to setting up an experiment. It is the quantification of the hydrogen ions in the soil-water medium. pH is defined as "the negative logarithm of concentration of H^+ ions" and the mathematical formula is

$$\mathbf{pH = -log\ a\ H^+}$$

The pH of the soil gives an idea of the nutrient availability. The value of the pH indicates the soil being acidic (pH<7), neutral (pH=7) or alkaline (pH>7) nature of the soil. This also dictates the nutrient availability being maximum at the neutral pH and decreases with increasing acidity or alkalinity of the soil. One of the important factor that impacts pH is the temperature. Therefore, for accurate measurements the pH measurements were carried out at room temperature i.e, 28^{o}C. pH meter with a hydrogen ion indicating glass electrode was used (Fischer Scientific). The machine was calibrated beforehand using standard buffer solutions (Himedia) of pH 4.0, 7.0 and 9.2.

The measurement of soil pH was done according to **Schofield & Taylor (1955)**. 20g of air dried soil was ground to fine powder and passed through 2mm mesh. The powder was then mixed with 40 ml of distilled water and stirred for 30 mins. The electrode was then immersed in the soil suspension the pH was recorded.

(b) **Electrical conductivity**: The measurement of total soluble salts present in the medium is termed as electrical conductivity. It is measured by the conductivity meter (Fischer Scientific) and is expressed as deciSiemens per meter (dS/m). The procedure for this

was same as the pH measurement. It indicates the salinity level of the soil (**Smith & Doran 1997**).

(c) **Organic carbon:** The organic carbon content is measured in percentage. The extent of organic nutrients present in the soil can be measured by quantifying the organic carbon (**Nelson & Sommers 1996**). The procedure involves digesting 1g of dried powdered soil in 10 ml of chromic acid (1N) and 20 ml of Sulphuric acid (1N). The acid digests the soil and oxidises the organic matter in it. The excess chromic acid left unreduced is then quantified by titration with Ferrous ammonium sulphate solution (0.5N) using diphenylamine indicator in presence of 10ml of 85%Phosphoric acid and 2% Sodium fluoride each. The quantification was done using the formula

$$\textbf{Organic Carbon (\%)} = \frac{10}{Blank}(Blank - Reading)\frac{0.003\times100}{wt.of\ the\ soil(g)}$$

(d) **Available Nitrogen:** Nitrogen is an essential element for plants. They acquire that from the soil mainly. Nitrogen is present in the soil in different forms like organic nitrogen or (NH_4^+), - N, (NO_3^-) - N or (NO_2^-) - N or their combinations. Hence, quantification of the different forms of nitrogen also differs. Here we estimated the total available nitrogen in the soil using the alkaline potassium permanganate method by using the method given by **Amule (2014)** with minor modifications. The soil samples were taken, air-dried, ground and passed through a 2mm stainless steel sieve.

The easily oxidisable and hydrolysable organic nitrogen can be extracted by mild oxidising agent like alkaline $KMnO_4$. A known quantity of soil is mixed with a known amount of alkaline $KMnO_4$ solution and distilled. The organic matter of the soil gets oxidised by the nascent oxygen released from the alkaline permanganate. The reaction releases ammonia which is absorbed by known volumes of boric acid in presence of a mixed indicator i.e., methyl red and bromocresol green. With absorption of ammonia,

the colour of the indicator solution turns green from pink. The greenish product is then titrated with 0.01N sulphuric acid to the point where the solution turns back to the original pinkish stage. The calculation was performed as the following.

Weight of soil taken = 20 g

Volume of 0.01 M H_2SO_4 = 25 cm^3

Volume of 0.02 M NaOH required for back titration = X cm^3

Volume of 0.01 M H_2SO_4 used by NH_3 evolved = (25 − X) cm^3

1 cm^3 of 0.01 M H_2SO_4 = 0.00028 g of nitrogen

Amount of nitrogen in 20 g of soil = (25 −X) ×0.00028 g = Z g

% available nitrogen (per 100 g of soil) = Z × 100/20 % = Z' %

Available nitrogen in ppm = Z' × 10,000

Available nitrogen in Kg/ha = Z' × 22400

(e) **Available Phosphorus**: Phosphorus is an essential nutrient for plants and it is abundantly present in various forms in the soil both in organic and inorganic forms. Organic phosphorus however, is not readily available as the extraction of the same is difficult. Inorganic phosphorus remains mainly as phosphates of Aluminium (Al), Calcium (Ca) and Iron (Fe). The Ca-P is predominant in neutral and alkaline soils while the other two forms Al-P and Fe-P are mostly present in acidic soils. Plants can take up the phosphates as orthophosphates and therefore, only a fraction of total phosphates is actually available to the plants. The amount of phosphorus that is actually available to the plants to take up from the soil is termed as Available phosphorus.

In our study we used the method of **Olsen and Sommers (1982)**. This method is mainly used to determine available phosphorus in neutral to alkaline soils. The

extracting agent contains dilute solution of Sodium bicarbonate (pH 8.5). The liberated phosphorous (present as orthophosphates) in solution is treated with ammonium molybdate under acidic conditions. It forms a complex called ammonium phosphomolybdate. The complex is then treated with a reducing agent like, stannous chloride or ascorbic acid to obtain intense blue colour compound called molybdenum blue. The intensity of colour of this complex is proportional to the concentration of phosphate and can be read with the help of a photoelectric colorimeter at a wavelength of 660 nm or 880 nm depending on the reducing agent used. The standard curve is prepared with known concentrations of phosphorus. Only limitation for this experiment is dark coloration that is imparted in the extract due to the organic matter that gets solubilised too. Activated charcoal is used to remove this colouration and make the extract colourless.

About 2.5g of ground soil is placed in a conical containing 50ml of $NaHCO_3$ for extraction. A pinch of activated charcoal is added to the solution remove any colouration. The solution is placed on a shaker for 30 mins and filtered with a Whatman 40. The Olsen reagent is prepared by mixing the solutions of ammonium molybdate and antimony potassium tartrate in 5N H_2SO_4 and ascorbic acid. 5ml aliquots of the extract filtrate was taken and acidified using 5N H_2SO_4 till pH lowers to 5. The volume is made upto 10ml with distilled water and 4ml of Olsen reagent is added. The final volume is made upto 25ml with distilled water. The blue coloration is then recorded at 660nm in UV-Vis spectrophotometer (Fischer Scientific).

(f) **Available Potassium**: Potassium is also an essential nutrient for the plants. The two forms of potassium i.e., exchangeable and soluble are measured by extracting with ammonium acetate (pH 7). The estimation of available potassium is performed using a flame photometer. The working principle here is that few elements emit radiation of

fixed wavelengths when excited. The Potassium emits radiation at 404.4 and 767 mμ. The flame photometer uses a red filter and a relatively low temperature (**Black 1965**).

Ammonium acetate is prepared by adding 70ml of concentrated ammonia to a solution of 58ml of glacial acetic acid in 600ml of distilled water. The pH is adjusted with either acetic acid or ammonia. A stock solution of 100 mg/kg KCl solution was prepared. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of this KCl solution was taken in 25ml volumetric flask. The volume was made up with ammonium acetate solution. The flame photometer is set to zero with the blank solution (without KCl) and to 100 at 40mg/kg. This gives the value of Factor (F) for 1 flame photometer reading = 0.4 mg/kg K.

5g of soil is sieved and extracted with 25ml of neutral ammonium acetate solution. The extraction is done by continuously shaking the mixture for 10 minutes. The extract is then filtered through Whatmann no. 1 filter paper and the potassium content in the filtrate is measured using the calibrated flame photometer. The calculation for potassium is given below.

$$\textbf{Available Potassium (kg/ha)} = \frac{R \times F \times 25 \times 100 \times 20 \times 1.121}{5 \times 1000}$$

5.4 Phytophysiological Parameters

Stress is an altered physiological response of living organisms caused by physical, chemical or biotic environmental factors that tend to shift their equilibrium away from its optimal thermodynamic state (**Gaspar et al. 2002; Strasser 1988**). Soil or water pollution, climate change or other anthropogenic effects can cause severe abiotic or biotic stress for both cultivated plants and natural vegetation. Understanding the effective plant resilience and adaptation to heterogeneous and changing environmental conditions is, therefore, in the forefront of agricultural, ecological or conservation research. An appropriate experimental design with a selection of the most sensitive parameters to be measured is a prerequisite for an

effective and time efficient study, although the right choice of these parameters is not always obvious. Regarding their relevance, applicability and the adequate number of parameters to be used, there are numerous examples of experimental approaches. Several groups of quantitative or qualitative parameters exist which have been applied to characterize plant development and growth, physiological status, symbiotic interactions, stress symptoms, photosysnthesis, etc. during or at the end of an experimental growth period (**Berger and Ludwig 2014; Grümberg et al. 2015; He and Dijkstra 2014; Kalaji et al. 2016; Latef and Chaoxing 2014; Munns 2002; Roger 2001; Salvatori et al. 2014; Talaat et al. 2015; Wehner et al. 2015; Zhang et al. 2015**). The simplest and most obvious parameters are: fresh and dry weight, root and shoot biomass production, root to shoot ratio, leaf area, grain yield, reproductive index. . The functioning of the photosynthetic apparatus can respond sensitively to environmental disturbances.chlorophyll content of plant leaves has been indicated as a good stress indicator (**Li et al. 2006; Mehta et al. 2010; Ueda et al. 2003; Chaves et al. 2009**). However, a stress-induced decrease of biomass may, therefore, mitigate the parallel decline of chlorophyll content. Therefore, the parameters chosen to determine the phytophysiological effects of the heavy metal stress namely, dry biomass and total chlorophyll content. Functional parameters of the photosynthetic apparatus: chlorophyll content was determined according to **Arnon 1949**. Total chlorophyll content was measured using the following equation.

$$\textbf{Chlorophyll (a + b) (mg/g tissue):}\ \frac{[20.21\ (A645) + 8.02(A663)] * V}{1000 * W}$$

A = Absorbance of specific wavelength; V = Final volume of Chlorophyll extract in 80% Acetone; W = Fresh weight of Tissue extract

5.5 Quantification of heavy metal uptake

The heavy metal concentration of the plant tissue as well as the soil was analysed using the MPAES. Plants were harvested after 60 days and separated into root and shoot. The plant parts

were washed repeatedly with distilled water as well as 0.01% EDTA solution to get rid of any heavy metal residue in them. The plant tissues were then oven-dried at 80^{o}C for 72 hours. The dried plant parts were then weighed, grounded and digested using the tri-acid mixture (**Hseu 2004**).

Table 5.1: The operating conditions for the MPAES analysis for quantification of heavy metals from plant tissue as well as medium.

Instrument model	**4210 MPAES (Aegilant Technologies Software 1.16.10384**	
Magnetron Voltage (V)	5	
Magnetron Temp.(^{o}C)	23.1	
Wavelength Temp. Front (^{o}C)	50.8	
Wavelength Temp. Back (^{o}C)	48.3	
Nitrogen generator pressure (psi)	140	
Uptake time (s)	20	
Rinse time(s)	20	
Stabilization time	25 s/ sample delay	
Pump speed (rpm)	15	
Read time (s)	3	
Resolution pixel	4	
Replication	3	
Background correction	Auto	
	Cd	**Pb**
Wavelength (nm)	228.8	405.8
Viewing position	-10	-20
Nebulizer flow (L/min)	0.5	0.75

5.6 Results and Discussion

5.6.1 Soil Parameters

The physicochemical characteristics of the soil pot mixture that was used in the experiment was recorded before the experiment set up. It was recorded as, pH 7.45± 0.05, organic carbon 0.83 ± 0.17 (%), phosphorous 11.3 ± 0.54 mg/kg, potassium 93.2 ± 1.21 mg/kg, available nitrogen 112 ± 1.93 mg/kg, Cd content of 3.875 ± 0.125 mg/kg and lead content of 3.75 ± 0.5

mg/kg. Physicochemical analyses of the soil (pot mixture) including pH, organic carbon, available NPK were carried out along with the Cd and Pb content after the period of 60 days incubation again to quantify any kind of changes that might have resulted during the experimental duration. The detailed quantities of the parameters are given in **Table 5.2 and 5.3**

Table 5.2: Soil parameters recorded after the treatment period of 60 days for Cadmium metal

Treatments (mg/ml) Cd	**pH**	**Organic Carbon (%)**	**Available Phosphorus (ppm)**	**Available Potassium (ppm)**	**Available Nitrogen (ppm)**
0	7.31	0.82	11.03	112.82	93.2
10	7.32	0.73	9.76	110.02	92.1
20	7.21	0.89	10.54	96.77	89.56
30	7.3	0.92	9.2	95.3	86.5
40	7.38	0.83	11.01	98	93.8
50	7.28	0.81	8.02	99.2	83.72
75	7.18	0.79	8.1	110.4	81.4
100	7.3	0.81	8.56	103.98	84.74
150	7.4	0.85	9.9	97.7	94
200	7.27	0.82	9.34	94.03	88.34
F(8,18)	0.535	0.391	0.350	1.022	0.139
p-value	0.747	0.846	0.843	0.447	0.980

Table 5.3: Soil parameters recorded after the treatment period of 60 days for Lead metal

Treatments (mg/ml) Pb	**pH**	**Organic carbon (%)**	**Available Phosphorus (ppm)**	**Available Potassium (ppm)**	**Available Nitrogen (ppm)**
0	7.27	0.71	9.348	104.32	94.37
10	7.2	0.82	8.56	113.02	93.55
20	7.19	0.88	10.86	98.66	85.67
30	7.23	0.8	8.22	92	89.32
40	7.31	0.88	7.65	89.1	77.98
50	7.32	0.82	9.23	99.54	81.54
75	7.21	0.89	8.03	100.4	88.43
100	7.28	0.81	9.34	83.8	73.21
150	7.3	0.83	8.3	91.88	82
F(8,18)	2.77	0.11	0.34	0.902	0.26
p-value	0.063	0.998	0.936	0.535	0.97

5.6.2 Phytophysiological effects of heavy metal toxicity on *C. rutidosperma*

Plant biomass and growth was significantly impacted with increasing heavy metal stress (**Figure 5.1**). In case of Cd, the dry wt of both root and shoot increased significantly at Cd treatment concentrations of 40mg/kg and 20-30 mg/kg respectively. The exact reason for such promotion in growth cannot be explained. However, such a response may be attributed to the phenomenon called hormesis in which a stimulatory effect in growth is noticed under the physiological toxic doses of heavy metal ions (**Poschenrieder et al., 2013, Tang et al., 2009**).

The Cadmium stress, however, did not show any significant effect on the total chlorophyll content of *C. rutidosperma* (**Figure 5.2**). The difference was not significant with respect to the control sets. On the contrary, the total chlorophyll content of *C. rutidosperma* exposed to lead stress did decrease significantly compared to the control in a dose dependant manner. ($y = -0.005x + 1.2256$; $R^2 = 0.957$) The plants treated with 200mg/kg Pb, in our studies, failed to survive. Therefore, we have the data of plants treated with 10 to 150mg/kg of Pb.

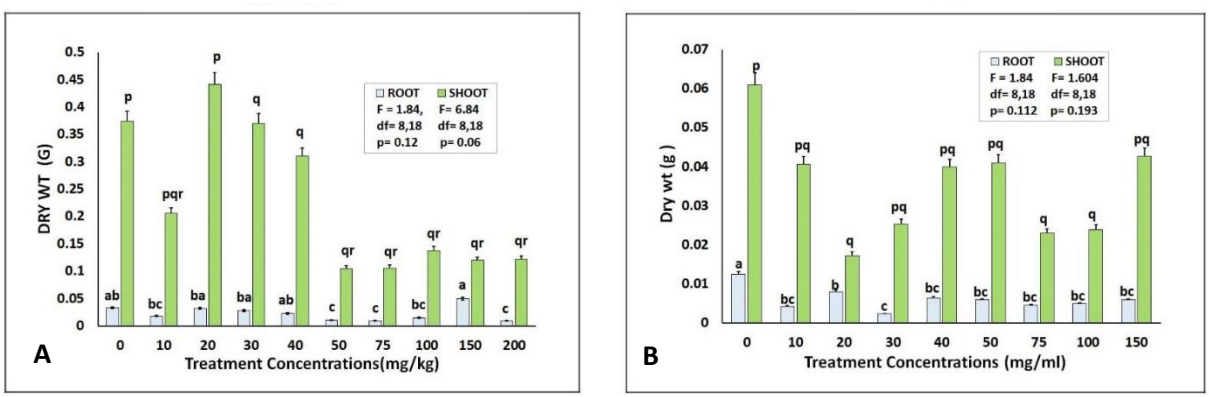


Figure 5.1.: Phytophysiological effects of heavy metal stress on *Cleome rutidosperma* at different tested concentrations of **(A)** Cadmium and **(B)** Lead in terms of biomass production measured as dry weight (g)

Similar observations were also reported in *Coronopus didymus* (**Sidhu et al., 2017**), *Brassica napus* (**Shakoor et al., 2014**) and *Eichornia crassipes (***Malar et al., 2014**) under Pb stress where a decrease in the total chlorophyll content was detected.

There was a linear increase in the uptake of both the metals in their shoots and roots (Equation 1 and 2) with increasing heavy metal exposure (10-200mg/kg). Equation 1 (a, b) and equation 2 (a, b) represent Cd and Pb content in roots and shoots respectively. R^2 represents the correlation between the content of metal in root-shoot tissues verses the soil at $p \leq 0.05$. There was a significant positive linear correlation between cadmium uptake and increasing treatment concentrations (Equation 1a and 1b) with an R^2 of 0.738 and 0.991.

$$y = 2.6079x + 215.39, R^2 = 0.738 \quad (1a)$$

$$y = 3.6667x + 9.5555, R^2 = 0.991 \quad (1b)$$

$$y = 93.732x - 52.267, R^2 = 0.933 \quad (2a)$$

$$y = 21.874x + 610.97, R^2 = 0.789 \quad (2b)$$

C. rutidosperma could efficiently accumulate as high as 639.07 mg/kg of Cd, 8726.03mg/kg of Pb in its roots while 752.83mg/kg Cd and 3732.64mg/kg Pb in its shoots (**Figure. 5.3**). The Cd content significantly increased from 256.5mg/kg to 639.07mg/kg in roots and from 90.93mg/kg to 752.83mg/kg in shoots. In case of Pb, *C. rutidosperma* could efficiently accumulate about 80 times more Pb in its roots as compared to the amount of Pb in the soil.

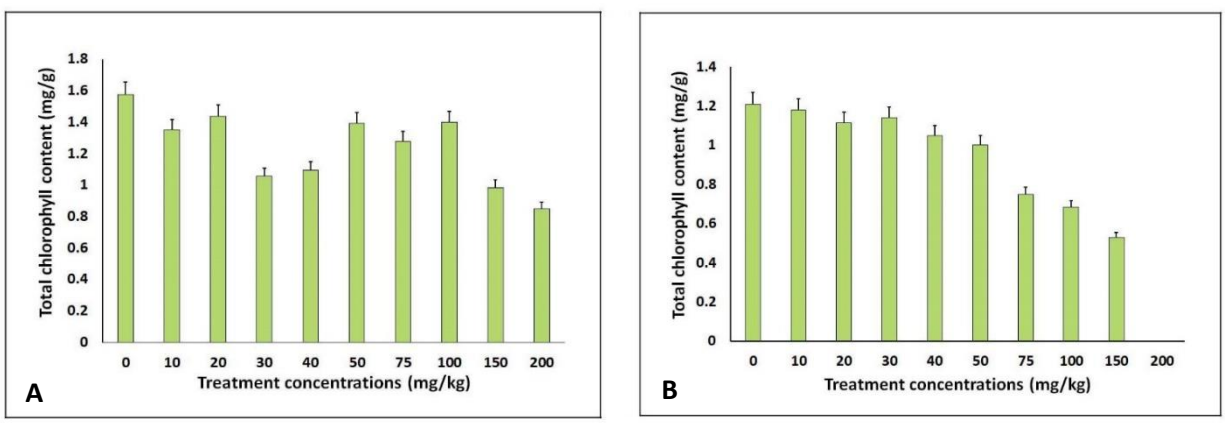


Figure 5.2: Phytophysiological effects of heavy metal stress on *Cleome rutidosperma* at different tested concentrations of **(A)** Cadmium and **(B)** Lead,on the total chlorophyll content measured as (mg/g)

The plant could accumulate a maximal amount of Pb i.e., 8726.03 mg/kg in its roots at the 100mg/kg concentration, while the plant accumulated 3732 mg/kg of Pb in its shoots at 150mg/kg treatment. There was a linear correlation in the Pb uptake for both the shoot and roots (equation 2a and 2b) with an R^2 values of 0.933 and 0.789.

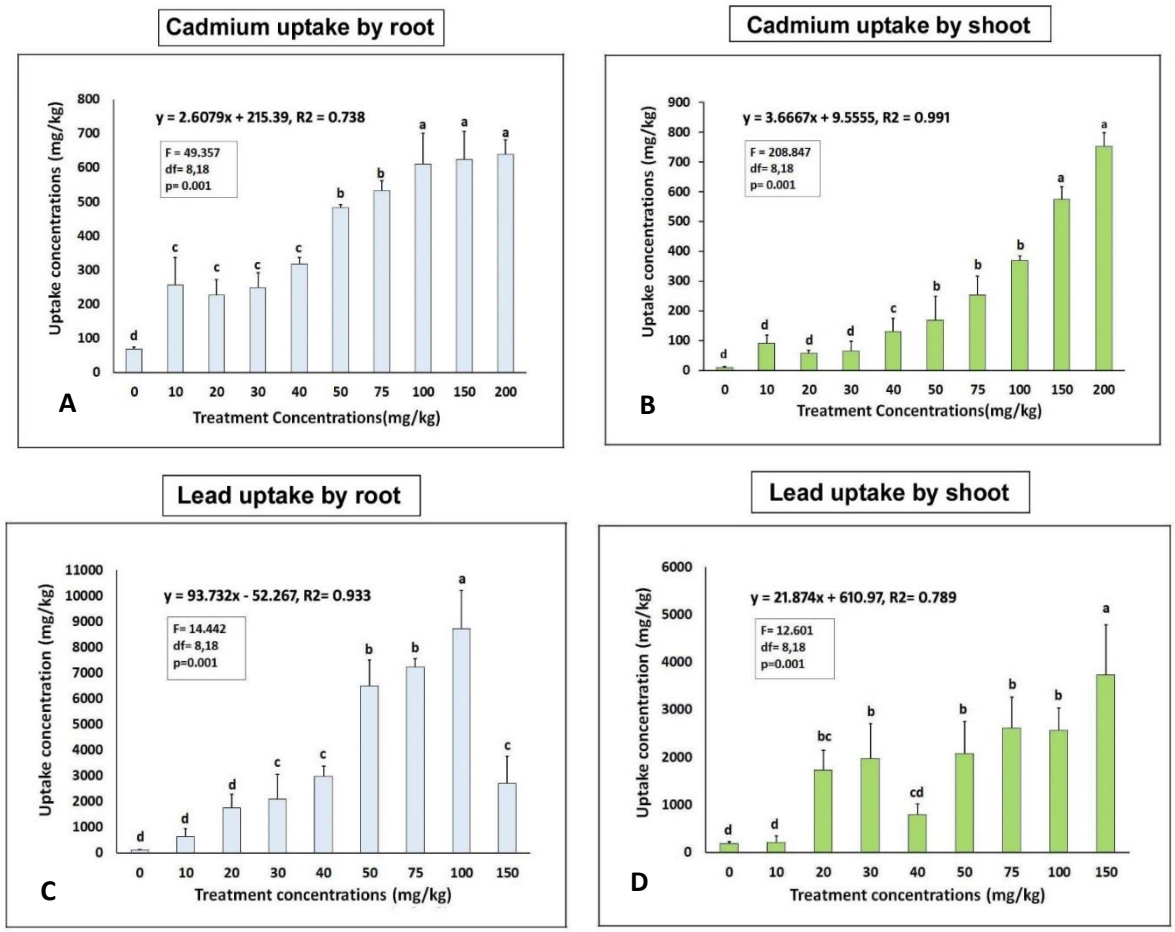


Figure 5.3: Heavy metal uptake by roots and shoots of *Cleome rutidosperma* recorded in pot experiments under different treatment concentrations of (**A**) and (**B**) cadmium, (**C**) and (**D**) lead in shoots and roots, respectively. The bars marked with different alphabets (a,b,c,d) are significantly different from each other (F values and "df" are given in the respective graphs). R^2 represents the correlation between the content of metal in root–shoot tissues versus the soil at $p \leq 0.05$.

In agreement to other findings, we observed that the roots of the plants that were primarily exposed to heavy metal stress retained significant amount of Cd as well as Pb (**Figure. 5.3**). As reported in Cd hyperaccumulator species namely *Arabis paniculata*,

Brassica napus and *Calendula officinalis,* the main organic compounds are metallothioneins and phytochelatins that help to sequester and accumulate Cd within the root cells (**Zeng et al., 2009, Ehsan et al., 2014, Liu et al., 2008**). *Celosia cristata pyramidalis,* an ornamental plant, has been reported to accumulate upto three times more Pb in its roots than shoots (**Cui et al., 2013**). As explained by the earlier studies, such accumulation of metals in roots could be attributed to the sub-cellular compartmentalisation of metals in vacuoles by the plant that helps it to cope with the possible toxicity imposed by increased heavy metal uptake. Another reason could be the formation of insoluble metal phosphates, carbonates and bicarbonates that precipitate in the intercellular root spaces (**Brennan and Shelly 1999**) which in turn reduces the translocation of the same from roots to the shoots (**Cunningham and Berti 2000**).

C. *rutidosperma* may employ such strategies to restrict the excess translocation of Cd and Pb, therefore protecting itself from metal-induced toxicity. However, *C. rutidosperma* also showed the tendency to translocate significant amounts of Cd and Pb in its aerial parts too which is a key criteria for a plant to have phytoextraction efficiency. This plant showed significant translocation at the higher treatment concentrations. The plant accumulated 752.8 mg/kg of Cd and 3732.63 mg/kg of Pb in its shoots at 200mg/kg and 150mg/kg treatment concentration respectively. Such translocation of metals may have occurred due to the increase in the internal transport of aqueous free Cd and Pb ions, a process mediated by xylem loading while being regulated by xylem flux and endodermis (**Uraguchi et al., 2009**). Uptake of these metals may takes place along with the essential metal nutrients like Zn, Cu and Fe via the membrane transporters (**Zheng et al., 2011**) and also by production of phytochelatins and formation of Pb-phytochelatin complexes within the vascular tissues (**Andra et al., 2009**).

In summary, the heavy metal content in roots and shoots of *C. rutidosperma* plants at all the treatments were well above the threshold level for Cd hyperaccumulators (>100 mg/kg) as well for Pd hyperaccumulators (>1000 mg/kg) (**Pollard et al., 2002**). Moreover, unpalatability, high biomass yield and shorter life span provide added advantages to make *C. rutidosperma* a novel and potential plant species to be exploited for Cd and Pb extraction from the polluted soils. These findings strongly support the potential of *C. rutidosperma* for both phytostabilisation and phytoextraction of Cd and Pb from the polluted soils.

Table 5.4: The residual heavy metal content in the treated soil and the removal percentage for the different treatments.

Treatments (mg/kg)	**Residual heavy metal concentration (mg/kg)**		**Heavy metal removal (%)**	
	Cd	**Pb**	**Cd**	**Pb**
0	3.27 ± 0.13	3.05 ± 0.75	10.7 ± 0.13	30 ± 0.75
10	8.92 ± 0.38	5.33 ± 1.15	10.83 ± 3.81	46.6 ± 11.54
20	13.25 ± 0.53	7.75 ± 1.29	34.23 ± 2.16	61.25 ± 6.49
30	14.84 ± 0.45	5.66 ± 1.37	38.97 ± 3.91	81.11 ± 4.58
40	18.95 ± 1.76	7.25 ± 1.32	42.43 ± 7.32	81.87 ± 3.31
50	23.75 ± 1.06	7.66 ± 0.63	54.1 ± 2.12	84.66 ± 1.25
75	27.63 ± 2.84	11.87 ± 2.12	51.86 ± 1.99	84.16 ± 2.83
100	39.92 ± 1.7	14.5 ± 2.5	60.08 ± 1.7	85.5 ± 2.5
150	51.61 ± 3.55	20.83 ± 1.18	64.88 ± 2.77	86.11 ± 0.78
200	60.01 ± 2.43	-	69.97 ± 0.22	-

The residual heavy metal content of the treated soils were also quantified using the same procedure as mentioned earlier in this chapter. The data revealed that at lower treatment concentrations, *C.rutidosperma* could only remove 10% of Cd while 46% of Pb. As the treatment concentrations were increased, the removal percentage also increased to

upto 69% for Cd and 86% for Pb (**Table 5.4**). This data supports the fact that there must be a threshold concentration of heavy metal that triggers the uptake of the same. In case of Pb, the percent removal increased from 61% to 81% at an increment of heavy metal treatment concentration from 20mg/kg to 30mg/kg. In case of Cd, however, there was a continuous increase in the removal percentage as the treatment concentrations were increased. This observation indicates that *C. rutidosperma* can efficiently remove heavy metals namely Cd and Pb at about 150mg/kg concentrations in the rhizospheric environment.

Chapter 6

Determination of BCF and TF

6.1 Introduction

Strategies of the plants grown on the metal containing soil (metallophytes), classified as accumulators and excluders, were published first by **Baker (1981)** based on the ratio between leaf:root metal concentration. Later, **Baker and Walker (1990)** improved this concept suggesting two groups of plants: metal excluders and metal non-excluders (indicators, hyperaccumulators). The plant strategy of hyperaccumulators was originally introduced to define plants containing >0.1% (1,000 μg g^{-1} d.m.) of Ni in dried plant tissues (**Brooks et al. 1977**). For other metals such as Zn and Mn the threshold is 10,000 lg g^{-1} (1%) of metal in aerial dry mass (**Baker and Walker 1990**); thereafter **Baker et al. (1994)** determined threshold for Cd as 100 lg g-1 d.m. (0.01%). Nowadays the accepted concentration defining hyperaccumulators for Cd is still above the mentioned 0.01% of this metal in the shoot (**Baker et al. 2000**). According to **Pollard et al. (2002),** hyperaccumulator plants can be regarded only as one subset of a larger category of metal-tolerant plants. However, the exact relationship between metal tolerance and metal hyperaccumulation has not been fully resolved.

Theory of plant strategy in response to metal excess was gradually enhanced. Recently, conditions for above-mentioned classification of plant strategies were improved by two further characteristics: bioaccumulation factor (in literature signified as BF or BAF) and translocation factor (TF). Both factors have to be considered for hyperaccumulator categorization (**Ma et al. 2001**). The term BF, defined as the ratio of metal concentrations in plant dry mass (lg g^{-1} d.m.) to those in soils (lg g^{-1} soil), has been used to determine the effectiveness of plants in removing metals from soils (**Tu and Ma 2002**). Similarly, for aquatic plants or plants cultivated in hydroponics BF expresses the ratio of metal concentration in plant dry mass (lg g^{-1} d.m) to that in external solution (lg cm^{-3}). It should be stressed that already **Kovalevsky (1969)** and later

Lee et al. (1998) named above-mentioned parameter as a biological absorption coefficient (BAC). However, BAC found more frequent application in the geological sciences (**Hemmati Ahoel 2006)**. Similarly, to determine the transfer of elements from soil into the plant the transfer factor was already defined by **Freytag (1986)** as a ratio of metal content in the plant to metal content in the soil. Later, **Cui et al. (2004)** stated that the soil to plant transfer factor (also termed uptake factor, accumulation factor or concentration factor) is an index for evaluation of transfer potential of metal from soil to plant. It should be stressed that factors affecting metal release from colloids and complexes in the soil influence the bioavailable metal concentration, whereby many different abiotic factors (e.g. pH, temperature, soil solution ionic strength, redox potential, cation exchange capacity, and organic matter content) influence the availability of metal to plants (**Greger 2004**). Moreover, it should be emphasized that bioaccumulation is a nonlinear process; bioaccumulation factors are generally highest at low concentrations and decrease with increasing media concentrations (**Suter et al. 2000; Greger 2004**).

The mobility potentials of plants for the heavy metals from the polluted growth media into the roots of the plants and the ability to accumulate in different parts, translocate the metals from roots to the harvestable aerial part were evaluated respectively by means of the bioconcentration factor (BCF), bioaccumulation factor (BAF) and the metal translocation factor (TF). These are the indices that are measured to assess the feasibility of the plant species for phytoremediation to provide insight for the use of native plants to remediate metal contaminated sites (**Shingadgaon et al 2018).**

6.2 Methodologies

In order to determine the type of remediation a plant performs, i.e., whether it has phytoextraction abilities or it immobilises the heavy metals by phytostabilisation, a range of indices are calculated based on the heavy metal contents of the plant parts.

1. **Bioconcentration factor**: It is the ratio of metal content in roots to that in the soil (**Zhuang, et al., 2007**). The plant species with both BCF and TF > 1 have the potential to extract metal(loid)s in their aerial parts and are employed for phytoextraction.

$$\textbf{Bioconcentration factor (BCF)} = C_{root}/C_{soil}$$

2. **Translocation factor**: It is the ratio of concentration of metal accumulated in shoots to the concentration in roots (**Zhuang, et al., 2007**).

$$\textbf{Translocation factor (TF)} = C_{shoot}/C_{root}$$

3. **Bioaccumulation factor:** It is calculated as ratio of heavy metal in shoots to that in the soil which signifies the levels of heavy metals stored in the shoots of plants.

$$\textbf{Bioaccumulation factor (BAF)} = C_{shoot}/C_{soil}$$

6.3 Results and Discussion

Plants can be considered as good phytoextractors if the BCF>1 and TF>1 based on the fact that those plants can translocate the heavy metals into its shoots. On the other hand if a plant exhibits the BCF>1 but TF<1, then those species can be categorised as having the ability to phytostablise and immobilise the heavy metal in its rhizosphere (**Zhuang, et al., 2007**).

6.3.1 Analysis for Hydroponic system

BCF was observed as greater than 1 for all the treatment concentrations in both the targeted heavy metals i.e., Cd and Pb. In the hydroponic experiments, with a treatment concentration of 10mg/kg, the plant showed the highest BCF of 16.12 for Cd and 57.82 for Pb (**Table.6.1**). However, the TF values were lesser than 1 for both the metals tested.

6.3.2 Analysis for Pot culture system

Pot culture experiments showed a similar trend. BCF values were all greater than 1 for both the metals at all the tested concentrations. The highest BCF recorded for Cd was 27 while for Pb 847.23 (**Table 6.1**). Here, an interesting fact observed was that the TF >1 at the highest treatment concentration. The TF was 1.18 at 200mg/kg concentration of Cd and 1.38 at 150mg/kg concentration of Pb. If we look closely, the metal uptake of Pb reached maximum at the preceding concentration of 100mg/kg in the roots. Interestingly, however, the uptake drastically decreased at 150mg/kg concentration.

Table 6.1: Bioconcentration indices calculated on the heavy metal concentration values. BCF=Bioconcentration factor; BAF= Bioaccumulation factor; TF= Translocation factor

TREATMENTS (mg/kg)	**BCF= ROOT/SOIL**		**TF= SHOOT/ROOT**		**BAF= SHOOT/SOIL**	
	Cd	**Pb**	**Cd**	**Pb**	**Cd**	**Pb**
Hydroponic experiment						
10	13.47	49.13	0.31	0.05	4.23	2.76
Pot experiment						
0	17.63	17.69	0.16	0.16	2.83	48.87
10	28.77	120.54	0.35	0.33	10.2	39.54
20	17.08	225.81	0.25	0.99	4.34	223.76
30	14.84	370.59	0.26	0.93	3.93	347.66
40	14.68	410.48	0.41	0.265	6.08	109.10
50	20.98	847.23	0.35	0.32	7.39	271.70
75	18.05	608.85	0.47	0.36	8.6	220.6
100	15.29	601.79	0.6	0.29	9.23	176.82
150	14.55	129.52	0.92	1.38	13.39	179.16
200	10.65	-	1.18	-	12.55	-

Additionally, the TF>1 at that concentration was observed which indicates that the plant was translocating the Pb taken up by the roots efficiently. According to the studies done on heavy metal sequestration and detoxification, it was reported that plants tend to cope the heavy metal stress by translocating and sequestering them in the aerial tissues (**Singh et al., 2016**). But, when exposed to even higher stress levels, i.e., 200mg/kg, the plant failed to survive.

The Bioaccumulation factor (BAF) are also greater than 1 for all the tested concentrations of cadmium and lead treatments. In case of cadmium, the BAF values range from 2.83 to 13.39 indicating that the target plant has a moderate rate of bioaccumulation tendency for cadmium. On the other hand, *C. rutidosperma* shows a very high BAF index for all the lead treatment concentrations ranging from 2.76 in hydroponic experiments to as high as 347.66 in pot culture experiments. This observation is in accordance with the heavy metal uptake and translocation factor of *C. rutidosperma* for lead. We could see that this plant is more efficient to translocate Pb to its shoots rather than Cd. Therefore, this plant would behave a better phytoextractor for Pb in heavy metal contaminated areas.

Chapter 7

Collection, purification and analysis of Root Exudates

7.1 Introduction

Root exudation is one of the ways of a plant to communicate with the plant and microorganisms present in the rhizosphere of the root. The chemical composition of the root exudates are specific to a particular plant species which also depend on the nearby biotic and abiotic environment. The chemical ingredient exuded by plant roots include amino acids, sugars, organic acids, vitamins, nucleotides, various other secondary metabolites and many other high molecular weight substances as primarily mucilage and some unidentified substances. Through the exudation of a wide variety of compounds, roots may regulate the soil microbial community in their immediate vicinity, cope with herbivores, encourage beneficial symbioses, change the chemical and physical properties of the soil and inhibit the growth of competing plant species (**Baker et al 2018**).

Root secretions may play both positive and negative communication in the rhizosphere. The positive communication includes symbiotic associations with beneficial microbes, such as mycorrhizae, rhizobia and plant growth promoting rhizobacteria (PGPR). Negative interactions include association with parasitic plants, pathogenic microbes and invertebrate herbivores. The rhizospheric bacteria are responsible for the elimination of the contaminants while the roots are responsible for providing nutrients (root exudates) used by the microorganisms to proliferate (**Bais et al., 2006**). Root exudates are defined as organic chemicals released by living and intact root system in certain stages of plant growth. The components of root exudates and their rhizosphere functions are summarized in **Table 7.1**

Table 7.1: The varied range of compounds that are released by the plants through their roots; their chemical nature and function along with the references

Chemical nature	Examples	Function	References
Organic acid	Acetic, aconitic, adipic, butyric, citric, cyclic, formic, fumaric, gluconic, glutaric, glycolic, glyoxylic, hydroxybutyric, indole-3-acetic, isocitric, lactic, maleic, malic, malonic, oxalic, piscidic, propionic, pyruvic, succinic, tartaric, valeric	Nutrient and energy sources, chemoattractant signals to microbes, chelators/adsorbents of insoluble mineral nutrients, acidifiers of soil, nod gene inducers, antibacterial agents	**Mucha et al., 2005; Magdziak et al., 2011; Ramachandran et al., 2011; Vranova et al., 2013**
Amino acids	a-alanine, b-alanine, g-aminobutyric acid, arginine, asparagine, asparticacid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan	Nutrient and energy sources, chelators of insoluble mineral nutrients, chemoattractant signals to microbes	**Yao et al., 2005; Ma et al., 2009a,b, 2011a; Ahemad and Kibret, 2014; Glick, 2014**
Saccharides	Arabinose, fructose, fucose, galactose, glucose, lactose, mannose, raffinose, rhamnose, ribose, sucrose, xylose	Nutrient and energy sources, anchoring of bacteria to plant surfaces	**Guibaud et al., 2005; Juwarkar et al., 2007; Sheng et al., 2008; Slaveykova et al., 2010; Venkatesh and Vedaraman, 2012**
Phenols	Caffeic acid, ferulic acid, flavonoids/bioflavonoids, *N*-hexanoyl-D,L-homoserine-lactone, 7-hydroxy-6-methoxycoumarin,isoflavonoids, neoflavonoids, pyrocatechol, quercetin, strigolactones, styrene	Nutrient and energy sources, chemoattractant signals to microbes, chelators of insoluble mineral nutrients, microbial growth promoters, *nod* gene inducers or inhibitors in rhizobia, inductors of resistance against phytopathogens	**Dakora and Phillips, 2002; Zhao et al., 2005; Steinkellner and Mammerler, 2007; Steinkellner et al., 2007; von Rad et al., 2008; Hofmann, 2013**
Enzymes	Amylase, DNase, phosphatase, polygalacturonase, protease, RNase, sucrase, urease, xylanase	Release of phosphorus from organic molecules, transformations of organic matter in soil	**Loh et al., 2002; Gonzales-Chavez et al., 2004; Ahemad and Kibret, 2014; Wu et al., 2014**

Chemical nature	Examples	Function	References
Vitamins	*p*-aminobenzoic acid, ascorbic acid, biotin, b-carotene, folic acid, niacin, pantothenate, pyridoxine, riboflavin, thiamin, thiocticacid,tocopherol,vitaminB12	Stimulation of plant and microbial growth, nutrient source, resistance to soil pathogens, facilitation of organic pollutant degradation, induction of plant–microbe symbioses	**Kafkewitz et al., 1996; Bertin et al., 2003; Vranova et al., 2013**
Others	Bilineurine, bradyoxetin, glomalin, inositol, nicotinic acid, rhamnolipids, somatropin, surfactants	Stimulation of plant and microbial growth, regulators of symbiotic expression of nodulation genes(nod, nol, noe)	**Loh et al., 2002; Vijayan et al., 2013**

7.2 Factors Affecting Exudation

The exudation of organic compounds by roots are influenced by either biotic (for example, soil microbial uptake) (**Kuzyakov et al., 2003**) or abiotic processes (**Hees et al., 2003**). In some instances, our knowledge is sufficient to explain why exudation is affected by the root environment, but often our ignorance of the physiological processes involved in exudation precludes a correct explanation. Some of the factors influencing exudation are listed as thus explained:

- **Plant species:** The amount, range and balance of compounds in root exudates differ for different plant species. Some worker found differences between wheat and barley (*Hordeum vulgare* L.) root exudates with respect to certain sugars (galactose, glucose and rhamnose), whereas other sugars occurred in similar amounts in exudates of both plants (**Vancura, 1964**). The specificity of root exudates from different plants in stimulating only certain groups of organisms is clearly demonstrated in the plant pathology literature, for example, the cysts of potato eelworm (*Heterodera*

rostochiensis) hatched when supplied the root washings of potato (*Solanum tuberosum* L.), tomato and some other solanaceous plants, but not the washings of beet (*Beta vulgaris* L.), rape (*Brassica napus* L.), lupin (*Lupinus lilosus* L.), mustard (*Brassica* sp.) or oats (*Avena sativa*) (**Xiaoe et al., 2005**).

- **Root age:** Research done with peas and oats indicated that more amino acids and sugars exuded during the first 10 days of growth than during the second 10 days (**Rovira, 1956**). **Vancura and Hovadik, (1965**) study found 3- pyrazolylalanine in root exudate of cucumber (*Cucumis sativus* L.) only at the early seeding stage. With tomato and red pepper (*Capsicum anznumm* L.), they found that tyrosine occurred in the exudate only at fruiting and not at any other stage of growth.
- **Microorganisms:** Microorganisms may affect the permeability of root cells, metabolism of roots, absorption and excretion of certain compounds in root exudates. It was reported that filtrates of cultures of some bacteria and fungi and also some antibiotics (penicillin), increased the exudation of scopoletin (6 methoxy -7 hydroxycoumarin) by oat roots (**Blaylock et al., 1997**). **Norman (1961)** found that certain polypeptide antibiotics, for example, polymyxin which is formed by Bacillus polymyxa from soil, altered cell permeability and increased leakage. There are two main difficulties in interpreting the significance of their results which show that culture filtrates or products increase the leakiness of plant roots. First, the conditions under which the organisms are grown are quite different both physically and nutritionally from those under which a rhizosphere population grows. Second, as it is not possible to calculate the concentration of biologically active substances in the rhizosphere, the concentrations used for "in vitro" experiments must of necessity be selected rather arbitrarily. A further point is that, any consideration of the significance of the rhizosphere population in modifying exudation must involve a concept of micro-

ecology, with a wide variety of organisms occupying different "niches on the roots and only those plant cells in the immediate vicinity of "exudation- promoting" organisms are likely to be affected. Microorganisms also influenced the exudation of organic materials into soil. A supplementary study showed that the exudation from wheat roots into synthetic soil was increased at least four fold by microorganisms (**Norman, 1961**). The magnitude of the effects of microorganisms upon exudation no doubt will depend on the species colonizing the roots (**Albert, 1969**). Some other plant biotic factors like developmental status, shoot herbivory, photosynthesis, supply of carbon from shoot to root, evaporation, transpiration, nutrient deficiency, root architecture, cytosolic concentration, membrane permeability, membrane electrochemical potential, release of microbial signal, allelochemical release, mychorrhizas, nodulation and some soil biotic factor are also influenced by the root exudation.

- **Temperature:** The release of amino acids and, especially, asparagine from roots of tomato and subterranean clover (*Trifolium subterraneum* L.) increased with rising temperature (**Rovira, 1959**). However, this effect is by no means universal, as some worker found more amino acids in exudates from strawberry plants (*Fragatia vesca* L.) grown at 5 to 10°C than at 20 to 30°C; this markedly influenced the pathogenicity of (italics) which attacks strawberries at low soil temperatures (**Husain and Mckeen, 1963, Hale et al., 1978**).
- **Light:** The light intensity at which plants are growing affects the amounts and balance of compounds exuded into nutrient solution by tomato and subterranean clover roots (**Rovira, 1959**). Clover grown at full daylight intensity exuded more serine, glutamic acid, and c-alanine than plants grown in 60% shade. With tomato, the levels of aspartic acid, glutamic acids, phenylalanine and leucine in exudate were reduced by shading. Beside these abiotic factors, few others such as moisture, humidity, wind speed and

light intensity, elevated CO_2 pesticides, available space, atmosphereic nitrogen deposition, ozone, physical disturbance, fire, irrigation, erosion, altitude and latitude are also influencing the exudation (**Torsivik et al., 1996**). Some soil abiotic factors resembling compaction, soil type, salinity, soil pH, metal toxicity, water availability, organic matter, cation and anion exchange, drainage, aeration, rooting depth, soil texture, soil structure and redox-potential influence the release of organic chemical from plant root (**Ross et al., 2000; Rangarajan et al., 2001**).

7.3 Collection of Root exudates by Root exudate trapping system

Cleome rutidosperma plants were grown in special root exudate trapping systems (**Tang & Young 1982**) which consists of Buchner funnel (dia=110 mm) and conical flasks (500 ml). The sieve inside the Buchner funnel was removed. The funnel was filled with soil after placing a piece of cotton cloth at the mouth of the funnel to hold the soil. The conical flasks were painted black to avoid growth of fungus or algae. The germinated seeds (6-10) of *C. rutidosperma* were sown in each funnel. An average of 5-6 plants depending on the growth, size and number of leaves, were allowed to grow in each set till maturity. Plant roots penetrated the soil in the funnel and extended into the flasks after 20-25 days. The flasks contained distilled water. The plants released compounds into the water and this water is further referred to as the root exudates (**Figure 7.1**). Root exudates were collected every 7 days and the flasks were filled with fresh distilled water. This procedure was continued for a period of 4 months.

Figure 7.1: Collection of root exudates from *Cleome rutidosperma* DC. (**A**) Total experimental setup, (**B**) root exudates trapping system, and (**C**) roots extend downward into the conical flasks containing distilled water.

7.4 Extraction and Purification of Root exudates

The root exudates were collected for a period of four months. A total amount of approximately 10 Litre was collected in the time period. The collected exudates were dried in vacuum evaporator to get the crude extract. This crude extract was then purified using the solvent extraction method based on the elutropic series. The extraction and purification is done by using the lowest polar solvents like Hexane followed by Ethyl acetate, Acetone and Methanol. The detailed flowchart is given in the **Figure 7.2**. The purified methanol fraction is then confirmed with Thin Layer Chromatography and further purified using column chromatography. The purified fraction was then sent to for identification using GCMS analysis.

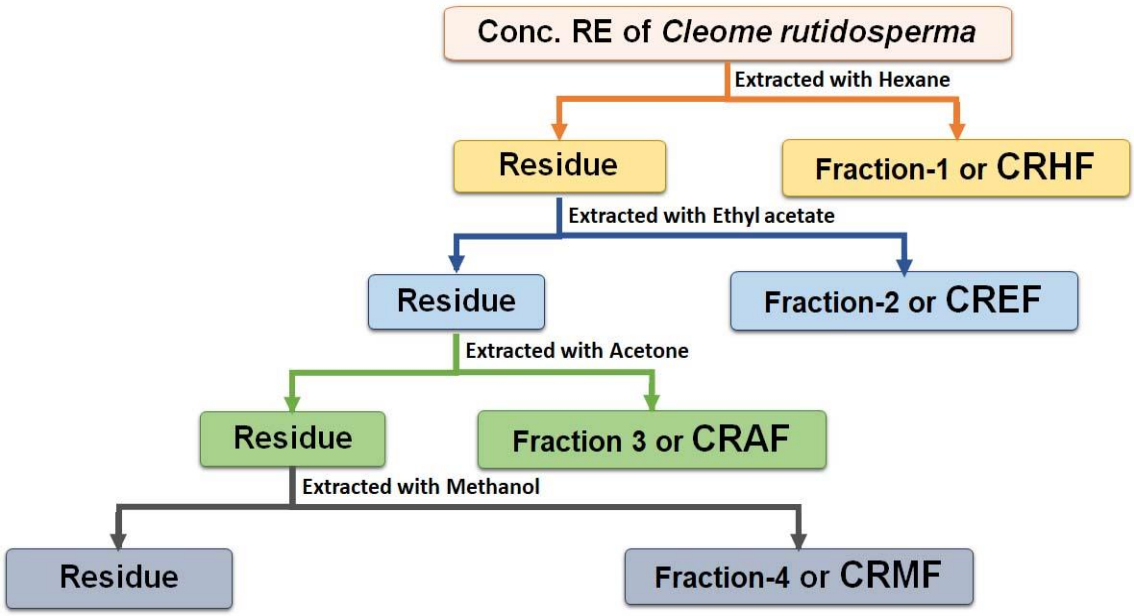


Figure 7.2: The flowchart of purification procedure. RE: root exudates. CRHF= *C. rutidosperma* Hexane fraction; CREF= Ethyl acetate fraction; CRAF= Acetone fraction; CRMF= Methanol fraction.

7.5 GCMS analysis of purified root exudate

Purified fraction of *C. rutidosperma* root exudate was subjected to GC-MS Analysis (Model No. Agilent Technologies, GC-6860N Network GC System with 5973 inert Mass Selective Detector) for detecting bioactive compounds. The GC-MS analysis was done at the National Test House, Salt Lake, Sector V, Kolkata 700091, INDIA. HP-1MS column (25 m x 0.33 mm, i.d. 0.25 µm) was used. 0.1 µL of purified sample (dissolved in chloroform) was injected into GC in the split mode for analysis at an injector temperature of 280 ^{o}C. A constant flow of helium as the carrier gas was maintained at a rate of 1 mL/min. The oven temperature was programmed as follows: 50 ^{o}C (1 min hold), 50 ^{o}C to 200 ^{o}C at 7 ^{o}C/min, 200 ^{o}C to 300 ^{o}C at 6 ^{o}C/min, 200 ^{o}C (2 min). The mass spectrometer employed the electron ionization mode with an ionization energy of 70 eV. A full scan mode was used with an ion source temperature of 280 ^{o}C and an acquisition rate of 0.2 s. The mass range was adjusted to 50-350 Da. The identification of compounds was done by comparing the mass spectra with the spectral data of the NBS75K library provided by the GC/MS control and data processing software.

7.6 Results and Discussion

7.6.1 Extraction and Purification of Root Exudates

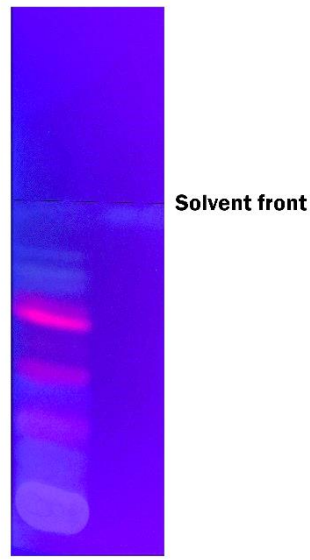


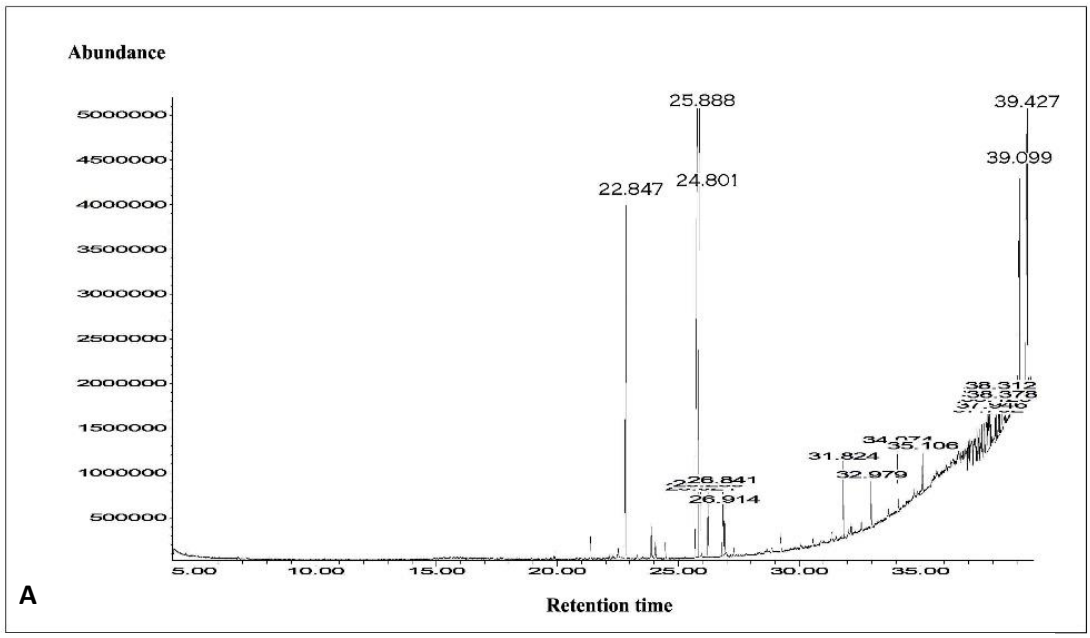
Figure 7.3: Thin layer chromatography of the purified CRMF extract of *C. rutidosperma* root exudates**.** The solvent system used for this TLC is Ethyl acetate: Hexane (70:30). The TLC was observed under UV light 365nm.

Root exudates of *C. rutidosperma* were purified using the solvent fractionation method (**Figure 7.2**). It was observed that most of the compounds were found in the methanol fraction of CRMF. The CRMF was further purified using column chromatography technique. The purified CRMF was then run on TLC (**Figure 7.3**). As it is shown in the figure, an approximate of 10-12 bands are visible in the TLC. This extract was then sent to GCMS analysis for further identification of the compounds.

7.6.2 GCMS analysis of Root exudate of *C. rutidosperma*

GCMS spectra of purified root exudates of *C. rutidosperma* revealed the presence of five major peaks along some minor peaks based on percentage area of the peak . Major compounds detected were palmitic acid (retention time = 22.847), linoleic acid (retention time = 25.888), Oleic acid (retention time=25.89), campesterol (retention time= 39.099) and stigmasterol

(retention time= 39.427). Minor compounds were stearic acid, ethyl lineolate, ethyl oleate, behenic acid, tricosanoic acid and lignoceric acid (**Figure 7.4)**.



Compounds	Area%	Retention time
Palmitic acid *	11.13	22.847
Linoleic acid *	25.57	25.801
Oleic acid *	17.89	25.890
Stearic acid	2.11	26.237
Ethyl Linoleate	2.02	26.839
Ethyl Oleate	1.44	26.912
Behenic acid	1.44	31.825
Tricosanoic acid	1.36	32.977
Lignoceric acid	1.36	34.072
Campesterol *	9.77	39.099
Stigmasterol *	15.32	39.427

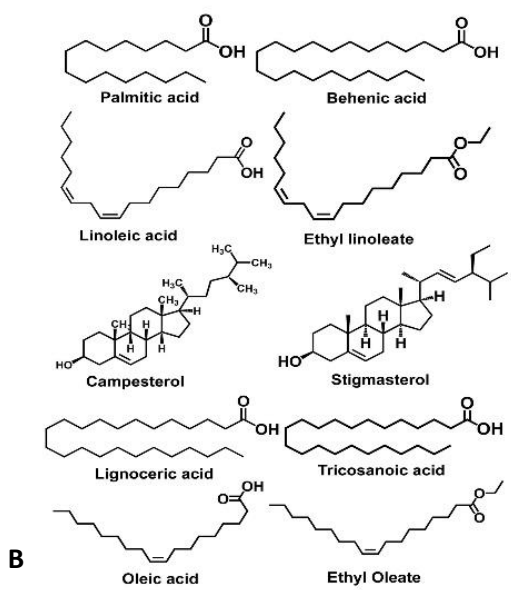


Figure 7.4: GCMS spectra for the purified root exudates (**A**). The list of the main compounds with the respective retention times is given in the table (inset) provided. The area% provides information about the abundance of the respective compounds. The compounds marked with (*) are the main compounds based on the abundance and sharp peaks. (**B**) The structures of the compounds that were identified in the GCMS spectra.

Heavy metal stress induces the plant cells to generate highly reactive Reactive oxygen species (ROS) which can oxidise and degenerate cellular macromolecules such as DNA, pigments, proteins, lipids, and other essential molecules irreversibly. In order to prevent that, plants enable their defence strategies mainly by producing some alternate bioactive molecules which act as reaction centers for the generated ROS (**Singh et al., 2016**). Unsaturated fatty acids and esters like linoleic acid, ethyl linoleate, oleic acid and ethyl oleate are such compounds which act as molecular targets that scavenge ROS (**De Bigault et al., 2016**). Presence of these compounds in the root exudates clearly indicate their increase production to cope the heavy metal stress (**De Bigault et al., 2016**). Peroxidation of reactive targets molecules like linoleic acid and its methyl ester derivatives through radicals generated by the heavy metal stress protects the cells from extensive injury to cellular DNA (**Guerzoni et al,. 2001**). Plants communicate with the environment using these chemical signals. In a study done by **Yi and Crowley in 2007**, it has been reported that fatty acids acts as metabolic marker which stimulates Polyaromatic hydrocarbon (PAH) degradation through roots. Therefore, this plant may also have a tendency to remediate such PAHs, which provides a future scope that require thorough investigation. Apart from fatty acids, the precursor of steroidal compounds mainly brassinosteroids i.e., campesterol and stigmasterol were also identified in the root exudates. There have been reports about roles of these compounds in detoxification mechanisms in heavy metal stress conditions. Studies done on Cd-treated plants of *Arabidopsis thalliana*, support this statements as concentrations of these compounds were observed to be considerably increased in the stress exposed plants compared to the untreated control (**Sun et al., 2010**).

Chapter 8

Discussion and Limitations of the Study

8.1 Discussion

A healthy ecosystem is the symbol of successful environment management, extremely vital to the society and economy. The burning issue of the today's environmental problem is the contamination of natural resources due to intense industrial and agricultural activities which creates pollutants that disturb ecosystems, global warming, human health hazards and infertile land. To overcome these situations, a much better perspective is to completely destroy the pollutants, or to transform them into some biodegradable substances. However, advances in science and technology enabled us to apply the potential of biological diversity for pollution abatement which is termed as Phytoremediation, is a relatively new approach to removing contaminants from the environment. It is considered as safer, cleaner, inexpensive and environment friendly technology which generally have a high public acceptance and can often be carried out at any site.

This research work is, therefore, aimed at proposing the species of *Cleome rutidosperma* DC as a novel species that has the potential for phytoremediation. The studies, therefore, suggest that *C. rutidosperma* DC is a candidate plant that can be explored for its heavy metal accumulating abilities as the plants do not exhibit any significant signs of heavy metal toxicities. In case of pot experiments, there was a linear increase in the uptake of both the metals in their shoots and roots with increasing heavy metal exposure (10-200mg/kg). *C. rutidosperma* could efficiently accumulate as high as 639.07 mg/kg of Cd, 8726.03mg/kg of Pb in its roots while 752.83mg/kg Cd and 3732.64mg/kg Pb in its shoots. This plant showed significant translocation at the higher treatment concentrations. The plant accumulated 752.8 mg/kg of Cd and 3732.63 mg/kg of Pb in its shoots at 200mg/kg and 150mg/kg treatment

concentration respectively. In summary, the heavy metal content in roots and shoots of *C. rutidosperma* plants at all the treatments were well above the threshold level for Cd hyperaccumulators (>100 mg/kg) as well for Pd hyperaccumulators (>1000 mg/kg) (**Pollard et al., 2002**). Moreover, unpalatability, high biomass yield and shorter life span provide added advantages to make *C. rutidosperma* a novel and potential plant species to be exploited for Cd and Pb extraction from the polluted soils. These findings strongly support the potential of *C. rutidosperma* for both phytostabilisation and phytoextraction of Cd and Pb from the polluted soils. The residual heavy metal present in the soil was also recorded. The data revealed that at lower treatment concentrations, *C.rutidosperma* could only remove 10% of Cd while 46% of Pb. As the treatment concentrations were increased, the removal percentage also increased to upto 69% for Cd and 86% for Pb.

In order to interpret the chemical vocabulary the plant communicates with the rhizospheric surroundings, we performed the root exudate collection, purification and analysis. GCMS spectra of purified root exudates of *C. rutidosperma* revealed the presence of five major compounds namely palmitic acid (retention time = 22.847), linoleic acid (retention time = 25.888), Oleic acid (retention time=25.89), campesterol (retention time= 39.099) and stigmasterol (retention time= 39.427). It has been reported earlier that unsaturated fatty acids and esters like linoleic acid, ethyl linoleate, oleic acid and ethyl oleate are such compounds which act as molecular targets that scavenge ROS. Presence of these compounds in the root exudates clearly indicate their increase production to cope the heavy metal stress. Additionally, Plants communicate with the environment using these chemical signals. In a study done by **Yi and Crowley in 2007**, it has been reported that fatty acids acts as metabolic marker which stimulates Polyaromatic hydrocarbon (PAH) degradation through roots. Therefore, this plant may also have a tendency to remediate such PAHs, which provides a future scope that require thorough investigation.

8.2 Limitations of phytoremediation

Although phytoremediation is a promising approach for remediation of heavy metal-contaminated soils, it also suffers from some limitations like

- Long time required for clean-up.
- Phytoremediation efficiency of most metal hyperaccumulators is usually limited by their slow growth rate and low biomass.
- Difficulty in mobilization of more tightly bound fraction of metal ions from soil i.e., limited bioavailability of the contaminants in the soil.
- It is applicable to sites with low to moderate levels of metal contamination because plant growth is not sustained in heavily polluted soils.

In our studies too, the limitations were evident. The experimental procedure was for 60 days which makes the process lengthy and time taking. This fact can be problematic if such remediation is done on a target site to get rid of heavy metals quickly. However, the process can be accelerated by treatment with some organic amendments like chelators. These mobilize the metals in the soil thus, speeding up the uptake by plants.

8.3 Future scopes of the study

The term ‘‘hyperaccumulator’’ was coined by **Brooks et al. (1977**) for plants that are different from others based on their metal uptake properties. In general, hyperaccumulator plants vigorously take remarkably large amounts of one or several heavy metals as well as other pollutants from the soils.

Plants that are able to accumulate heavy metals or Polyaromatic hydrocarbons belong to different phylogenetic groups, and it seems that the ability to hyperaccumulation appeared many times independently in the course of evolution. To date, there are no comprehensive studies of evolutionary relationships between hyperaccumulators of different pollutants. Such

studies could help to understand better the mechanisms of hyperaccumulation and to create optimal strategies of bioremediation.

Consequently, advancements in molecular science would accelerate our knowledge of adaptive plant remediation/resistance and plant production in the context of global warming. Genome modification using artificial nucleases has the potential to enhance phytoremediation by modifying genomes for a sustainable future.

In our study, we propose *C.rutidosperma* DC as a potential phytostabilizer when the cadmium and lead metal contamination in the target area is at a low to medium levels. Nevertheless, at higher levels of metal contamination, this plant can efficiently translocate metals from its roots to its shoots. Another interesting observation was made during the study, that this plant releases certain molecular signalling compounds in the rhizosphere which can play an important role in helping this plant survive in the highly contaminated soil. *C. rutidosperma* is a plant that naturally grows and proliferates in disturbed and contaminated lands. Therefore, it must have a strong coping mechanism that enables it to survive in those unfavourable conditions. The root exudate analysis revealed that *C. rutidosperma* releases fatty acids like linoleic acid that have been identified as a metabolite marker for plants that have the ability to degrade Polyaromatic hydrocarbons. Based on this observation, the future research can be done with the aim of unravelling the role of this plant as PAH accumulator.

Chapter 9

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Chapter 10

Publications

1. Dutta, R., **Bhattacharya, E**., Pramanik, A., Hughes, T. A., & Biswas, S. M. (2022). Potent nutraceuticals having antioxidant, DNA damage protecting potential and anti-cancer properties from the leaves of four Ficus species. *Biocatalysis and Agricultural Biotechnology*, 102461.
2. **Bhattacharya, E**., & Mandal Biswas, S. (2022). First Report of the Hyperaccumulating Potential of Cadmium and Lead by *Cleome rutidosperma* DC. With a Brief Insight into the Chemical Vocabulary of its Roots. *Frontiers in Environmental Science*, *10*, 830087.
3. **Bhattacharya, E**., Saha, S., Dutta, R., Dutta, M., & Biswas, S. M. (2022). Fractionation based evaluation of phytochemical constituents, antimicrobial and allelopathic potential of *Piper chaba*, Hunter. stem and identification of "Pipercyclobutanamide-A" as a strong allelopathic agent. *Biocatalysis and Agricultural Biotechnology*, *42*, 102356.
4. **Bhattacharya, E**., Mandal Biswas, S., & Pramanik, P. (2021). Maleic and l-tartaric acids as new anti-sprouting agents for potatoes during storage in comparison to other efficient sprout suppressants. *Scientific reports*, *11*(1), 1-12.
5. **Bhattacharya, E**., & Mandal Biswas, S. (2021). Role of Tartaric Acid in the Ecology of a Zoochoric Fruit Species, *Tamarindus indica*. L. *International Journal of Fruit Science*, *21*(1), 819-825.
6. **Bhattacharya, E**., Pal, U., Dutta, R., Bhowmik, P. C., & Mandal Biswas, S. (2022). Antioxidant, antimicrobial and DNA damage protecting potential of hot taste

spices: A comparative approach to validate their utilization as functional foods. *Journal of Food Science and Technology*, *59*(3), 1173-1184.

7. Bose, R., **Bhattacharya, E**., Pramanik, A., Hughes, T. A., & Biswas, S. M. (2021). Potential oil resources from underutilized seeds of *Sterculia foetida*, L.-Quality assessment and chemical profiling with other edible vegetable oils based on fatty acid composition, oxidative stability, antioxidant activity and cytotoxicity. *Biocatalysis and Agricultural Biotechnology*, *33*, 102002.
8. **Bhattacharya, E**., Dutta, R., Chakraborty, S., & Biswas, S. M. (2019). Phytochemical profiling of *Artocarpus lakoocha* Roxb. leaf methanol extract and its antioxidant, antimicrobial and antioxidative activities. *Asian Pacific Journal of Tropical Biomedicine*, *9*(11), 484.
9. **Bhattacharya, E**., Bose, R., & Mandal Biswas, S. (2019). A comprehensive study on occurrence records of African neglected and underutilized weed species, *Cleome gynandra L.*(cat's whiskers) validating the ecogeographical range expansion in West Bengal, India (Vol. 19, No. 4, pp. 129-134). Melbourne: John Wiley & Sons Australia, Ltd.

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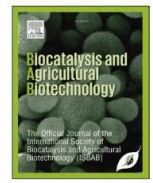

Potent nutraceuticals having antioxidant, DNA damage protecting potential and anti-cancer properties from the leaves of four *Ficus* species

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ABSTRACT

In the present study, we have evaluated fractionation based phytochemical constituents, antioxidant activity, DNA damage protecting potential and anticancerous properties of leaves of common *Ficus* species (namely *Ficus virens, Ficus benghalensis, Ficus religiosa, Ficus elastica*) along with GCMS analysis for identification of major bioactive constituents. Methanol fraction of *F. virens* contained maximum amount of phenolics (1267.35 mg GAE/g dry extract) and flavonoids (1080.61 mg QE/g dry extract) whereas hexane fraction of *F. religiosa* possessed highest amount of tannins (123.76 mg TAE/g dry extract). Least amount of phytochemicals was recovered from *F. elastica*. Highest DPPH radical scavenging activity (IC_{50} = 108.28 μg/ml) was detected by methanol fraction of *F. benghalensis* whereas highest ABTS activity (IC_{50} = 105.56 μg/ml) by *F. benghalensis* and highest ferric reducing power by *F. virens* (359.44 mg QE/g dry extract). Leaf methanol fraction of *F. virens, F. religiosa* and *F. elastica* were able to prevent oxidative DNA damage at 0.1 mg/ml,0.2 mg/ml and 0.3 mg/ml respectively. Viability of normal breast cells was unaffected by methanol fraction of tested *Ficus* species at doses less than 160 μg/ml, whereas survival of breast cancer cells was decreased by *F. benghalensis* at 5 μg/ml. GCMS analysis of the purified methanol fraction of tested species revealed the presence of potent bioactive compounds such as carvacrol, phytol, tocopherol, benzophenone, dibutyl phthalate, lycopersen etc. All our experimental results along with the identification of the bioactive compounds supported the fact that leaves of tested *Ficus* species as rich source of phytochemicals with nutraceutical potentialities.

1. Introduction

Phytochemicals are secondary metabolites which not only have physiological functions in plants but also exert significant pharmacological effects especially for preventing oxidative damage to cells. Extensive research is going on in plant derived natural

Abbreviations: Ficus virens, FV; *Ficus benghalensis*, FB; *Ficus religiosa*, FR; *Ficus elastica*, FE; Hexane Fraction, HF; Ethyl acetate Fraction, EF; Acetone Fraction, AF; Methanol Fraction, MF.

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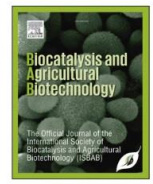

Research Paper

Fractionation based evaluation of phytochemical constituents, antimicrobial and allelopathic potential of *Piper chaba*, Hunter. stem and identification of "Pipercyclobutanamide-A" as a strong allelopathic agent

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ABSTRACT

Piper chaba, Hunter. is a less known medicinal spice. Its medicinal properties have been studied thoroughly but its role as a source of agrochemicals has not been studied yet. So, in the present work, we are interested to explore its other biological activities. Fractionated quantitative biochemical analysis revealed that all fractions contained substantial amounts of phenolics (ranging from 863.75 mg to 1073.11 mg GAE/g of dry extract), flavonoids (164.01 mg to 244.57 mg QE/g of dry weight) and tannins (42.86 mg to 64.88 mg TAE/g dry extract) except acetone fraction. All fractions of *P. chaba* revealed strong fungicidal activity with Inhibition Zone Diameter [IZD] ranging from 32.49 to 36.66 mm except acetone fraction. In case of bactericidal activity, all the fractions except hexane fraction exhibited strong effect with IZD ranges from 14.96 to 30.39 mm against all the tested species. Dose dependent bioassay experiment on rice showed that methanol fraction of *P. chaba* possesses strong allelopathic activity with IC_{50} value 202.77 μg/mL for root length and 509.1 μg/mL for shoot length. As methanol fraction of *P. chaba* exhibited strong allelopathic activity, we were interested to identify the actual compound responsible for that activity. LCMS analysis of purified methanol fraction of *P. chaba* revealed the presence of "Pipercyclobutanamide-A" as a major compound– and this is the first report of this compound from stem of *P. chaba*. Therefore, methanol fraction of *Piper chaba* could be used as natural source of compounds with agrochemical and antimicrobial potential for sustainable agriculture and pharmaceutical purposes.

1. Introduction

Plant bioactive compounds (BACs) provide infinite opportunities for development of natural agrochemicals such as biopesticides, bioherbicides or biofertilizers due to vast diversity of secondary metabolites (Guerriero et al., 2018; Isah, 2019). Secondary metabolites of plants are metabolic intermediates or products which are not essential for growth and development but play various important functions in the living plants such as resistance against pathogens, pests, and repulsion against herbivores; reaction to environmental stresses, and mediating ecological interactions (Yang et al., 2018; Jain et al., 2019; Aguirre-Becerra et al., 2021). These compounds are

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First Report of the Hyperaccumulating Potential of Cadmium and Lead by *Cleome rutidosperma* DC. With a Brief Insight Into the Chemical Vocabulary of its Roots

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Phytoremediation is gaining interest in recent years as it is a simple and effective strategy for heavy metal decontamination. The most straightforward strategy for successful heavy metal clean-up is searching for efficient hyperaccumulator species that grow naturally in contaminated sites. The present study, therefore, is the first detailed account of hyperaccumulator potentialities of a neglected and underutilized (NUS) species, *Cleome rutidosperma* DC. Hydroponic screening experiment against cadmium and lead revealed that even at 10 mg/kg concentration, it could accumulate 42.49 mg/kg of Cd and 27.79 mg/kg of Pb in shoots, while it could accumulate 134.71 mg/kg Cd and 491.35 mg/kg of Pb in its roots, and these values were significantly higher than those of the control plants. This plant could efficiently accumulate as high as 639.07 mg/kg of Cd, 8,726.03 mg/kg of Pb in its roots, while it could accumulate 752.83 mg/kg Cd and 3,732.64 mg/kg Pb in its shoots as evident from the pot experiments. In the case of Cd, there was no significant effect of toxicity on the phytophysiological parameters. But increasing concentrations of Pb did have toxic effects on the total chlorophyll content. This plant showed to have a BCF >1 in most of the tested concentrations. At the highest treatment concentration, however, both the BCF and TF were found to be greater than 1. This indicated that *C. rutidosperma* can accumulate and translocate the heavy metals to its aerial parts when the metal concentration is extremely high, proving itself to be an efficient hyperaccumulator. In order to decode the chemical signals, this plant may emit through the roots to cope with stress; root exudates were collected, purified, and analyzed through GCMS. This revealed the presence of five major compounds, namely, palmitic acid, linoleic acid, oleic acid, campesterol, and stigmasterol, which mainly are metabolic markers for detoxification mechanisms triggered by various stresses. Therefore, based on this study, *C. rutidosperma* can be termed a potent hyperaccumulator and can further be exploited for remediation of other classes of environmental pollutants.

Keywords: phytoremediation, phytoextraction, chemical signaling, heavy metal, campesterol

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ORIGINAL ARTICLE

Antioxidant, antimicrobial and DNA damage protecting potential of hot taste spices: a comparative approach to validate their utilization as functional foods

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Abstract Hot taste spices have enormous health benefits starting from kitchen to pharmaceutical laboratories. Our present study is focused on phytochemical and pharmacological screening of six hot taste spices namely *Zingiber officinale* (ginger), *Capsicum annuum* (chilli), *Piper chaba* (java long pepper), *Piper nigrum* (black pepper), *Syzygium aromaticum* (clove), *Trachyspermum ammi* (carom). Among all six spices, clove and ginger exhibited strong antioxidant activity owing to higher phytochemical contents. Significant antifungal activity (IZD $\geq$ 11 mm) was revealed by all six spices except hexane fraction of carom whereas strong antibacterial activity with lowest MIC was displayed by clove, ginger and chilli. DNA was successfully protected from oxidative damage by clove, ginger followed by chilli, java long pepper and carom but black pepper could only partially protect DNA damage even at 4 mg/ml concentration. Based on the DNA damage protecting potentials and antioxidant activities clove, ginger, java long pepper and carom may be utilized for neutraceuticals development. Antimicrobial activities suggested that clove, ginger, java long pepper and chilli may be useful as food preservatives. Fractionated bioactivity of the all the six HTS would help for targeted extraction and development of nutraceuticals from these commonly used medicinal spices.

Keywords Spices · Antiradical potential · Oxidative DNA damage · Bioactive · Nutraceuticals

Abbreviations

HTS	Hot taste spices
ZO	*Zingiber officinale*
CA	*Capsicum annuum*
PC	*Piper chaba*
PN	*Piper nigrum*
SA	*Syzygium aromaticum*
TA	*Trachyspermum ammi*
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
RPA	Reducing power assay
LPA	Lipid peroxidation assay
MDA	Malondialdehyde
IZD	Inhibition zone diameter
MIC	Minimum inhibitory concentration

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Maleic and L-tartaric acids as new anti-sprouting agents for potatoes during storage in comparison to other efficient sprout suppressants

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Inhibiting sprouting of potatoes is an interesting subject needed for potato storage and industry. Sprouting degrades the quality of tuber along with releasing α-solanine and α-chaconine, which are harmful for health. Sprout suppressants, available in the market, are either costly or toxic to both health and environment. So, there is a need for developing countries to explore new sprouting suppressant compound which is cheap, non-toxic and reasonably efficient in comparison to commercial ones. We have established that simple maleic acid and L-tartaric acid are effective sprout suppressing agents. Both can hinder sprouting up to 6 weeks and 4 weeks post treatment respectively at room temperature in dark. These do not affect the quality parameters, retain the moisture content and maintain the stout appearance of the tubers along the total storage period. Thus maleic acid and L-tartaric acid would qualify as alternative, cheap, efficient sprout suppressant for potato storage and processing.

Potato (*Solanum tuberosum* L.) ranked fourth as the world's main food crop, following maize, wheat, and rice[1]. Being the world's number one non-grain food crop, its shelf-life is of great concern. Potatoes are generally stored at a low temperature for several months as a measure to delay sprouting[2]. Based on the geographical region, fresh potatoes are available only for a few months. Thus storage of potatoes is necessary to maintain supply throughout the year. Germination and growth of the eyes of potatoes, is a significant factor that contributes towards the weight loss of the potato and facilitates production of toxic α-solanine and α-chaconine with symptoms ranging from nausea, vomiting, diarrhea, and fever to delirium, coma, and even death[3,4]. It also alters the taste of the potato. Upon sprouting, respiration as well as transpiration increase rapidly which in turn increases the rate of physiological weight loss of stored tubers. Besides weight loss, sprouting also affects the nutritional values and quality of potatoes[5,6]. Sprouting causes higher rate of respiration, remobilization of storage compounds in the potato tubers mainly starch and proteins, besides shrinkage, due to loss of water[7]. Higher level of reducing sugar content also causes lower processing quality of potato tubers[8]. It also increases sugar concentrations through hydrolysis[9,10]. Sprouting also denatures potato quality parameters such as firmness and content of vitamin C[11].

The most widely used sprout suppressant on potatoes all over the world for more than 50 years is CIPC [isopropyl *N*-(3-chlorophenyl) carbamate][12]. The cost of CIPC treatment per kg of potato in cold storage houses ranges from 0.14 to 0.54 INR[13]. But continuous and long term use of CIPC leads to some toxicological effects on health and environment. It was reported that up to 45% of applied CIPC is persistent in the soil besides adhering to tubers[14]. It was observed that the CIPC residue in peel samples were fairly high about 15–85 mg/kg[15,16]. CIPC renders sprout growth by blocking the spindle formation during cell division[17,18]. For that reason its uptake into the body can also cause alteration in cellular structure and functions. In addition, due to the low solubility in water (89 mg/l), organic solvents (like; methanol or dichloromethane) are needed for its application as a fogging treatment which not only creates pollution to the environment but also imposes the risk on the personnel involved in treating/fogging application[19].

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Role of Tartaric Acid in the Ecology of a Zoochoric Fruit Species, *Tamarindus indica*. L

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ABSTRACT

Zoochoric plants usually produce fruits with a mechanical barrier in the exocarp, or chemical inhibitors in the fruit pulp (mesocarp) or seed coat (endocarp) to achieve required dormancy. *Tamarindus indica* L. is one such zoochoric tree species, but the role of possible bioactive chemicals in the ecology of its fruit and seed dispersal has not been explored before. We investigated the germination inhibitory effects of *T. indica* fruit pulp by comparing germination of intact *T. indica* fruits with depulped *T. indica* seeds. This experiment revealed that attached pulp (including exocarp) delayed or decreased germination via an unverified mechanism. We then analyzed the pulp (including exocarp) to find out if there was a dominant bioactive chemical, by creating extracts using both alcohol and aqueous fractionation, and then analyzed them using GLC-MS analysis. We discovered that tartaric acid was the main bioactive chemical present in the methanol fraction (but not aqueous), so decided to test its effect on *T. indica* seed germination. We exposed viable depulped *T. indica* seeds to varying concentrations of pure tartaric acid in distilled water and found germination inhibition was observed at a concentration of 0.4 mg/ml. Therefore, since tartaric acid is the major chemical component in *T. indica* fruit pulp, it is possible that it could play a crucial role in the zoochoric dispersal of seeds by inhibiting germination while they are still attached to the tree or have just fallen close to the parent tree so that they have an opportunity to reach distant sites favorable for germination.

KEYWORDS

Chromatography; fruit pulp; India; seed dormancy; *Tamarindus indica* L.; tartaric acid; zoochory

Introduction

Many plants produce fruits that are dependent on zoochoric agents (e.g. birds or mammals) for dispersal of their seeds, and can have unique mechanisms to delay the germination of seeds until they can reach distant sites that are favorable for germination and growth (Mayer and Poljakoff-Mayber, 1982; Sato, 2012). Such plants usually have mechanical or chemical inhibitors in the fruit, either in the exocarp, pulp or seed coat, to achieve the needed dormancy (Jordano, 2000). Once the fruit is consumed by the zoochoric agent, these inhibitors are removed either through digestion by saliva or passage through their alimentary canal and thus improve germination potential when finally dispersed to distant locations (Kiepiel and Johnson, 2019; Kissmann and Habermann, 2013; Pegman et al., 2017; Yagihashi et al., 1998).

Tamarindus indica L. is a leguminous tree (Fabaceae) that is solely dependent on animals for long distance seed dispersal and is a native and widely distributed species in Asia, with immense economic as well as traditional importance, and produces edible pod-like fruits. It is used for food, medicinal, cultural, social, environmental amelioration and income generation purposes (Havinga et al., 2010). The fruit pulp is thick, paste-like, strongly sour and highly acidic in nature and is used as food

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Potential oil resources from underutilized seeds of *Sterculia foetida*, L. - Quality assessment and chemical profiling with other edible vegetable oils based on fatty acid composition, oxidative stability, antioxidant activity and cytotoxicity

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ABSTRACT

Vegetable oils are integral part in production of manufactured food both in domestic and industrial scale. Vegetable oil market is increasing upward with a CAGR of 3.25% during the forecast period (2019–2024) all around the world. Therefore, it is necessary to find alternative sources of vegetable oil to fulfil the scarcity in the market. Seeds of *Sterculia foetida*, L. yielded considerable amount of oil (58.7 g/100g) as compared to other vegetable oils (such as sunflower, ground nut, mustard, soybean). Fatty acids composition of all the five tested oils showed that total fatty acid as well as unsaturated fatty acid percentage is higher in Sterculia seed oil. Proximate and mineral composition analysis suggested that Sterculia oil is a good source of protein, lipids, macro and micronutrients. Lowest TOTOX value (2.67) and higher iodine value (132–144) indicated its higher oxidative stability and presence of greater number of unsaturated bands in the fatty acid moieties which is also beneficial for human health. Sterculia oil exhibited lower IC_{50} values in DPPH (825.73 μg/ml), and NO (111.98 μg/ml) radical scavenging assays. In case of ABTS radical scavenging activity, no significant differences were observed in groundnut, mustard, sterculia and soybean except sunflower. Sterculia oil did not exhibit any cytotoxic effect on both normal and cancerous cell lines even at concentrations of 40 μg/ml as evident from MTT assay. Thus, seed oil of *Sterculia foetida* may be a cost-effective and viable source of safe nutritious edible oils to combat the present market demand.

1. Introduction

With a rising standard of living as well as industrial growth, demand for vegetable oils (VO) is also soaring in global market. It is expected that in 2014, global vegetable oil market is subjected to exceed 275 million metric tons due to the growing health consciousness of the consumers. Palm oil mainly dominated the world vegetable oil market by more than one-third of the total vegetable oil consumption and rest of the market occupied by soybean oil, canola oil and sunflower seed oil (Mielke, 2018). Though palm oil represents one potential source to meet this demand yet its consumption is associated with high risk of cardiovascular disease (World Health Organization, 2003; Ismail et al., 2018) and also it is eco-destructive. In Asia-Pacific regions, vegetable oil market extended upward steadily with a CAGR of 5.4% over the analysis period due to the factors such as population growth, simultaneous growth of food commodities, changing dietary habits; rapid urbanization; improving living standards; increasing crop yields and oil production and growing biofuel production in countries such as Indonesia, Malaysia, Thailand, Philippines, China and India. At present, there is a great demand of VO internationally, but alternative sources are very few. Therefore, it is necessary to find alternative sources of VO from underutilized oil resources to fulfil the global scarcity of VO applying non-agriculture land (Shi et al., 2019).

Sterculia foetida, L. commonly known as bastard poon, java olive, hazel sterculia, wild almond, is a large, straight, deciduous tree. It grows up to 40m in height and 3m in girth and its branches are

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TECHNICAL REPORT

A comprehensive study on occurrence records of African neglected and underutilized weed species, *Cleome gynandra* L. (cat's whiskers) validating the ecogeographical range expansion in West Bengal, India

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Cleome gynandra L., commonly known as cat's whiskers, is an erect, branched, annual herb, belonging to the family Cleomaceae. The species is thought to have originated in tropical Africa and Southeast Asia, and thereafter have spread to other tropical and subtropical countries in the Northern and Southern hemispheres. Cat's whiskers is a highly nutritious vegetable and also possesses numerous medicinal values, yet it is considered as a neglected and underutilized weed species (NUS) in most of the world. In India, *C. gynandra* is recorded in Assam, Gujarat, Kerala, Maharashtra, Rajasthan, Tamil Nadu, and Uttar Pradesh. There is no earlier report of this species in West Bengal, India. This report is the first record of natural occurrence of *C. gynandra* in West Bengal, India. The main objective of this report is to provide valid information about the invasion and naturalization of this species in a new geographical area supported with suitable data. Global distribution data of cat's whiskers was retrieved from available web resources and mapped using Quantum GIS software in order to validate the *de novo* nature of occurrence of this species in this region.

Keywords: Cat's whiskers, *Cleome gynandra* L., global distribution, NUS, Quantum GIS.

Cat's whiskers (*Cleome gynandra* L.), is described as a NUS, i.e., neglected and underutilized species by Hammer *et al.* (2001). The species is thought to have originated in tropical Africa, from where it has spread to other tropical and subtropical countries in the Northern and Southern hemispheres (Chweya & Mnzava 1997). Brown *et al.* (2005) and Feodorova *et al.* (2010) have recognized the species to have a C_4 photosynthetic pathway, an adaptive mechanism that enables it to survive in drier and hot environments. It persists well in semiarid, subhumid, and humid climates, and is suited to many soil types, but grows luxuriantly around rubbish dumps and soils supplied with organic manure.

The plant is a highly nutritious leafy vegetable with rich source of vitamins A and C, calcium, iron, and proteins. It is consumed by local people throughout Africa (Jinazali *et al.* 2017; Omondi *et al.* 2017). In some African countries such as Zambia, Zimbabwe, Botswana, Malawi, Uganda, Tanzania, and Kenya, the leaves and young tender shoots are sold in rural and urban markets by rural women (Van den Heever & Venter 2007). This vegetable resource plays a significant role in nutrition, food security, and income generation for the poor and the unemployed people in rural populations in the Third World Country (Onyango *et al.* 2013). Ethanol and aqueous extracts of *C. gynandra* leaves possess the highest antinociceptive activity (Ghogare *et al.* 2009). Flavonoids and tannins observed in the active extracts are responsible for the

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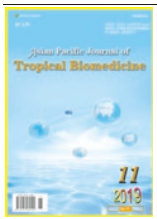

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Phytochemical profiling of *Artocarpus lakoocha* Roxb. leaf methanol extract and its antioxidant, antimicrobial and antioxidative activities

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ABSTRACT

Objective: To explore the phytochemical profile of *Artocarpus lakoocha* Roxb. leaves both qualitatively and quantitatively, and validate its role as a potent antioxidant and antimicrobial agent.

Methods: Extraction and isolation of different compounds were done from the leaves of *Artocarpus lakoocha* based on solvent fractionation method. Subsequently, quantitative and qualitative phytochemical profiling along with antioxidant, antimicrobial and antioxidative activities were tested following standard protocols.

Results: Among the five fractions, methanol fraction of *Artocarpus lakoocha* exhibited higher content of phytochemical compounds [phenols = (3 175.21±290.43) mg GAE/g dry extract, flavonoids = (1 173.15±47.52) mg QE/g dry extract and tannins = (923.53±95.21) mg TAE/g dry extract] as compared to other fractions. The methanol fraction showed the highest antioxidant activity in DPPH and ABTS radical scavenging assays with IC_{50} of (111.98±34.20) μg/mL and (138.26±0.66) μg/mL, respectively, and the best reduction potential with a value of (316.81±2.96) mg QE/g dry extract in reducing power assay. There was significant correlation between the amount of phytochemicals and antioxidant activities. Moreover, the extract successfully protected Lambda phage DNA from damage at 5 and 6 mg/mL concentration and exhibited substantial bactericidal as well as fungicidal activity. The GC-MS analysis of methanol fraction of *Artocarpus lakoocha* revealed diethyl phthalate as the main phytochemical compound, along with 3,4-dihydroxymandelic acid, 9-octyl eicosane and 7,8-didehydro-3-methoxy-17-methyl-6-methylene morphinan.

Conclusions: The methanol fraction of *Artocarpus lakoocha* could be used as a potent antioxidant and antimicrobial agent for sustainable agriculture and pharmaceutical purposes.

1. Introduction

Plants are rich sources of secondary metabolites, such as phenols, tannins, and flavonoids, which have been found to have *in vitro* antimicrobial and antioxidant properties. Control of plant pathogens and combating plant's stress by biological means is of great significance for sustainable agriculture and restoring healthy ecosystems. At present, major research emphasis has been given to discover biologically active natural products from various plants that

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