Allelopathic Activity Evaluation in Selected Aromatic Plants and its Application as Bio-pesticide

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In the name of Allah, the most beneficent, the most merciful.

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Thesis submitted to Department of Environmental Science, International Islamic University, Islamabad as a partial fulfillment of the requirement for the award of the Degree of PhD in Environmental Science

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DEDICATION

Dedicated to my beloved mother, father, husband and sons.

DECLARATION

I hereby declare that the work present in this thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition.

No part of the thesis has been previously presented for any other degree.

Date: 13-09-2022

Sajida Begum

FORWARDING SHEET
BY
RESEARCH SUPERVISOR

The thesis entitled "Allelopathic Activity Evaluation in Selected Aromatic Plants and its Application as Bio-pesticide" submitted by Sajida Begum in partial fulfillment of PhD degree in Environmental Science has been completed under my guidance and supervision. I am satisfied with the quality of student's research work and allow her to submit this thesis for further process to graduate with PhD degree from the Department of Environmental Science, International Islamic University, Islamabad, as per IIU rules and regulations.

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Dated: _____

ABSTRACT

Allelochemicals present in the aromatic plants can be used for protection of crops and natural ecosystem. This study assessed allelopathic potential of selected aromatic plants. For this purpose, 121 plants were collected from different regions of Pakistan and their allelopathic potential was studied based on lettuce hypocotyl and radical growth inhibition of *Lactuca sativa* through dish pack method. Among identified plants *Justicia adhatoda* was top indigenous plant screened out due to its maximum allelopathic potential among wild plants. Hence, *J. adhatoda* was further analyzed for identification of allelochemicals through GC-MS analysis. Phytochemical analysis revealed the presence of 14 allelochemicals in the dried leaves of *J. adhatoda* and prominent compounds include1,2-Benzenedicarboxylic acid, diisooctyl ester (80.93 %) and *n*-hexadeconoic acid (11.31 %). The cytotoxic activity of 1,2-benzenedicarboxylic acid, diisooctyl ester is the cause of hypocotyl and radical growth inhibition of *L. sativa* Twenty-six allelochemicals were identified in the methanolic leaf extract of *J. adhatoda* through GC-MS analysis of which N, N-Dimethylglycine (19.82%), 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro- (12.67%), Octadecanoic acid (7.45%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (6.98%), Oleic Acid (6.745), 9-Octadecenoic acid (*Z*)-, 2,3-dihydroxypropyl ester (5.84%), *n*-Hexadeconoic acid (5.09%) were the prominent compounds. The methanolic leaf extract of *Justicia adhatoda* was applied against three fungal species of Aspergillus i.e., *A. niger, A. flavus* and *A. terreus* (common crop pests) The extract was most effective against *A. terreus* (24mm) followed by *A. niger* (22mm) and *A. flavus* (16mm) at the concentration of 200 mg/ml. The allelochemicals identified in the methanolic leaf extract of *J. adhatoda* were the cause of fungal growth inhibition.

In addition to the above cited results, another experiment was carried out to check allelopathic potential of remaining top plants in aquatic environment. Methanolic leaf extracts of experimental plants were used for identification and quantification of allelochemicals by HPLC and LC-MS analysis. Through HPLC, the detected allelochemicals include caffeic acid, rutin, trans-ferulic acid and naringin. In *M. royleana*, naringin concentration was 4.77±0.48 µg/mL followed by caffeic acid 4.67±0.03 µg/mL and rutin 4.22±0.31 µg/mL. The leaf extracts of above-mentioned plants were applied on *C. reinhardtii* to check their allelopathic inhibitory potential. Leaf extracts of plants significantly inhibited growth of Chlamydomonas ($p<0.05$). The p value against each plant species were: *M. royleana* ($p<0.05$), *M. azedarach* ($p<0.05$), *C.*

citratus ($p<0.05$), *M. philippensis* ($p<0.01$) and *R. sativus* ($p<0.01$); thus, indicating that number of cells of *C. reinhardtii* were significantly inhibited.

The inhibitory effect of methanolic leaf extracts on *C. reinhardtii* strongly supports the hypothesis that experimental plants have ample potential to be used as algicides due to the presence of allelochemicals in their leaves. The most prominent compound identified in the *J. adhatoda* was 1,2-Benzenedicarboxylic acid, diisooctyl ester having strong herbicidal and antifungal properties. In view the aforesaid findings, this research is useful to comprehend the role of allelopathic potential in the plants to control the weed, fungi and algal blooms. *J. adhatoda* has previously been used for medicinal purposes, however, in this research an endeavor is made to explore application of *J. adhatoda* to control the weeds and fungal infection; thus, giving research a new direction to discover allelochemicals present in the aromatic plants especially *J. adhatoda* as a natural source for control of weed and fungal growth.

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Abbreviations and Acronyms

AJK	Azad Jammu and Kashmir
ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionization
ATP	Adenosine Triphosphate
DMSO	Dimethyl Sulfoxide
EIC	Extracted Ion Chromatogram
ESI	Electrospray Ionization
GC/MS	Gas Chromatography–Mass Spectrometry
GC-FID	Gas Chromatography Flame-ionization Detection
HAB	Harmful algal blooms
HABs	Harmful algal blooms
HPLC	High-performance liquid chromatography
HPTLC	High Performance Thin-Layer Chromatography
IAA	Indole acetic acid
ICT	Islamabad Capital Territory
LCMS	Liquid Chromatography Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MAPs	Medicinal and Aromatic Plants
MIC	Determination of minimum inhibitory concentration
MRSA	Methicillin Resistant and sensitive Staphylococcus Aureus
MS	Mass Spectrometry
PTFE	Polytetrafluoroethylene
SDV	Standard Deviation Variance
TAP	Tris-Acetate-Phosphate medium
UCR	University of California Riverside
UV	Ultra Violet
UV-VIS	Ultraviolet–Visible Spectroscopy

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CHAPTER 1

INTRODUCTION

Allelopathy is the ability of plants which causes growth inhibition or stimulation for the neighboring plants due to discharge of chemicals in the environment. The concept of allelopathy was introduced by Hans Molisch who described both the harmful and beneficial chemical interaction of plants and microorganisms (Narwal & Jain, 1994). Afterwards, the term "allelopathy" has undergone various changes and now it is described as any type of direct or indirect beneficial or detrimental effects of a plant on other plants by producing chemical compounds called allelochemicals released into the environment (Rice, 1984). Tukey (1969) describes it a phenomenon through which allelopathic plants discharge chemical compounds via exudation from leaves, roots and stems as well as by volatilization from decaying tissues. Thus, the likely channel of allelopathy could happen in three ways: exudation, leaching and volatilization (Weston & Duke, 2003). Allelopathy is a known biological phenomenon in which an organism produces chemicals which effects the growth, reproduction, development and survival of other organisms. These biochemicals have both positive and negative effects on other organisms. For sustainable agricultural, it is crucial to develop cultivation systems by taking advantage of the stimulatory/inhibitory properties of allelopathic plants for the regulation of plant growth and development and to evade allelopathic autotoxicity of these chemicals. Allelochemicals can be used as herbicides, insecticides, growth regulators, antimicrobial for plant protection (Cheng and Cheng, 2015). The subject of allelopathy is receiving ample attention from scientists due to its agro-ecological applications, which can provide substitutes to synthetic herbicides for weed control.

1.1 Aromatic Plants

Plants that have aroma characteristics are called aromatic plants. These aromatic properties in the plants are due to the presence of essential oils known as volatile compounds. Aromatic plants generally produce and exude aromatic substances which are mostly in the form of ether oils which are not only used in foods and making perfumes but are also used in the liquor and pharmaceutical industries. Most aromatic plants species belong to Lauraceae, Myrtaceae, Umbelliferae and Labiatae families. Aromatics plants have been used traditionally in the folk medicine and also to extend foods shelf life due to inhibition ability of aromatic compounds against bacteria, yeasts and fungi. Most of these properties are due to the presence of essential

oils which are produced by secondary metabolites i.e., allelochemicals (Sartoratto et al., 2004). Some plants are used for their specific medicinal or aromatic properties in pharmaceutical and perfume industry are termed as Medicinal and Aromatic Plants (MAPs) (Lubbe & Verpoorte, 2011).

1.2 Allelochemicals

The biochemicals are considered as allelochemicals and could have beneficial (positive allelopathy) or detrimental (negative allelopathy) effects on the target plants (Reigosa et al., 2006; Cheng & Cheng, 2015). Allelochemicals are produced by the plants as by-products, end-products and metabolites, and these allelochemicals naturally exist in the stems, leaves, flowers, inflorescences, fruits, seeds and plants roots (Sisodia & Siddiqui, 2010). Release of these allelochemicals into the environment affects other organisms such as plants, weeds and microorganisms either by inhibiting or stimulating their activities (Fujii et al., 2003). It is evident from the literature that these chemicals produced by the plants have the ability to suppress germination and growth of various weed species (Singh *et al.*, 2003; Turk & Tawaha 2003; Sampietro & Vattuone 2006; Mohsenzadeh et al., 2011). Allelochemicals can be classified into different categories according to their different properties and structures (Soltys et al., 2013) such as straight-chain alcohols, water-soluble organic acids, aliphatic aldehydes, simple lactones and ketones phenolics, cinnamic acid and its derivatives, flavonoids, coumarins, steroids, tannins and terpenoids, long-chain fatty acids, polyacetylenes and quinines.

Rutin and quercetin in high concentration inhibit the germination of many plant species by impacting the respiratory process and the concentration of ATP during embryonic stage of plant due to the inhibition of phosphate uptake or substrate oxidation by uncoupling oxidative phosphorylation (Macias et al., 2004). Moreover, treatment of Arabidopsis with rutin was observed as a significant reduction of the cell's protein content (Hussain & Reigosa, 2016). Caffeic acid inhibits the growth of plants as it inhibits leafy spurge (*Euphorbia esula*) seed germination, root length and growth of callus culture (Barkosky et al., 2000).

Singh et al. (2014) stated that ferulic acid showed substantial allelopathic potential in the form of inhibitory effects on various biophysical and biochemical characteristics of tomato. The inhibitory effect of ferulic acid was observed on fresh weight, dry weight, root and shoot length of tomato seedlings. Allelopathic inhibition was also observed on the chlorophyll-a, chlorophyll-b and carotenoids content with increase in the concentration of ferulic acid. High

concentration of ferulic acid significantly reduce protein, nitrate reductase and sugar contents. However, high concentrations of Ferulic acid significantly enhance antioxidant enzymes such as superoxide dismutase, catalase and peroxidase which is the appearance of tolerance or a defense mechanism to allelochemicals. Ateyyat et al. (2012) reported that significant allelopathic effect of quercetin hydrate, rutin and naringin was observed by the increased mortality rate of nymphs after 24 hours of application, and it also increased with the increase of concentration.

1.3 Phenolic Compounds

Secondary metabolites present in the plants are the group of organic compounds with low molecular weight. These metabolites help plants to make a defense mechanism by interacting biotically or abiotically with the environment (Murthy et al., 2014). Their function is not vital for normal growth and developmental process, but their synthesis is necessary for plants defense and protection (Bennett & Wallsgrove, 1994). The unique secondary metabolites are produced in the specific plant species which are beneficial for humanity (Dixon, 2001). Many kinds of secondary metabolites are manufactured by plants and are being used by mankind since the ancient times for various needs (Bourgaud et al, 2001). Biosynthesis of secondary metabolites from the primary metabolite originates by sharing substrates of the primary metabolite (Yamazaki et al., 2003a). According to Cheynier et al., (2013), it is necessary to maintain a balance between the growth and defense mechanism by regulating the carbon fluxes between primary and secondary metabolites for effective adaptation to environmental stresses and for protection mechanism. The quantity of metabolites in the plants is normally very low and depends on the plant physiological and developmental stages (Facchini, 2001). Secondary metabolites consist of phenolic compounds, nitrogen-containing compounds and terpenoids. Secondary metabolites may denote chemical adaptations for broader range of stress conditions of environment and act as chemical agents (protective and defensive) against microorganisms, insects and herbivorous (Namdeo, 2007). When microorganisms invade the plants, then it causes production of secondary metabolites which are anti-microbial in a similar manner as abiotic stress agents such as osmotic shock, radiation, inorganic salts, fatty acids and heavy metal ions cause the production of secondary metabolites for protection in plants. Ultra violet radiations, nutrient deficiencies, temperature, high light and use of herbicide are source of abiotic stresses and become the cause of increased synthesis and accumulation of phenylpropanoids. Both biotic and abiotic stresses cause the synthesis oof secondary

metabolites in plants (Akula & Ravishankar, 2011). The effect of biotic and abiotic stresses and their function in the synthesis of secondary metabolites have been extensively reported in the literature (Naik & Al-Khayri, 2016). Secondary metabolites synthesis can be enhanced by the induction of elicitors in the cell cultures through the culture medium (Srivastava & Srivastava, 2014; Ahlawat *et al.*, 2014; Shakeran et al., 2015; Hashemi & Naghavi 2016). Several secondary metabolites are present in the extract of *Asperigillus niger* (Ahmed & Baig, 2014), which are also present in the yeast extract (Deepthi & Satheeshkumar, 2016). Secondary metabolites can be classified on their biosynthetic origin, plants secondary metabolites are placed in three main groups

which are phenolic compounds, terpenoids and nitrogen-containing compounds. The main group of secondary metabolites include the Phenolic compounds and are synthesized by plants which have many applications (see Figure 1.1) for different purposes (Kabera et al., 2014).

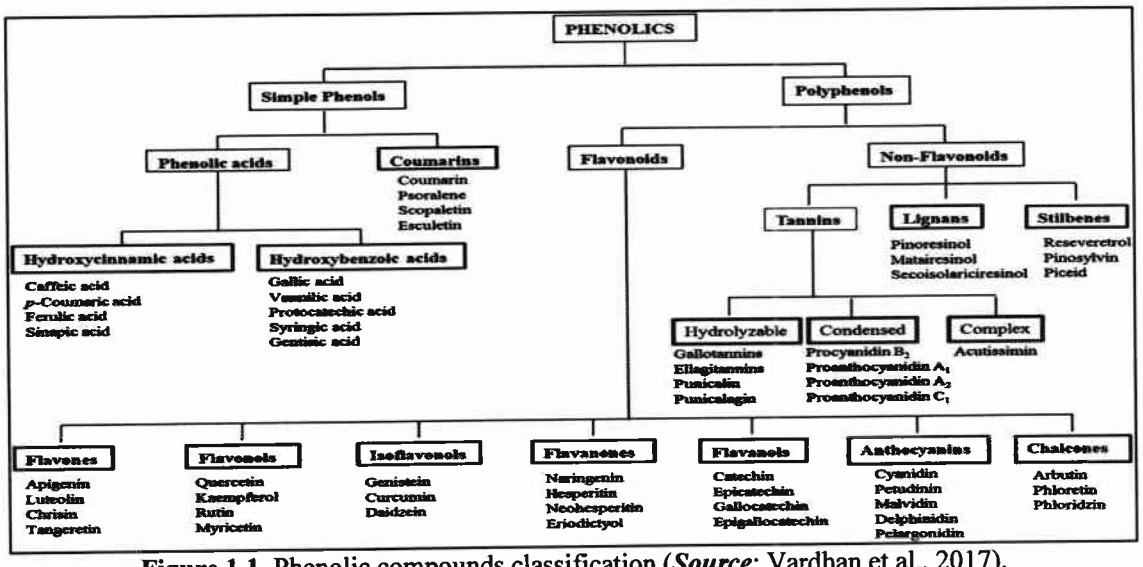


Figure 1.1. Phenolic compounds classification (***Source***: Vardhan et al., 2017).

Phenolics are normally present in the form of esters or glycosides and are conjugated with other natural compounds, for example flavonoids, sterols and alcohols (Dai & Mumper, 2010). Several phenolic compounds contain anti-inflammatory, antiseptic, antioxidant, anthelmintic, anti-bacterial and other pharmacological and biological properties (Stankovic, 2011; Cueva et al., 2010). Polyphenols are principal compounds among phenolic compounds owing to their abundant presence in nature and also their diversity and their apparent preventive function from various ailments e.g., cancer, neurodegenerative and cardiovascular diseases (Scalbert et al., 2005). Polyphenols make an extensive group of secondary metabolites which contains about 8000 identified compounds. Polyphenols can be categorized in to two main classes i.e.,

flavonoids and non-flavonoids, and this classification is based on the chemical structure differences (Galeotti et al., 2008). Moreover, flavonoids can be placed in three groups which are anthocyanins, flavones and flavanols. Flavones are colorless crystalline water-insoluble aromatic ketones. Flavanols is a subgroup of flavonoids and includes kaempferol and quercetin.

1.4 Importance of Allelopathy

Use of synthetic chemicals in the form of herbicides in agriculture sector has caused numerous ecological and environmental problems. These synthetic chemicals are destructive to ecological balance and become the cause of introducing various ailments which could be fatal. Due to these detrimental effects, there is crucial need of sustainable agriculture by using eco-friendly options. Allelopathic potential of plants is a great alternative of synthetic herbicides in agroecosystem for weed management (Asaduzzaman et al., 2010).

1.4.1 Way of Allelopathic Action

Competition is an important phenomenon in the biosphere. Plants compete for their survival e.g., they compete for water, sunlight, nutrients and space as other living organisms compete for their survival and this competition becomes the base for allelopathy. Plants having allelopathic characteristics use their chemical power to win the competition and use efficiently available natural resources. Allelopathy way of action goes through the following process:

- Allelopathic plants discharge chemicals into the soil from their roots, and these chemicals cause the suppression of growth and development of neighboring plants. These allelochemicals inhibit the chlorophyll production of the neighboring plant and slow down the photosynthetic process which ultimately leads to death of plants (Yu et al., 2006).
- Various allelopathic plants secrete allelochemicals in gaseous form. These allelochemicals are discharged from the small pores present in their leaves. Neighboring plants absorb these gaseous biochemicals and their growth and development are inhibited due to suppression of photosynthetic and other biological process (Yu et al., 2006).
- When leaves of allelopathic plants drop on the ground/soil, they decompose and release chemicals into the soil which are absorbed by the neighboring plants and ultimately become the cause of their growth/development inhibition or death (Shiraishi et al., 2002).

1.4.2 Importance of Allelopathy in Weed Control

There is a worldwide use of chemical herbicides in enormous amount for the management of weeds present in different crops. However, synthetic herbicides like insecticides, fungicides, herbicides and pesticides etc., which are usually toxic and deteriorate the environment (Sodaeizadeh et al., 2009; Scavo & Mauromicale, 2021). The extensive use of synthetic herbicides has led to the development of resistance in the weed biotypes (Sodaeizadeh et al., 2009). Worldwide efforts are being made to reduce the use of chemicals in agriculture practices by swapping with biological and ecological methods for sustainable production of crops. The best and possible solution to replace synthetic chemicals with natural compounds extracted from plants i.e., use of allelopathic potential by exploring the negative interactions among plants (Azizi & Fujii, 2005). The use of allelopathic potential is highly recognized in sustainable production of crops (Khan et al., 2009). The trend of use of allelopathic potential is increasing (Anjum et al., 2010). Various methods are developed to identify the allelopathic potential of plants for the use of allelopathy to control the weeds naturally (Taiwo & Makinde, 2005; Terzi, 2008). To attain sustainable growth, a comprehensive weed management system is crucial to cater dependency on synthetic herbicides and manual removal of weeds from the fields.

A bioherbicide is a biologically based control agent for weeds. Dhima et al. (2009) state that the use of cover crop mulches is a new strategy in agricultural sector for suppressing weeds and reducing the chemical inputs. The focus of their study was based on the use of cover crops in the summer crops for weed control. The study indicated that some winter cereals and legumes are used as living mulches which were grown under the crop simultaneously or incorporated as green manure into the soil or used as a stubble or bio-herbicide. This strategy can considerably lessen the biomass and density of many weed species present in maize and soybean crops. Cover crops have the ability to discharge toxic materials into soil and make the environment hostile for germination and growth of weeds and this method becomes the reason for weed growth inhibition (Ben-Hammouda et al., 2001).

1.4.3 Algal blooms and Allelopathic Control

An algal bloom (or algae bloom) is considered as a prompt increase or buildup in the concentration of algae in both freshwater and marine water systems. It is generally distinguished due to staining in the water from algae pigments. The algal blooms are increasingly becoming a genuine concern for the public health and resulting as an ecological issue in wetlands and waterways such as canals, ponds, lakes, rivers and creeks. Harmful algal blooms (HAB) are increasing both in intensity and frequency for the last few decades. It is

deliberated that disproportionate nutrient enrichment through anthropogenic inputs are main cause of the amplified pervasiveness of HABs (Millie et al., 1999).

Typically, algae are produced in nutrient-enriched lakes almost throughout the year, particularly in the summer season and more specifically in the month of September. Mostly, the water surface is covered with abundance of algae that looks blue-greenor green powder is generally considered as water bloom. These water bloom algae cause pungent nasty smell and yield toxin resulting in various kinds of diseases in humans and animals. This contamination of water can directly result of in death of living stocks who drink it. Also, water blooms result in reduction of oxygen in the water reservoirs, which severely affects and may cause death of the aquatic animals. Presence of detrimental algae blooms have been reported as the foremost environmental issue in almost 50 states of USA. *Chlamydomonas reinhardtii* is a single-cell green alga. It is approximately 10µm in diameter and swims on the water with two flagella. Its cell wall is made of hydroxyproline-rich glycoproteins (a large-shaped chloroplast) which is a huge pyrenoid and an eyespot that can sense light. The bloom-forming Chlamydomonas has relatively extreme initial enzyme activities and can cause disturbance in nutrient (Kruskopf & Plessis, 2004).

Chlamydomonas frequently develops blooms and harmful algal blooms are the reason for bad water smell and produce toxins which become the cause of many human and animal ailments or directly affect livestock due to drinking the contaminated water. *Chlamydomonas* utilized a K-strategy in its growth pattern by showing slow growth rate during optimal conditions and high growth rate during nutrient limitation. It has capability to form algal blooms when conditions are not favorable to other algal species. *Chlamydomonas* members can be classified as harmful algal bloom species due to their ability to frequently clog the water purification filters (Kruskopf & Plessis, 2004).

Protection of water environment from algal bloom is an important issue because algal bloom has many negative impacts on aquatic ecosystem, aquaculture, human health as well as landscaping. It is crucial to develop an environment friendly, expedient and cost-effective method to control the algal bloom, which is becoming major concern. Use of allelopathic potential of aquatic and terrestrial plants as a protection from the destruction of algal bloom is new and secure method. Ziwen et al. (1992) showed that growth of *Chlamydomonas reinhardtii* was inhibited by the use of leachates of *Pistia stratiotes, Alternanthera philoxcroides, Azolla imbricate, Lemna minor* and *Spirodela polyrhiza* by different rates. *Eichhornia crassipes* showed more inhibition as compare to the other species. They also showed in their study that

broken cells formed irregular fragments when growth of *Chlamydomonas reinhardtii* was inhibited by the application of *Pistia stratiotes* root exudates.

Wenhao et al. (1993) also describe that there is significant inhibition of *Chlamydomonas reinhardtii* by the use of root exudates of *Eichhornia crassipes.* Ziwen et al. (1992) reported that *Chlamydomonas reinhardtii* was inhibited with different rates when treating with the leachates of *Alternanthera philoxcroides, Lemna minor, Pistia stratiotes, Spirodela polyrhiza, Azolla imbricate* and stimulated by leachate of*Nasturtium officinale* liquor.

Controlling eutrophication caused by excessive nutrients input into the aquatic ecosystem is a big challenge. Eutrophication enhances the growth of green algae and cyanobacteria resulting in formation of blooms. Chlamydomonas reinhardtii Dangeard is a unicellular green alga found in temperate regions in pelagic zone lakes and occasionally develops the blooms (Krivtsov et al., 2000). The *C. reinhardtii* is photosynthetic algae but in stressed conditions, due to K-strategy, its growth is increased which causes Harmful Algal Blooms (HABs) (Kruskopf et al., 2004). Increase in algal blooms is a serious threat to ecosystem and water quality (Dodds et al., 2009). Algal bloom control is an important concern to protect the aquatic ecosystem, because floating algal blooms suffocate plants and animals present in the aquatic environment and reduce aquatic biodiversity (Verschuren et al., 2002). Globally, the aquatic ecosystem is badly affected by devastating impact of algal blooms. HABs can cause serious threat to the aquatic ecosystem and use of allelochemicals obtained from the plants is an environment friendly approach. *Mallotous philpinensis* parts are used to prepare dyes, pesticide and religious rituals, etc. and also have medicinal uses as vermifuge, purgative, antibacterial, anticancer and wound healing (Buha et al., 2020). *Melia azedarach* L. timber has multiple uses such as construction, furniture, farm tools, boats and musical instruments (Kumar et al., 2017). Besides, it has high potential to control pests and medicinal properties (Khoshraftar et al., 2020). *Mentha royleana* is a food ingredient with medicinal properties (Majeed et al., 2021). Radish is part of food and has medicinal properties (Do et al., 2021).

To control HABs, we need sustainable methods. Allelopathy is an evolving field with its sustainable approach to control weeds and algae. Allelochemicals inhibits the growth of algae by impairing structure of its cells, inhibiting enzymes activity and affecting the rate of photosynthesis (Tan et al., 2019). Zuo et al. (2014) investigated allelopathic potential of aquatic plant species and inferred that these plants could play an important role to control harmful algal bloom. Allelochemicals are naturally present in the plants and are effective to inhibit the growth of neighbouring plants and act as algaecides (Ni et al 2012). Zuo et al. (2011) reported that various plants showed inhibitory effects on the algal growth e.g., Chlamydomonas reinhardtii

Dangeard growth was inhibited by application of grape extracts; the authors also studied effects of garlic juice on harmful algal species e.g., *Alexandrium tamarense*, *A. catenella*, *A. satoanum* and *Scrippsiella trochoidea* and reported that garlic has inhibited the growth of algae. Aragão et al. (2015) found that allelochemicals from eucalyptus may be developed as ecofriendly herbicide for sustainable ecosystem.

In this study, Chlamydomonas algae is selected because it is common and is broadly distributed throughout the world and is present in the fresh water worldwide. *Chlamydomonas reinhardtii* is a biological model and is a widely studied organism as it can be easily cultured and its genetics can be manipulated.

1.5 Gas Chromatography Mass Spectrometry (GCMS)

Gas chromatography–mass spectrometry (GC-MS) is a hybrid analytical technique and it joins the separation abilities of GC with the detection capabilities of MS to provide a greater efficiency for sample analysis. GC can separate volatile components in a sample and MS helps break the components and identify them on the basis of their mass. GC-MS offers enhanced sample identification with effective analysis, higher sensitivity, quick and better results. This technique is a useful method for the analysis of volatile components. The working principle of this technique is that a mixture separates into individual substances when heated. The heated gases run through a column containing inert gas (such as helium). As the substances is separated after emerging from the column opening, these substances move into the MS.

1.6 Antifungal Effects of *Justicia adhatoda*

Justicia adhatoda belongs to the family Acanthaceae and its local names are Baikar and Vasaka. It is found in Pakistan, India, Indonesia, Malaya, Panama and South East Asia. Its leaves and roots are used for rheumatism, pneumonia, cough, snake-bites, eye and ear ailments, asthma and tuberculosis (Haider et al., 2011), but the allelopathic potential of *Justicia adhatoda* for weed control has not yet been investigated extensively. Although some studies have shown that *Justicia adhatoda* could potentially be a good source of functional groups to control weeds. Justicia genus has been revealed to contain a variety of functional substances comprising vitamin C, triterpene acids, amino acids, fatty acids, polyphenols, polysaccharides, triterpene acids, flavonoids, saponins, alkaloids and indole derivatives (Khanom, 2017). The use of compounds present in this plant especially for crop protection was not a focused area as for the medical treatment.

Fungi cause 20–40% of known plant diseases. Usually, application of chemical fungicides is the most common method to control fungal infections in the plants to obtain better quality yield, but there could be substantial harmful effects in the future due to gradual development of resistance in the fungi. Moreover, synthetic fungicides have the ability to trigger lethal medical consequences for human beings such as cancer (Elshafie et al., 2017; Da et al., 2019). *Aspergillus terreus* is a filamentous pathogenic fungus which is harmful to the animals, plants and humans. There is a general consensus among the research community that *Aspergillus terreus* can affect potato (*Solanum tuberosum* L.) by causing foliar blight disease which results in abnormalities in the epidermal cells and leaf tissues (Louis et al., 2014). *Aspergillus niger* and *Aspergillus flavus* are also responsible for post-harvest loss in vegetables like potato, cucumber, carrot, okra, eggplant, round gourd, turnip, cauliflower and chilli. There is a need to overcome crop loss due to infection cause by these harmful fungi (Perveen et al., 2021).

1.7 High Performance Liquid Chromatography (HPLC)

HPLC is a foremost analytical technique which is generally used for the identification and quantification of components of liquid samples. In this method two phases namely mobile phase and stationary phase are used. A solution (liquid) is pumped through a column having a packaging of tiny porous particles which increase the surface area for the interaction of two phases and separates the components from the parent compound on the basis of their polarity. The column is considered as the main part of this technique. Different solubilities of the compounds (sample components) in the two phases trigger the components to move within the column with various average velocities, thus establishing a separation of the components of the sample. The solution which is pumped is called the mobile phase and the phase present in the column is known as the stationary phase. HPLC technique works with high flow rate so the pressure is high.

There are various modes of liquid chromatography, which depend on the nature of stationary and mobile phase used in the method. In reverse phase chromatographic technique, mobile phase is polar and stationary phase is not polar. The mobile phase is composed of an aqueous buffer added with acetonitrile which is an organic modifier and its addition is according to the eluting strength. The stationary phase to be used is C18 hydrocarbon groups bonded to 3 μm silica particles. In this form, the silica which is water soluble can be used for providing a wide range of applications.

During an HPLC analysis, a pump with high-pressure procures the mobile phase from a

reservoir via an injector. Then it passes through a C18-packed column (reverse-phase) for component identification. At last, the mobile phase goes into detector cell and the absorbance of components is measured at specific wavelength e.g., 220 nm, and at the end all the waste passes to a waste bottle. Time required for the movement of component from the injector to the detector is known as retention time.

1.8 Problem Statement

The use of allelopathic potential is required for a sustainable environment. Synthetic herbicides, fungicides and algicides are the major cause of environmental toxicity. The harmful effects due to gradual development of resistance in the weeds, fungi and algae against synthetic herbicides has become a major challenge. Moreover, synthetic chemicals used in herbicides, fungicides and algaecides have the ability to trigger various environmental problems and lethal medical aftermath. Hence, there is a need to replace synthetic herbicides with natural alternatives such as allelochemicals.

1.9 Hypothesis

The core hypothesis formulated for this thesis is:

"The allelochemical exudates from aromatic plants into the environment affect the associated/neighboring plants and other living organisms."

1.10 Research Objectives

The objectives of this study include the following:

1. Screening of selected aromatic plants for their allelopathic potential through bioassay evaluation.
2. Isolation and identification of active compounds in the selected aromatic plants using the GC-MS and HPLC.
3. Assessing potential inhibitory effects against selected plant pathogens and their impact on the biotic environment.

1.11 Research Significance

Aromatic plants are a group of plants having sustainable source of natural compounds that endure numerous benefits. Since the ancient times, natural compounds found in the plants are commonly being used for healing ailments, foods flavoring and fragrance formulation. Nowadays, these plants are grown and used as natural herbicides for the treatment of weeds in a sustainable way. There is a commercial production of variety of constituents from plants on commercial scale. There is a need to screen the natural flora (aromatic plants) of Pakistan for getting promising (biological) activity. In this study, 121 plants belonging to 100 genera and 50 families were selected to screen the allelopathic potential present in them through dish pack method. This research is useful for understanding the role of allelopathic management studies for protection of agroecosystem against weeds. This study could also be useful to develop awareness in formers and may be helpful for researcher by developing baseline data.

Fungi cause 20–40% of known plant diseases. The use of chemical fungicides is the most common method to control fungal infections for better produce. There could be significant harmful effects in the future due the development of resistance in the fungi and these chemicals also cause medical problems in have the ability to trigger lethal medical consequences for human beings such as cancer (Elshafie et al., 2017; Da et al., 2017).

Aspergillus terreus is a filamentous pathogenic fungus which is harmful to the animals, plants and humans. The *Aspergillus terreus* can affect potato (*Solanum tuberosum* L.) by causing foliar blight disease and cause in abnormalities in the plant growth (Louis et al., 2014). *Aspergillus niger* and *Aspergillus flavus* can cause post-harvest loss in vegetables like potato, cucumber, carrot, okra, eggplant, round gourd, turnip, cauliflower and chilli. It is necessary to overcome crop loss due to these harmful fungi to overcome the food insecurity problem in future (Perveen et al., 2021).

Plant extracts and other natural products are getting popularity to control fungal infections in crops because they neither have a health risk nor cause environmental pollution. These products are also not expensive as synthetic chemicals, and also have minor negative side effects on the host species. On the other hand, synthetic fungicides have various problems e.g., acute and chronic toxicity, long half-lives, accumulation of harmful substances in the food chain and killing beneficial pests along with the pathogenic pests. The use of plant extracts having antifungal compounds reduces these negative impacts (Alotibi et al., 2020), so recent research emphasis is to employ biological control of plant diseases and to execute biological control programs (Elshafie et al., 2017; Meena et al., 2021).

Harmful Algal Blooms (HABs) are a big threat to aquatic ecosystem, and is increasing worldwide in range of fresh waters, coastal estuarine, wetlands and marine waters (Van Donk et al., 2002) and a major cause of enormous harmful impacts in the aquatic ecosystems in which they are present.

HABs produced by Chlamydomonas is the cause of bad smell of water due to toxins produced which are harmful for human and animals' health due to drinking of poisonous water. *Chlamydomonas* has characteristic of utilizing a K-strategy growth pattern by slowing growth rate during optimal nutrient and environmental conditions but showed high growth rate during limited nutrients. It has capability to form algal blooms when conditions are not favorable to other algal species. *Chlamydomonas* members are harmful algal bloom species because of having ability of clogging the water purification filters (Kruskopf & Plessis, 2006).

Water protection from algal blooms is an important issue for the safety of human and animal health, landscaping and also for aquatic ecosystem. There is a critical need to develop a cost effective and environment friendly method for the control of HABs, which is becoming a major research interest. Allelopathic potential of plants can be used for the inhibition of HABs growth. The experimental results were statistically analysed using ANOVA and Tukey test to assess the % age of inhibition and determine index response of allelopathic potential of experimental plants. Quantification of phenolic compounds was obtained by drawing calibration curve for each standard by using the peak areas obtained through HPLC for the standard against its three different concentrations.

CHAPTER 2

LITERATURE REVIEW

The aromatic properties of aromatic plants are due to the presence of various essential oils, known as volatile compounds, in them. Aromatic plants produce and exude aromatic substances, largely ether oils, which are not only used in the food, cooking items and making perfumes but are also used in the pharmaceutical and liquor industries. The allelopathy term was first introduced in 1937 and the chemical interaction of plants with each other is known for thousands of years. Theophrastus found the allelopathic effects of chick pea plants on other plants in 300 BC. Allelopathic effects were first recorded by Pliny in walnut tree. Leaves, roots and fruits of the walnut plant produce hydroquinone which is oxidized in the environment and it has toxicity effects onthe other plants (Kocacaliskan & Terzi, 2001).

Many of the aromatic plant species belong to Lauraceae, Umbelliferae, Myrtaceae and Labiatae families. Essential oils from aromatic plants can be used as pre-emergent weed for controlling the weeds through seed germination inhibitors due to the fact that this approach can reduce the application of chemical herbicides (Ramezani et al., 2008). Weeds are detrimental to the agriculture sector as they undermine the quality and productivity of agricultural yield. For the last few decades, efforts have been made by the researchers to eradicate the invasive plants using synthetic herbicides, which consequently result in damaging the environment since they cause considerable toxic effects on the living organisms including humans (Bhatt et al.,2011). With the passage of time, the invasive herbs have improved their resistance against the synthetic herbicides due to their excessive and disproportionate use in the agriculture sector. This fact necessitates using bio-herbicides as a potential environmentally and economically sustainable alternative (Batish et al., 2008). The allelochemicals obtained from the aromatic plants can be formed through various types of plant tissues. The action of these allelochemicals can be due to different means such as residues decomposition, root exudation and volatilization (Weston and Duke, 2003). Most of the invasive weeds are known due to their allelopathic capability. Majority of the weeds vie for all types of nutrients with their neighboring plants resulting in serious inhibition of development and growth of cultures (Cheng and Cheng, 2015).

2.1 Allelopathic Effects of Plants due to Release of Aromatic Compounds

Classification of allelochemicals into different categories such as straight-chain alcohols, water-soluble organic acids, aliphatic aldehydes, simple lactones, ketones, phenols, cinnamic

acid and its derivatives, coumarins, flavonoids, tannins, steroids and terpenoids (sesquiterpene lactones, diterpenes, and triterpenoids), quinines (benzoquinone, anthraquinone and complex quinines) and long-chain fatty acids and polyacetylenes can be performed on the basis of their structure and allelopathic properties (Soltys et al., 2013). It has been explored in a study that *Conyza sumatrensis* of family Asteraceae is an herb which is common all over Tunisia but has been ignored by the researchers and has never been studied. *C. Sumatrensis* is useful for extraction of essential oils from its different parts e.g., stem leaves, flower heads and roots. These plants are collected from Monastir, Tunisia in autumn, which is their flowering stage. In total, 98 compounds were recognized by GC-MS and GC-FID analyses, with 88.1%–99.3% of the oil composition. The distinction is made on the basis of essential oil contents and roots have high content of acetylenes in essential oil whereas there is high content of oxygenated sesquiterpenes in the essential oil obtained from the flower buds and leaves. The oils extracted from *C. Sumatrensis* are from Matricaria ester. The essential oils obtained were assessed for the allelopathic properties i.e., antifungal and antibacterial. The study results showed that the use of oil extracted from leaf *in-vitro* exhibited considerable antibacterial activity against *Enterococcus faecalis, Proteus mirabilis* and *Staphylococcus aureus.* The *C. Sumatrensis* oils extracted from the aerial parts showed significant inhibitory effects to mycelial growth of filamentous fungi and *Candida albicans*. Furthermore, the essential oils extracted from various plant parts had inhibitory effect on the *Raphanus sativus* (radish) seedlings; root and shoot growth. Further, the inhibitory effect on growth of the plant radicle can vary (Mabrouk et al., 2013)

Fialová et al. (2015) reported the importance of mint belonging to family Lamiaceae; various beneficial phenols e.g., flavonoids and phenolic acids present in the mint plants give it medicinal properties. The presence of active phenolic compounds was confirmed in plants which were 3 to 4 years old. The authors analyzed different phenolic compounds for several years continuously and determined the polyphenols and tannins (at λ= 760nm) as rosmarinic acid and flavonoids as quercetin (at λ= 420nm).

A study conducted by Kapoor et al. (2019) found that *Psidium guajava* and *Artemisia absinthium* contained secondary metabolites with allelopathic potential. Weed (*Parthenium hysterophorus*) growth was inhibited, leaf extract of these plants affects the seed germination, root and shoot length of *P. hysterophorus* by altering the enzymatic and non-enzymatic activities negatively and decreasing the photosynthetic pigment. An important finding is that *A. absinthium* is more effective than *P.guajava.* It is evident from the study that *Psidium guajava* and *Artemisia absinthium* are strong candidates for bioherbicide against weeds (*P.*

hysterophorus).

Justicia adhatoda which belongs to Acantharean family is native to Indian subcontinent and possesses antimicrobial properties. It was reported in a study by Shukla et al. (2017) that essentials oils from the fresh leaves of *Justicia adhatoda* have antimicrobial potential. Components of essential oils was analyzed through GC-MS which showed the presence of phytol, heneicosane, n-hentriacontane, nonacosane, β-Eudesmol and pentacosane in different concentrations. Antimicrobial activity of *Justicia adhatoda* was checked by application on Methicillin-resistant and sensitive *Staphylococcus aureus* (MRSA) and inhibited its growth by blocking the retinol teratogenic effects (Shukla et al., 2017). In another study conducted by Sharma and Kumar (2016) on *Justicia adhatoda*, it was determined that bioactive compounds present in the leaf extract showed inhibitory potential against bacteria and fungus (*Pseudomonas aeruginosa* and *Proteus vulgaris*). By using the GC-MS techniques on phytochemicals from *Justicia adhatoda,* nine phytochemical components were identified, which confirmed that *Justicia adhatoda* leaves can be herbal alternatives for curing various diseases and is especially used for mosquitocidal activities (Jayapriya & Shoba, 2015). Various ailments are treated with *Justicia adhatoda* and number of medical formulations are acquired by using different parts of this plant (Sharma & Kumar, 2016).

2.2 Mode of Action of Allelochemicals

There are different modes of action of allelochemicals and it is important to understand the mode of action of allelochemicals. An allelopathic effect of plants is usually due to the negative or positive interaction of plants (De Albuquerque et al., 2011; Radosevich et al., 2007); and positive interactions depends on the concentration of allelochemical of the target species of plant (Eichenberg et al., 2014). Certain herbicides are derived from the allelochemicals of plants or microorganisms (Dayan et al., 2012; Duke et al., 2002). The secondary metabolites produced by the plants are important for their defense mechanism, pollination and seed dispersal (Kroymann, 2011). In agroecosystem, secondary metabolites from plants play an important role for weed management by rotation of crops, mulching and plant reproduction etc. (Chon et al., 2006). These secondary metabolites (allelochemicals) have the ability to use them for the production of natural herbicides. To increase the production of desired allelochemical, use of molecular biology (Transgeny) and classical breeding techniques are helpful. Knowledge about genetics is helpful and necessary to identify the role of allelochemicals in interactions of plants to other plants and assess their positive and negative ecological effects in the environment.

Production of allelochemicals is a burden on plants and it is difficult to keep a balance between plant yield and allelopathic use of plants (Wink, 2010).

According to Rizvi (2012), the allelochemicals affect the cytology, ultra-structures, membranes permeability, phytohormones production and balance, uptake of minerals by plants, movement of stomata, pigment synthesis, germination of spores and pollen, protein synthesis, respiration, nitrogen fixation, plants conducting tissues enzymes activity, genetic material (RNA and DNA), and water relations in plants. Allelochemicals are cause of mitosis suppression, which can become the cause of plant growth inhibition (Duke et al., 2000). Allelochemicals also disturb arrangement of cell organelle and their structures and became the cause of damage to root cells (Li et al., 2010). Furthermore, phytotoxins directly damage the roots growth. The roots have less cuticle protection so it leads towards accumulation of high concentration of allelochemicals in the root tissues and act strongly as compared to the shoot growth (Yoshimura et al., 2011).

A single phytotoxin/allelochemical is not only responsible for allelopathic activities, it is a joint endeavor of various compounds due to intervention of surrounding plants (Weston, 2005). Allelopathic inhibitory effects on the growth of other plants/weeds is concentration dependent and it increases with the increase in the concentration of the allelochemical (Mushtaq *et al.*, 2019; Mushtaq et al., 2018; Gulzar et al., 2016; Ishak & Sahid, 2014). The action of allelochemical from trees on crops is dissimilar for different plant species (selective action). It is described in a study by Singh et al. (2008) that elder plants leachate inhibited the growth of pangola grass and the same leachate augment the growth of pasture grass i.e., bluestem.

It was proposed in a research study that certain process takes place when an allelochemicals secreted from a plant into the environment and these processes are (a) movement of allelochemical through different media like soil, water and air is termed as retention of allelochemical, (b) alteration in the structure of the compound, change into different fraction and leading toward its decomposition is termed as transformation, and (c) movement of the allelochemical in the environment is called transport (Cheng & Koskinen. 2002). Environmental factors, nature of compound and properties of soil effects the abovementioned process. The fate of allelochemicals is affected by the individual process's kinetics and interaction under explicit natural conditions (Rizvi, 2012).

The mode of action of allelochemicals can be direct and indirect (Rizvi, 2012). The indirect action involves change in the properties of soil, nutritional conditions, altered population of soil fauna like nematodes and soil microorganisms. The direct action includes physiological/biochemical impacts on metabolism of plants. There are different processes by

which allelochemicals affect the growth of plants. Allelochemicals affects the absorption of minerals and the ion absorption rate of minerals by plants can be altered by allelochemicals (Baar et al., 1994). Phenolic acids present in allelochemicals inhibit the macro and micro nutrients uptake (Mohammadkhani & Servati., 2018). Cell structure and ultra-structures can be affected by different types of allelochemicals and inhibits the mitosis in root cells of plants (Gulzar et al., 2016; Mushtaq et al., 2019; Celik & Aslanturk, 2010; Mohamadi & Rajaie, 2009). Growth hormones (phytohormones) present in plant which affect the growth of plants e.g., Indole Acetic Acid (IAA) and gibberellins are responsible for the shoot length of plants. IAA activity was inhibited by IAA oxidase and changed into inactive form by certain allelochemicals (Chou, 1980). Abscisic acid and ethylene synthesis were enhanced under stressed allelopathic conditions (Bogatek et al., 2005). Allelochemicals damage the cell membrane and affect its permeability (Wang et al., 2017). Allelochemicals are secreted from the plant roots, however, assessments of allelochemicals effects on the roots is difficult, so the root cuts can be used to analyze the damage index (Gniazdowska & Bogatek, 2005).

Photosynthesis is also inhibited by the allelochemicals because cinnamic acid and benzoic acid cause reduction in chlorophyll content and inhibit the rate of photosynthesis (Li et al., 2010). Allelochemicals affect the electron transport change by inhibiting the electron acceptors (Ye et al., 2013). Respiration is also affected by the allelochemicals and it can be enhanced or inhibited which is dangerous for the energy producing system (Batish et al., 2001). It was reported by Bertin et al. (2007) that protein synthesis is hindered by allelochemicals and it was studies by using sugar containing C_{14} and amino acids and their assimilation to proteins. Enzyme's activity is also affected by allelochemicals which inhibit the activity of enzymes present in plants (Muscolo et al., 2001). Allelochemicals obtained from *N. plumbaginifolia* stimulate the activity of catalase (CAT) and also affect the activity of superoxide dismutase (SOD) (Singh et al., 2015). It was reported in a research study of Hayat et al. (2012) that proline content accumulated when plants are in stress conditions. Allelochemicals also affect the conductive tissues of plants (Gniazdowska & Bogatek, 2005). Effects of allelochemicals on plant water balance was also reported in a study conducted by Sheteawi & Tawfik (2007). Plants growth and development was affected by allelochemicals and showed obvious and immediate effects e.g., seed germination inhibition and root growth (Abenavoli et al., 2008). Allelochemicals impacts the genetic material of plants (Jensen et al., 2001).

2.3 Methods used for Screening of Plants

There are two popular methods, Dish Pack method and Sandwich method usually used for screening of plants for their allelopathic potential. Fujii et al. (2005) developed Dish Pack method which is a new bioassay technique; this method is used to analyze volatile compounds present in the plants. In this technique, a multi dish with six wells is used; Grounded Leaves are placed in one well and filter paper is placed in the other five wells with seven lettuce seeds and 0.7 mL of distilled water is added. After sealing the dish with aluminum foil, it is placed in the incubator and growth of hypocotyl and radical are recorded after four days. This method is considered the best to screen the plants according to their allelopathic potential, Appiah et al. (2015) also used dish pack and Sandwich method with *Lactuca sativa* as target plant for screening 251 plant species to check their allelopathic potential. The authors performed their research due to taxonomic richness of plants which are endemic species and survived many years of extreme climatic conditions.

Shinwari et al. (2013) examined the effects of dried leaves and root exudates of plant species by conducting experiments on the growth of lettuce (*Lactuca sativa*) by using dish pack and sandwich method. The study reports that phytotoxicity altered phytotoxins, mode of release as emitted by root residues, exudates or volatilization. Among the species investigated in the study, the *Tagetes minuta* demonstrated the highest inhibitory activity by using the Plant box method. The next strongest inhibition was observed for *Setariaverti cillata* and *Mirabilis jalapa*. Though it was observed for the Sandwich method, that radical growth of *Pyrus pashia* is maximally inhibited followed by the inhibition in *Solanum surattense* and *Solanum villosum*. Whereas, in the dish pack method, the maximum radical growth inhibition was observed in *Tagetes minuta* followed by *Prosopis juliflora* and *Lantana camara*. A study conducted by Begum et al. (2020) using dish pack method for the assessment of 103 plant species indicated that the pericarp of *S. mukorossi* (Sapindaceae) inhibited the radicle elongation (97%) of *L. sativa* due to release of volatile allelochemicals.

In the research conducted at Plant Chemical Ecology Laboratory, National Institute of Agro Environmental Science, Japan during 2008-2009, three different types of dimensional assessments were made to determine allelopathic potential of 38 communal weeds and invasive plants of Pakistan and Japan which are also found in the five continents. The plant species were examined by using their leaf litter, root exudates and volatiles by applying Plant box, dish pack and sandwich methods. The results of all these three different methods were analyzed statistically by calculating the mean ($\bar{x}$), standard deviation (σ) and standard deviation variance

(SDV) to determine the inhibitory effect pattern on radical growth of the lettuce seedlings (test plant) for risk assessment. After interpretation of all results, *Melilotus officinalis* among all the species was noticed for maximum toxic effect due to strong inhibitory effects on *Lactuca sativa* radical growth which was followed by *Melilotus alba*, *Datura stramonium* and *Mirabilis jalapa*. Whereas, *Plantago lanceolata*, *Rumex crispus*, *Trifolium pratense* and *Rumex conglomerates* have shown minimum inhibitory effect.

2.4 Harmful Algal Blooms and Allelopathy

Eutrophication of water bodies due to human activities is becoming serious problem worldwide. It causes extensive growth of algal bloom due to enriched nutrients in sewage water, which have many negative effects on aquatic environment as it spoils water quality due to release of toxins which could have poisonous effects on both the humans and animals (Yang et al., 2008). Algae is microscopic organism that grow and live in water and produces chemical energy through photosynthesis like higher plants. Algal blooms formed by harmful algae reproduction and some of them produce toxins in aquatic environment. Algal blooms can be seen with naked eye and are in the form of red, blue, brown or green layers according to the type of algae present. The production of toxins depends on the environmental conditions like temperature and nutrient content etc. The release of toxins from algal blooms is harmful to aquatic environment and adversely affect the fish population, aquatic and terrestrial animals and humans (Berdalet et al., 2016). HABs are present in the fresh water areas and also in the marine ecosystem. This is the cause of dreadful harm to the industries related to fisheries and aquaculture, like prawn breeding culture, abalone breeding and fish breading in cages etc.

It is essential to understand the characteristics of species which are more efficient to cause HABs by using their competitive dominance. It is also very important to understand the existence of certain species in different ecological environment with metabolic and physiological process in different ecosystem at different trophic levels (Smayda & Reynolds., 2001). Presence of green algae and cyanobacterial caused the eutrophication of river Vaal in South Africa and became the matter of great concern. Kruskopf & Du Plessis (2004) showed in their study that various concentrations of nitrogen and phosphorus affect the growth, concentration of Chlorophyll-a, phosphate activity acidic and both of Chlamydomonas and chlorella. Their studies showed that Chlamydomonas and chlorella both developed algal masses in the river but later did not show any reason of problem related to its presence. After the collection and isolation of these two strains from the river, these were treated with nitrogen

and phosphorus limiting conditions and after treatment when the enzymatic activity was checked then both the strains showed very different results. Chlamydomonas adopt K-strategy, due to which the growth rate is minimum/slow when growth conditions are optimum and have excellent growth when conditions for growth are not good e.g., limited growth. The authors concluded that algal mass occurrence in river Vaal in a competitive condition is due to this property (K- growth strategy) and become the cause of HABs.

Algal bloom control is an important issue for the protection of aquatic ecosystem, because the floating algal blooms suffocate the plants and animals present in the aquatic environment and reduce the aquatic biodiversity (Verschuren et al., 2002). Three methods, physical (radiations), chemical (synthetic algaecides) and biological (biological manipulation) are used for the control of algal blooms, which are expensive and unsafe for aquatic environment (Xia & Guisen., 2002). There is a need to develop cost effective and environmentally friendly method to control the algal blooms in aquatic ecosystem; hence, use of allelopathy approach is the best option. Ferrier et al. (2005) used barley straw (Hordeum vulgare) a macrophyte to control the algal blooms and noticed its effect on certain algal species.

Ridge et al. (1996) reported in their study that the application of leaf litter had inhibitory effects on algal growth, and was not effective as barley straw. In another study it was shown that the application of decomposed rice straws also has similar inhibitory effects as shown by barley grass on *Microcystis aeruginosa* which is a cyanobacteria (Su et al., 2014). Interactions in the aquatic ecosystem play an important role to maintain its structure and allelopathy is also a factor which has a key role for the regulation of population of phytoplankton (Gross, 2003; Van Donk & Van de Bund, 2002). Therefore, the excessive use of macrophytes for the control of algal blooms is becoming another concern for the environment.

Allelopathy gives a new direction to solve the problem of algal blooms which is a major threat to the economic growth by effecting the aquatic ecosystem which is ultimately harming fisheries. Allelopathy could inhibit growth of algae by harming the structure of cells and inhibiting the enzymes activity and effecting the rate of photosynthesis (Tan et al., 2019). Ziwen et al. (1992) based on the lab experiment analyze that growth of *Chlamydomonas reinhardtii* was inhibited when they applied the leachates of *Pistia stratiotes*, *Alternanthera philoxcroides*, *Azolla imbricate, Lemna minor* and *Spirodela polyrhiza.*

There is extensive research on the use of allelochemicals for sustainable ecosystem. Though, most of the research focused on the control of weeds present in crops, e.g., cineole (Romagni et al., 2000). However, there is need to use the allelochemical present in terrestrial or aquatic plants (macrophytes) to control the algal blooms. Barani et al. (2014) assessed the allelopathic

activity of *Zataria multiflora, S. rechingeri* and *Satureja khuzistanica* to control the growth of algae. Biocontrol of algae with these plant species is appropriate and efficient due to their antialgal activities and easy cultivation. It was also investigated in their study that antialgal compounds present in the essential oils (e.g., Carvacrol and thymol) can be used as antialgal compounds which inhibit the growth of algae. Oils prepared from *Z. multiflora* showed the highest growth inhibition to algae and the rate of inhibition is dependent on the doses of essential oils. The best absorption of the essential oils and lipophilic oils present in the extract could be the possible reason for the antialgal activity (Yi et al., 2012). The characteristics of a compound due to which it can be a good candidate for growth inhibitor are its low toxicity, high effectiveness and cost effectiveness (Zhou et al., 2007). The volatile plant materials are less stable, so these are considered safe for the aquatic environment. This characteristic of the compound can be its strength or weakness because water tides can affect its algicidal effect, so the stability of the inhibitor compound is important. Interestingly, strength and weaknesses of the compounds lies in this feature (Zhou et al., 2007). It is not easy to understand the allelopathic effects under natural conditions (e.g., in the aquatic ecosystem) due to natural factors such as temperature, pH, light and nutrient competition. Any change in these conditions halt their allelopathic effect. However, the identification of such natural compounds is hard for the identification of natural products (Wang et al., 2007).

Shao et al. (2013) reported in their research that bioactive substances derived from plants have potential to inhibit the growth of harmful algae. Terrestrial plants extract has allelopathic potential and inhibit the growth of algae e.g., cyanobacteria which is blue green algae. These allelopathic terrestrial plants belong to families Rutaceae (Purcaro et al., 2009; Cantrell et al., 2005; Meepagala et al., 2010; Meepagala et al., 2005a), Papaveraceae (Jancula et al., 2007), Asteraceae (De Melo et al., 2011), Apiaceae (Meepagala et al., 2005b) and Ephedraceae (Yan et al., 2012). Extract from *E. equisetina* root showed good allelopathic potential which inhibited the growth of cyanobacteria (Yan et al., 2012). Jiang et al. (2014) isolated an unknown allelochemical from *Ficus microcarpa* which inhibited the growth of *Chlorella pyrenoidosa* by damaging the PSII system centers (Photoinhibition) and altering the photosynthetic activities of *C. pyrenoidosa* due to allelopathic compounds effects on the antioxidant activities of photosynthetic enzymes. The identification of the primarily active fraction (2-Propyl phenol) from *Ficus microcarpa* extract which has a significant inhibitory effect on the photosynthetic activities, antioxidant activities of the enzymes and ultimately showed growth inhibition of *C. pyrenoidosa*. There is a history of allelochemicals isolation from submerged plants to treat the algal blooms (Gross 2003; Nakai & Hosomi 2002; Mulderij et al., 2003) and allelopathic

compounds were also isolated from the submerged plants i.e., macrophytes (Hong et al., 2010; He & Wang, 2001; Li & Hu, 2005). Terrestrial plants also showed allelopathic inhibitory effects on alga e.g., decomposed barley straw showed growth inhibition to algae (Ball et al., 2001) and allelopathic effects of *Lantana camara* (Kong et al., 2006) which cause reduction in of some algal species in lab or field conditions. Woody plants are enriched with allelochemicals and provide good amount of raw material for the control of algal blooms (Pohjamo et al., 2003); however, not enough research is done on the use of woody plants as an allelopathic to algae bloom. Artemisinin from extracted from *Artemisia annua* L could inhibit the growth of algae (Soltys et al., 2013). A research study conducted in Southern China revealed that the extracts obtained from the leaves of Ficus *microcarpa* have significant potential to inhibit the growth of *Chlorella pyrenoidosa* (Li et al., 2010).

2.5 Phenolic Compounds

The contemporary demand for substantial food production has significantly increased the use of synthetic pesticides in the agriculture sector, but ample efforts have been done to decrease their use, both to diminish their levels in foods and to decrease the environmental impact on the agriculture. Phenolic compounds present in plants are very important in human diet due to their antioxidant properties. These compounds are composed of an aromatic ring with one or more hydroxyl groups and structurally these compounds are from simple phenolic molecule to complex polymer. Flavonoids have the C_6–C_3–C_6 structure, which describes more than half of the phenolic compounds. Their antioxidant activity varies according to structure; particularly due to hydroxyl group's position, number and nature of substitutions present on the aromatic rings (Balasundram et al., 2006). Classification of plants phenolic compounds is categorized into groups can be done on the number of carbons present (Bhuyan & Basu, 2017).

Phenolic compounds from plants are actually secondary plants metabolites which are derivatives of the pentose, shikimate, phosphate and phenylpropanoid pathways of plants (Randhir et al., 2004). These compounds are considered as one of the most extensively studied groups of phytochemicals due to substantial physiological and morphological value in the plants. In spite of their structural diversity, this group of compounds is insinuated as polyphenols. Usually, phenolic compounds which naturally exist as conjugates with mono- and poly-saccharides, with one or more of the phenolic groups, and may also found as functional derivatives e.g., esters and methyl esters (Harborne et al.,1999). However, such structural diversity is the cause of presence of wide range of phenolic compounds in nature. Classification

of phenolic compounds based on number of carbons is shown in Table 2.1. Flavonoids and tannins are key dietary phenolic compounds (King & Young, 1999).

Table 2.1. Classification of phenolic compounds. (Bhuyan & Basu, 2017).

Class	Number of carbon atoms	Basic structure	Examples
Simplephenolics Benzoquinones	6	C_6	Catechol, hydroquinone 2,6-Dimethoxybenzoquinone
Phenolic acids	7	C_6 –C_1	Gallic, salicylic acids
Acetophenones Phenylacetic acids	8	C_6 –C_2	3-Acetyl-6-methoxybenzaldehyde p-Hydroxyphenylacetic acid
Hydroxycinnamic acids Phenylpropanoids Coumarins Isocoumarins Chromones	9	C_6 –C_3	Caffeic, ferulic acids Myristicin, eugenol Umbelliferone, aesculetin Bergenin Eugenin
Napthoquinones	10	C_6 –C_4	Juglone, plumbagin
Xanthones	13	C_6 –C_1 –C_6	Mangiferin
Stilbenes Anthraquinones	14	C_6 –C_2 –C_6	Lunularic acid, resveratrol Emodin
Flavonoids Isoflavonoids	15	C_6 –C_2-C_6	Quercetin, cyaniding Genistein
Lignans Neolignans	18	$(C_6$-$C_3)_2$	Pinoresinol , Eusiderin
Biflavonoids	30	$(C_6$-C_3-$C_6)_2$	Amentoflavone, agathisflavone
Lignins	Many	$(C_6$-$C_3)_n$	Pinoresinol
Condensed tannins (proanthocyanidins or flavolans)	Many	$(C_6$-C_3-$C_6)_n$	Selligueain A, prodelphinidin

2.5.1 Allelopathic Potential of Selected Phenolic Compounds

Rutin (quercetin-3-rutinoside) is a flavonoid present in a number of plants especially in citrus (Musallam et al., 2012). Rutin has allelopathic potential (Pereira et al., 2018). A study proved that rutin caused reduction in the process of photosynthesis. The treatment of plants (Arabidopsis) with rutin for seven days reduced the Chl–a content significantly as compared to the control (Hussain et al., 2016). In pharmaceutical and cosmetic industry, it is commonly an active compound of products, which are for oral or cutaneous use. Studies showed that rutin has scavenging ability for oxidizing species e.g., hydroxyl radical, peroxyl radicals and superoxide radical (Calabro et al., 2005). Due to antioxidant properties, rutin affects the stratum

corneum, and proposing a potentiality for reducing aging process (Baby et al., 2008).

Fahmy et al. (2012) showed that rutin, catechol and syringic acid significantly inhibited the *Corchorus olitorius* seedling growth. Some flavonoids involving quercetin and rutin showed radish seed germination inhibition and decline in seedling growth (Basile et al., 2000). Golisz et al. (2007) analyzed in their study that rutin has a major contribution in the allelopathy of buckwheat. Rutin play a major role in inhibitory activity among flavonoids existing in *S. oleraceus*. The inhibition to seedling growth may be assigned to the phenol's involvement, which have the ability to suppress the protein and nucleic acids synthesis and cause the deactivation of growth enzymes essential for plant growth (Chou, 2006). Rutin and quercetin have major role in growth suppression of seedling (Kuamr et al., 2011). Different analytical methods for determining the rutin including ultraviolet detection (UV), high-performance liquid chromatography (HPLC) with mass spectrometry (MS) etc. (Ishii et al., 2001; Chen et al., 2000, 2001; Song & Hou 2002; Rijke et al., 2006; Legnerova et al., 2003).

Quercetin is a plant flavonol present in various plants and foods including cabbage, cauliflower, berries, apples, nuts, tea, onions, red grapes, citrus fruit, tomato, broccoli and other leafy green vegetables as well as in a number of berries including raspberries and cranberries etc. (Lakhanpal & Rai, 2007). It has biological activities with relevance to plant physiology and development. Flavonoids including quercetin are helpful in protection of plants and animals against detrimental abiotic factors, but also assists interactions with other microorganisms and plants (Mierziak et al., 2014).

Caffeic acid is a phenolic acid and is found in various plants (Chiang et al., 2015). It showed allelopathic activity by showing particular behavior towards target species by stimulating lettuce plants growth and inhibiting the *Chlorella vulgaris* (Bravo et al., 2013). Caffeic acid has been investigated for its allelopathic potential and it is observed that there exists moderate inhibitory effect on germination rate of lettuce (Li et al., 2010). Caffeic acid has allelopathic activity and is capable to inhibit seed germination of many plants (Ishikura *et al.*, 2001). Caffeic acid also inhibits the rate of photosynthesis by effecting the electron transport chain process (Zhuo & Yu, 2006). Barkosky et al. (2000) stated in their study that application of caffeic acid resulted in a major increase in diffusive resistance of leaf and rate of transpiration after 12 days of the treatment.

Trans-Ferulic acid acts as allelopathic agent and is commonly reported as an inhibitory allelopathic substance which affects many plant species (Singh et al., 2014). Ferulic acid shows diversity of biological activities e.g., anti-inflammatory, antioxidant, antiallergic, antimicrobial properties (Kumar and Pruthi, 2014). Ferulic acid is one of the dominant allelochemicals which

were found through HPLC in black mustard and the application of plant extract and root exudates on *Triticum aestivum* and *Trifolium alexandrinum* (Crop species), *Phalaris paradoxa* and *Sisymbrium irio* (weed species) showed inhibitory effect to the target plant species (Al-Sherif et al., 2013). Phenolic compounds such as ferulic, syringic, caffeic and *p-Coumaric* were noted as allelopathic compounds (Einhellig et al., 2004; Rice, 1984).

Extracts of different parts of the plants contain several phenolic acids called as allelochemicals e.g., 4-hydroxybenzoic acid and *p-Coumaric* (Fernandez et al., 2006). The presence of *p-Coumaric acid* is observed in the needles of *P. halepensis* (Pasqualini et al., 2003). The *p-Coumaric* showed a significant effect on the growth of of *Linum usitatissimum L* roots and its above-ground organs (Ray & Hastings, 1992).

Hydroxytyrosol is a phenolic phytochemical and has antioxidant properties in vitro present in olive leaves (Martínez *et al.*, 2018). It has phytotoxic properties and also algicidal (Scognamiglio et al., 2013). It has been assumed that hydroxytyrosol have a broad range of biological effects (Parkinson & Cicerale, 2016). It is present in leaves and fruits of olive (Vilaplana-Pérez et al., 2014).

Phytochemicals present in *Jatropha curcas* Linn (Kernel meal) detected by RP-HPLC were gallic acid, rutin, pyrogallol, naringin, and vanillic acid (Namuli et al., 2011). Rutin hydrate, quercetin dehydrate and naringin can be considered as botanical herbicides and is useful for integrated management for pest control (Ateyyat et al., 2012). Naringin due to its phytochemical properties can act as herbal insecticides and used in programs of integrated management of the aphid control (Ateyyat *et al.*, 2012). A study conducted by Nakai et al. (2000) and their study results showed that protocatechuic, ellagic acid, gallic acid, vicinal, protocatechuic acid and triphenol are produced by *Myriophyllum spicatum*, which inhibit *Microcystis aeruginosa* growth.

Furthermore, a study conducted by Yang et al. (2005) showed that some of phenolic acids types e.g., gallic acid, vanillin and catechin have a prominent inhibitory effect on the *Tamar alexander* growth. Ding et al. (2007) reported in their study that gallic acid and diphenol inhibited the growth of *M. aeruginosa*. Allelochemicals released from *A. corniculatum* were identified and isolated by Xu & Long (2009) and Zhang et al. (2005). Gallic acid is a secondary metabolite and is from the phenolic acid group, which is present in a various plant stems and roots. Its formula is $C_6H_2(OH)_3COOH$ and molecular weight is 170.12. It is also known as 3,4,5-trihydroxy benzoic acid and is easily soluble in water. The results of the study conducted by Liu & Huang (2013) provided the basic data of relationship between algae and mangroves

and also presented valuable information of complex impacts of mangroves on microbial communities especially micro ecology in rhizosphere system.

According to Liu & Huang (2013), microscopic observation which showed that cell shape started to deform with the effect of gallic acid; the changes in the cell structure were mainly noticed with elongated cells, chlorophyll color was faded, lessened the oil droplets and chloroplast bodies, and rest of the cell organelles became unclear. Nakai et al. (2001) observed in their study that the gallic acid was also responsible for inhibiting the growth of Microcystis aeruginosa.

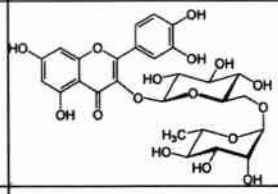
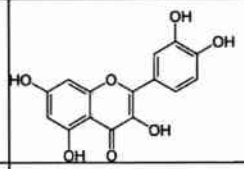
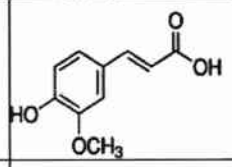
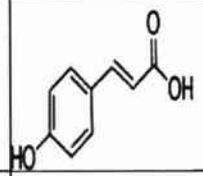
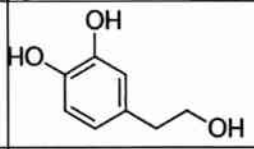
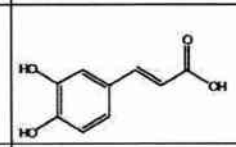
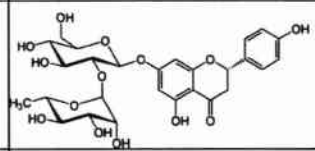
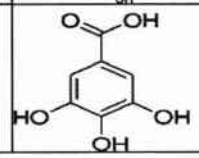
Reigosa et al. (1999) identified phenolic compounds of low molecular weight in the leaves of different *Capsicum annuum L* varieties. Gallic acid, ferulic acid, vanillic acid, p-coumaric acid, p-vanillin and p-hydroxybenzoic acid were examined in laboratory bioassays to check the allelopathic potential effects on seed germination and seedling growth of weeds. To check the allelopathic effects of these phenolic compounds in laboratory, six weeds naming *Chenopodium album L., Solanum nigrum L., Plantago lanceolata L., Amaranthus retroflexus L., L. Cirsium sp and Rumex crispus* were chosen for allelopathic effects. The inhibitory effect on seed germination and growth of seedling were observed at highest concentrations but no effect at low concentrations was noticed. According to dos Santos et al. (2008), ferulic acid is a derivative of cinnamic acid and is an allelochemical that is present in plants. Various biochemical and physiological process such as foliar expansion, water absorption and utilization, photosynthesis, root growth, cell respiration, nutrient uptake of plant is affected when plant is put under ferulic acid stress through its roots and restrict the plant growth.

Plants are the main source for getting new natural products (Boligon & Athayde, 2014). Natural products from plants have earned popularity in the recent years because ofhaving ability as an antioxidant, herbicides, enzyme inhibitors and anti-parasitic agents (Yang et al., 2009).

Green leafy vegetables have antioxidant properties due to presence of phenolic compounds. There is increase in use of plants containing phenolic compounds in diet and as well as in sustainable agriculture to control the weeds and replace the synthetic herbicides with natural herbicides. Kumar (2017) showed the use of versatile and susceptible and highly selective analytical techniques for identification, extraction and quantification of phenolics from extracts of different plants which is very helpful for using their important biological properties for sustainable ecosystem. The author described in the study that the advanced pretreatment methods, separation and spectrometry techniques for qualitative and quantitative analysis of phenolic compounds. The use ofHPLC and LCMS is a beneficial tool for metabolic profiling of various plant extract samples. The molecular formula, molecular weight and structural

formula of the selected phenolic compounds analysed in this study are shown in Table 2.2.

Table 2.2. Selected phenolic compounds

Compounds	Molecular formula	Molecular weight	Structural formula
Rutin	$C_{27}H_{30}O_{16}$	610.5	
Quercetin	$C_{15}H_{10}O_7$	302.23	
***trans*-Ferulic acid**	HOC6H3(OCH3)CH=CHCO2H	194.18	
p-Coumaric	$C_9H_8O_3$	164.04	
3-Hydroxytyrosol	$C_8H_{10}O_3$	154.16	
Caffeic acid	$C_9H_8O_4$	180.16	
Naringin	$C_{27}H_{32}O_{14}$	580.5	
Gallic acid	$C_7H_6O_5$	170.12	

2.6 Effects of Fungi on Crops

Crop growth and yield is vulnerable to various biotic and abiotic stresses. Therefore, it is important to improve the quality of crops by protecting the them from various fungal infections. Fungi cause 20–40% of known plant diseases. The use of chemical fungicides is the most

common method to control fungal infections in the plants for better yield, but continuous use of these synthetic herbicides could be dangerous in the future due to steady development of resistance in the harmful fungi. Moreover, synthetic fungicides can elicit the toxic effects for human and become the cause of different diseases such as cancer (Elshafie et al., 2017; Da et al., 2017).

The genus Aspergillus is located worldwide and consists of more than 180 recognized species, *Aspergillus terreus* is a filamentous pathogenic fungus which is harmful to the animals, plants and humans. There is a general consensus among the research community that Aspergillus terreus can attacks potato (*Solanum tuberosum* L.) and effects its yield by causing foliar blight disease by causing abnormalities in the epidermal cells and leaf tissues of the plant (Louis et al., 2014). *Aspergillus niger* and *Aspergillus flavus* are also harm the yield and responsible for post-harvest loss in vegetables like potato, cucumber, carrot, okra, eggplant, round gourd, turnip, cauliflower and chilli (Perveen et al., 2021).

2.7 GC-MS Analysis

Chromatography is a separation technique that has a mobile phase containing a mixture which moves in contact with a stationary phase having selective absorbent. It also plays an essential and basic role as an analytical technique for Phyto therapeutics and quality control in standardization of phytochemical properties of plants. In the recent years, GC-MS studies have been used for the analysis of medicinal plants as this technique is a valuable method for the analysis of non-polar components and volatile essential oils, lipids, fatty acids etc. (Hameed *et al.*, 2016). There are many different kinds of chromatographic technique based on the mobile and the stationary phases. Gas chromatography is particularly gas-liquid chromatography which involves a sample certainty vaporized and then injected onto chromatographic column head. The experimental sample is transported via the column with the flow of inert gas i.e., mobile phase. The column itself has a liquid stationary phase and is adsorbed onto the inert solid surface. The gas chromatography work on a principle of adsorption and partition. In chromatography family, the gas chromatography (GC) is one of the most extensively used techniques. James and Martin in 1952 first defined GC and it became one of the most important tools used for the separation of volatile compounds (Bartle and Myers. 2002).

Plants are a good source of secondary metabolites with biological activities. Usually, these secondary metabolites are valuable source with a various structural arrangements and characteristics (De-Fatima et al., 2006). The phytochemicals were identified through GC-MS

analysis by different researchers and showed many biological activities which can be helpful in present study and are listed in Table 2.3.

Table 2.3 Allelopathic potential of different allelochemicals from different plants.

Plant	Plant extract	Phytotoxin	Crop/weed/fungi/living organism	Inhibitory effect	Reference
Flaveria bidentis	Dichloromethane extract of root exudates	Decane,2,4,6-trimethyl			Xing *et al.*, 2014
Echinochloa crus-galli (L.) (Barnyard grass)	Root exudates	Decane	Alfalfa, lettuce, rice, monochoria and paddy weeds	Inhibition of germination and seedling growth	Xuan *et al.*, 2006
Spartina alterniflora	Aqueous extract	Cyclohexane, Heptane	*M.aeruginosa*	Weakening photosynthesis by decreasing Chl-a and effecting plant growth	Yuan *et al.*, 2020
Chenopodium murale	Methanolic leaf extract	o-xylene p-xylene	*Fusarium oxysporum*	Inhibition to fungul biomass	Naqvi and Qureshi, 2019
Sugar cane (postharvest residue)	Water soluble extract from postharvest sugarcane residue	Decane	Sugar cane	Inhibition to leaf development	Viator *et al.*, 2006
Conyza sumatrensis	Essentialoils extracted from the different parts of C. sumatrensis plants	Nonane	*Raphanus sativus* (radish)	inhibited the shoot and root growth	Mabrouk *et al.*, 2013
Prangos ferulacea	Essential oils extract	Mesitylene	Application at different egg stages of *Ephestia kuehniella*	mortality rate increased with the increasing concentration of essential oil	Ercan *et al.*, 2013
Eucalyptus grandis	Rhizosphere	Decane	*Eisenia fetida*	significant DNA damage to E. fetida	Zhiqun *et al.*, 2017
E. salubris	Essential oil	p-cymene	*Solanum elaeagnifolium*	stronger inhibitory effects on germination and seedling growth of Solanum elaeagnifolium (synergistic effects of	Zhang *et al.* (2012)

				several bioactive compounds including p-cymene)	
Piper nigrum	Ethanolic plant extract	Naphthalene	Oral bacteria	synergistic effect (antimicrobial activity	Ali *et al.*, 2019
Sonchus arvensis	Essential oils	cis-1,3-Dimethylcyclopentane	Bacilus cereus and Staphylococcus aureus	Growth inhibition of Bacilus cereus and Staphylococcus aureus	Kanaani *et al.*, 2015
Artemisia absinthium	Essential oils	cis-1,3-Dimethylcyclopentane	S. aureus and L. monocytogenes	Antibacterial activity agains S. aureus and L. monocytogenes	Hasannezhad *etl al.*, 2016
Spartina alterniflora	Plant extract	octane	Microcystis aeruginosa	Growth inhibition	Yuan et al., (2020)
Nauclea latifolia	Essential oils	1-ethyle-2-methyl-benzene, Decane, mesitylene, undecane, nonane, p-xylene	Gram negative bacteria	Antibacterial activity against gram negative bacteria	Morah *et al.*, 2017
Verbena officinalis, V. supina	Essential oils	undecan	wheat, maize, linseed and canary grass	root growth and seed germination inhibition	Dallali *et al.*, 2014
Cassia angustifolia	methanolic leaf extract	7- Oxabicyclo [4.1.0] heptan-2-one,6-methyl-3-(1-methylethyl)-,	*Aspergillus terreus*	Antimicrobial activity	Al-Marzoqi *et al.*,2016
Mentha piperita	Essential oils	Oxabicyclo [4.1.0] heptan-2-one,6-methyl-3-(1-methylethyl)-,	*Staphylococcus aureus, Escherichia coli, Aspergillus niger*	antibacterial, antifungal and antioxidant activities.	Afridi *et al.*, 2016

Moroccan seaweeds	Essential oils	Toluene	*Microcystin aeruginosa*	antialgal activity	(El Amrani & Mauromicale, 2021)
Sarcopoterium spinosum	Hexane extract of the stem, leaves and roots	1-Ethyl-4-methylbenzene	Staphylococcus aureus and Bacillus cereus	Antimicrobial activity	Sipahi *et al.*, 2017
Moringa olifera)	Hexane seed extract	Undecane	*Sinapis arvensis*	Seed germination inhibition	Tahir *et al.*, 2018
Lycopersicon esculentum	Leaf extract	Ethylcyclohexane	*Botrytis. cinereal* and *Fusarium. oxysporum*	spore germination and hyphal growth	Zhang and Chen, 2009
Bryophyllum pinnatum	Essential oils from leaves and stem	*2,5-Dimethylheptane*	-	Antioxidant activity	Adibe *et la.*, 2019
Cryptocarya massoy	Essential oils	4-Propylcyclohexene	L. esculentum and C. sativus	Growth inhibition pre and post-emergence stage	Rolli *et al.*, 2016
Olea europaea	Extra virgin olive oil	1,2,4-trimethylBenzene	-	-	Cecchi & Alfei, 2013
Platanus orientalis	Flower extract	7-Oxabicyclo 4.10] heptane,1-methyl-4-(1-methylethenyl)-	-	ethnomedicatio n	Zhang et al., 2020
Protea odorata	Essential oils	3- Methylhexane,	*Amaranthus hybridus* and *Dactyloctenium austral*	inhibition of seed germination	Almarie et al., 2016
Parthenium hysterophorus	methanolic flower extract	3 Methylcyclohexane oxide	*Vigna radiata*	inhibited the growth and agronomic parameters of mung bean under field conditions.	Kumar & Khari, 2020
Tipuana tipu	Pod essential oil	1-Methylnaphthalene	*Lactuca sativa*	Seed germination and seedling growth inhibition	El Ayeb - Zakhama et al., 2016
Aloe dawei	Roots extract	2-Methylnaphthalene	MCF-7 breast cancer cells	cytotoxic	Abdissa *et al.*, 2014

				activity	
Ipomoea cairica	Leaf extract	Dodecane	*Chrysanthemum coronarium*	Growth inhibition	Ma *et al.*, 2020
Chenopodium murale	Silver nanoparticLes prepared from leaf extract	trans-p-Mentha-2,8-dien-1-ol	*Staphylococcus aureus*	Antimicrobial activity	Abdel-Aziz *et al.*, 2014
Cymbopogon nervatus	Essential oils	trans-p-Mentha-2,8-dien-1-ol	*S. aureus, B. subtilis* and *E. coli*	Antimicrobial activity	Ahmed 2018

CHAPTER 3

MATERIAL AND METHODS

3.1 Study Area

The plant samples used in this study were collected from different regions of Pakistan particularly from Margalla Hills (ICT), Haripur, Abbottabad, Mardan, Lower Dir, Swat and Bagh district during May to July 2018. The regions from where the samples were collected are shown in Fig. 3.1. Plants were identified with the help of flora of Pakistan and reconfirmed by the specimen available in the Herbarium of Pakistan, Quaid-i-Azam University, Islamabad. Plant names were authenticated from the flora of Pakistan. Experimental work was conducted at Ecology and Biodiversity Laboratory of International Islamic University Islamabad, National Institute of Lasers and Optronics (NILOP) Islamabad and Department of Chemical and Environmental Engineering, University of California Riverside (UCR), USA. The aim of this study is to evaluate the allelopathic potential and screening of selected aromatic plants collected from different regions of Pakistan through bioassay (dish pack method) along with isolation and identification of active compounds in the selected aromatic plants. Identification of aromatic compounds were performed by using GC-MS. Identification and quantification of allelochemicals (phenolic compounds) was performed by using the high-performance liquid chromatography (HPLC) and Liquid chromatography–mass spectrometry (LC-MS). Assessment of potential inhibitory effects on algal growth i.e., *Chlamydomonas reinhardtii* were undertaken at UCR.

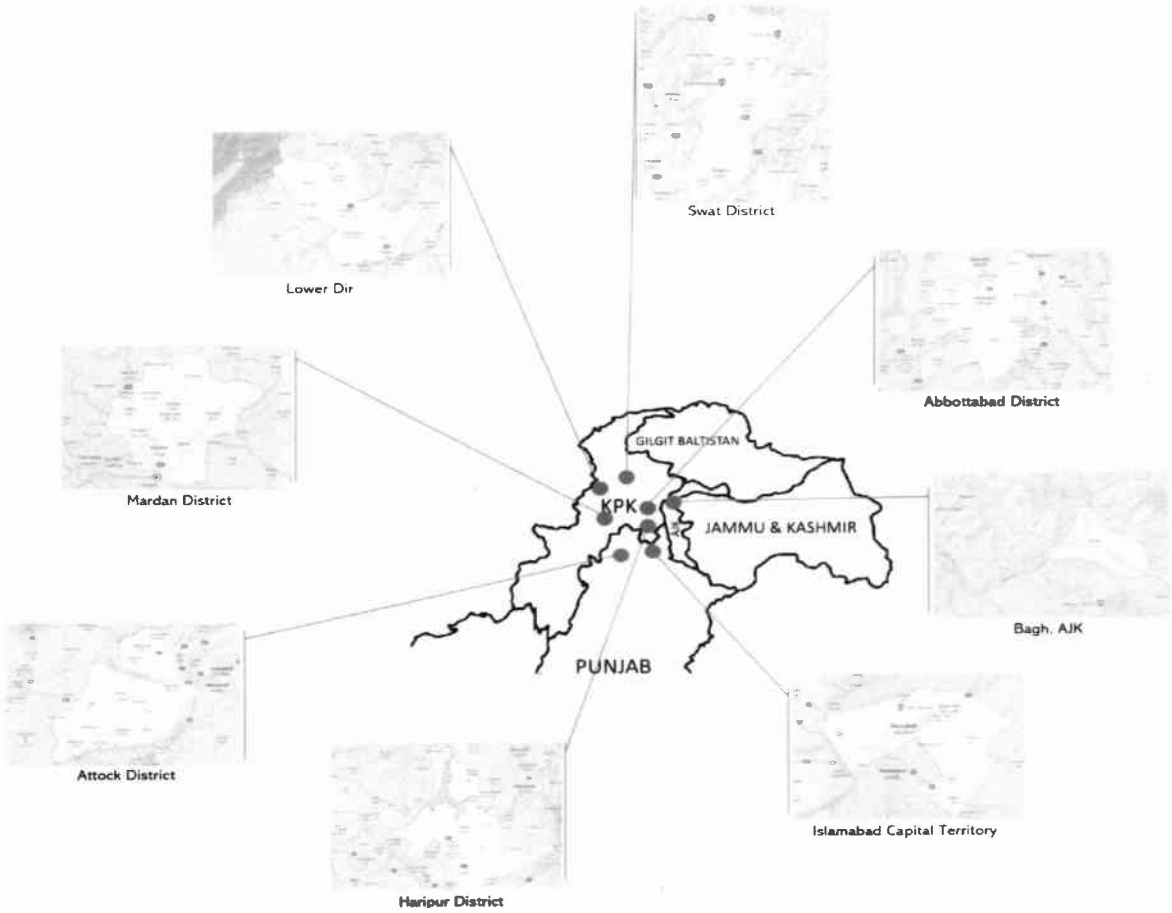


Fig. 3.1. Site maps of sample collection.

3.2 Instruments used in the study

- 6-well-multi-dish made of plastic
- Filter paper (Grade 1, 33mm)
- Incubator
- Amber-colored polypropylene centrifuge tube
- Distilled water fully automatic (HWSFA30)
- Biosafety cabinet
- BOD Incubator (NB-2201 LF)
- Oven (BJPX-SUMMER)
- Vertical autoclave fully automatic (CLASSIC 1050)
- Weighing balance
- Haier refrigerator (HRF-420 FDX)
- Ceramic Tweezer

- Gloves
- Cellophane tape
- Aluminum foil
- Sterilized syringes
- Polythene bags
- Paper bags and news papers
- Logarithmic graph
- HPLC apparatus (Shimadzu HPLC-2020)
- LCMS apparatus (Shimadzu LC-MS-2020)
- Kinetex ® 2.6 umXB-C18 100 ALC Column 100 x 2.1mm.
- Vortex Thermo Scientific
- Thermo Scientific Heraeus Pico 17 microcentrifuges
- Ohaus weighing balance
- Heraeus Multifuge X1R Centrifuge
- Automated cell counter Bio RAP TC 20TM.
- Grinder by Grainger
- Common laboratory glass ware
- Gas Chromatography (Agilent 6890 series)

3.3 Chemicals and Biological Samples

Following HPLC standards are used in this study:

- Hydroxy benzoic acid 99% (Arcos organics) bought from Fisher Scientific
- Rutin 97% (Arcos organics) bought from Fisher Scientific
- Quercetin (Arcos organics) bought from Fisher Scientific
- *trans*-Ferulic acid (Sigma Aldrich)
- *p-Coumaric* $\geq$ 98% (Sigma Aldrich)
- 3-hydroxy Tyrosol (Sigma Aldrich)
- Caffeic acid (MP Biomedicals) bought from Fisher Scientific
- Naringin (Alfa Aesar) bought from Fisher Scientific

Other chemicals used in experimentation include the following:

- Tris-Acetate-Phosphate medium (TAP)
- Acetonitrile (Sigma Aldrich)
- Formic acid (Sigma Aldrich)

- Ammonium acetate (Fisher Scientific)
- Acetic acid (Sigma Aldrich)
- Methanol (Fisher Scientific)
- *C. reinhardtii* CC-5119 21 gr mt- wild type
- Plant samples
- *Lactuca sativa* seeds (TAKI II Seed G-LEO 1)
- Fungal strains *(Aspergillus terreus, Aspergillus niger* and *Aspergillus flavus*) from NARC

The main steps involved in this research work are: plants collection, preparation of plant samples, measurement of length of radical and hypocotyl of germinating seeds, aromatic compounds isolation, identification and assessment of potential inhibitory effects against plant pathogens and data analysis.

3.4 Screening of Aromatic Plants on the basis of Allelopathic Potentials

3.4.1 Collection of Samples

The field work was based on collection of aromatic and medicinal plants. Fresh samples of 121 plant species were collected from different regions of Pakistan and leaves of these plants were used as samples. The allelopathic potential of these plants was determined through analysis of their leaf litter through Dish Pack Method. Lettuce (*Lactuca sativa L.*) was used as test plant due to its fast germination and high sensitivity.

3.4.2 Preparation of Plant Samples

The leaves were washed with fresh water to remove dust and other unwanted materials. These leaves were then oven dried at 60°C. Properly oven dried leaves were stored in brown envelops for further use and 0.10g of dry leaves of each plant was used for experiment.

3.4.3 Dish Pack Method

Dish Pack Method was used to analyze allelochemicals. In this method, the sample leaves were cut into pieces of 2 mm to enhance the release of volatiles and 100mg of dried sample leaves were put into lower left well of each plate in a multi dish (6 well with dimension 36 mm×18 mm). In other 5 holes, 0.75 mL of distilled water was added on the filter paper (Grade 1, 33 mm), and 7 seeds of lettuce were placed on the filter paper. The distances from the center of the source well (where plant sample was placed) to the center of other wells were 41, 58, 82, and

92 mm. multi-well dishes were sealed with cellophane tape for air tighten purpose to avoid desiccation and volatile compounds loss. Multi-well dishes were wrapped in aluminum foil. Dishes were placed in an incubator (Biobase Model BJPX-HI10) for the incubation process at 25°C. Radicle and hypocotyls length of five lettuce seedlings from each well were measured after three days and seed germination (%) against each sample was recorded (Fujii et al., 2005).

3.4.4 Growth measurements

After three days, germinated seeds of lettuce were counted. Hypocotyl and radical lengths were measured in mm. Statistical analysis of results was done through ANOVA by using Microsoft Excel 2013. % age inhibition was calculated by using the following equations:

$$IR = (Lc - Lt) / Lc \times 100 (\%) \ldots\ldots (Eq. 1)$$

where, IR; Inhibition rate (%), Lc: Radicle or top length of a control plant

Lt; Radicle or top length of a test plant

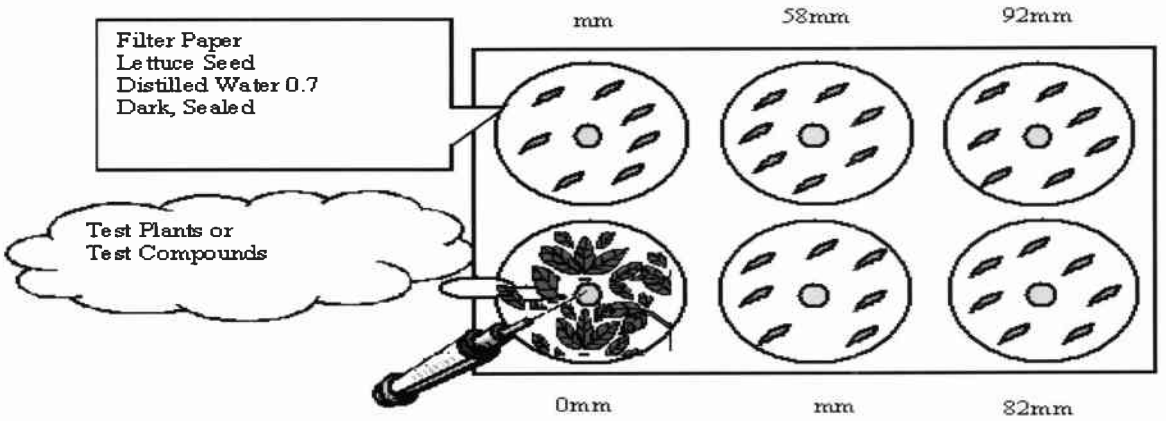


Fig. 3.2. Dish pack method (Fujii et al., 2005).

3.5 Identification of Volatile Compounds in the Dried Leaves of *Justicia adhatoda* (screened through dish pack method) using GC-MS techniques

GC-MS analysis of dried leaves of *Justicia adhatoda* and methanolic leaves extract of *Justicia adhatoda* was performed through GC-MS analysis

3.5.1 Sample preparation

For GC-MS analysis, 2 g of grounded leaves were placed in a glass bottle (100 mL) which has an air tight rubber lid through which a needle of the air tight syringe can pass. After 15 minutes, 0.5 mL of internal gas was taken out with an air tight syringe and gas was analyzed using GC-MS proposed by (Fuji et al., 2005).

3.5.2 Compounds identification by GC–MS

GC-MS analysis was carried out by using a Trace 1310 Gas Chromatograph thermo Scientific, ISQ 7000 Single Quadrupole Mass Spectrometer, Column HP-5ms with helium gas carrier having flow rate 1 mL/min, Oven conditions: 40°C – 5 min (hold), Ramp 2°C/min up to 60°C (0 min hold), Ramp 10°C/min up to 300°C (3 min hold), Inlet: 270°C, Split Ratio: 10:1, Mass Range 35-500. The compounds were identified by comparison of retention time, spectra and molecular weight with published data and libraries.

3.6 Identification of Allelochemicals in the Methanolic Leaf Extract of *Justicia adhatoda* using GC-MS Technique

Justicia adhatoda screened out on the basis of allelopathic potential through dish pack method as discussed in the previous section was then further analyzed for volatile compounds through GC-MS analysis. The details of these procedures are described in this section and the subsequent section.

3.6.1 Leaf Sample Preparation

Plant leaves were grounded into fine powder with coffee bean grinder and placed in aluminum foil to avoid light.

3.6.2 Preparation of Methanolic Leaf extract

The finely grounded homogenized sample (5 g) was drawn in a 50 mL amber-colored polypropylene centrifuge tubes by adding a quality control standard (Forchlorfenuron, 1 mg/L). It was instantly extracted with methanol (20 mL, 1% formic acid) by vortexing at 2000 revolutions per minute (rpm) for 2 minutes in the dark conditions. Then it was followed by centrifugation (10000 rpm for 10 minutes) at 10°C. Dilution of the supernatant was done with methanol (1:2, v/v) and injected (5 µL) into GC-MS for further analysis.

3.6.3 Identification of compounds by GC-MS

The presence of aromatic compounds in plants is identified by using the following steps:

- Comparison of Retention time with the authentic standard used.
- Comparison of MS Chromatograms and MS spectrum and with authentic data (provided in the literature) and NIST library.

3.7 Fungicidal Activity of Leaf Extract of *Justicia adhatoda*

Antifungal activity of *Justicia adhatoda* was checked against *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus flavus*.

3.7.1 Preparation of Methanolic Leaf Extract for Antifungal Activity of *Justicia adhatoda*

In this study, 100 gm of powdered leaves of *Justicia adhatoda* were soaked in 500 mL of analytical grade methanol in 1000 mL flask for 24 hours. By using rotary evaporator, extract was evaporated by vacuum evaporation. The methanolic leaf extract was then filtered by using Whatman filter paper number 41 (filter paper wetted with ethanol), 2 gm of sodium sulfate was added to remove traces of water and any other sediment in the filtrate. The extract (filtrate) was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phytochemicals in the *Justicia adhatoda* material. This extract was used for checking the antifungal activities.

3.7.2 Collection of Fungal Strains

The three fungal strains *Aspergillus terreus* , *Aspergillus niger* and *Aspergillus flavus* were isolated from rice seeds at Seed Health Lab at PGRI, NARC, Islamabad.

3.7.3 Purification of Fungal Cultures

Isolated fungus was repeatedly cultured on potato dextrose agar (PDA) media plates to attain pure cultures. Table 3.1 shows the composition of PDA. The PDA media was autoclaved at 121° C for 15 minutes.

Table. 3.1 PDA media composition

Potatoes, infusion	200g
Dextrose	20g
Agar	15g
Distilled water	1 L (Total volume)

3.7.4 Antifungal Potential through Agar Well Diffusion Method

The antifungal efficacy of various doses of Justicia extract was examined through agar well diffusion method. Pure cultures that had been maintained on PDA for seven days yielded fungal spores. In autoclaved distilled water, spore suspension (10^5 - 10^6/mL) was prepared using fungal spores. A haemo-cytometer was used to count the number of spores. With the use of a spreader, an equal number of spores (n=500) were dispersed on PDA plates. A sterilized cork borer was used to make six 5 mm diameter wells on every agar culture plate. Justicia extract in four different concentrations (50 ppm, 100 ppm, 150 ppm, and 200 ppm) was placed into the wells. Fluconazole at 200 ppm concentration and DMSO (Dimethyl Sulfoxide) was also poured in one well each. Fluconazole was used as a positive control and DMSO as negative control. After 7 days of incubation at 25° C, inhibitory zones (mm) were determined. Every test was carried out in triplicate.

3.7.5 Determination of minimum inhibitory concentration (MIC)

The agar plug method was utilized to assess the MIC of Justicia extract against every fungal strain. Before solidification, serial dilutions of 100 – 150 ppm of extract of Justicia were mixed to 20 mL of PDA media. When the fungal cultures became seven days old, one disc (10 mm in diameter) of mycelial plug was placed into PDA plates that contained Justicia extract. For next seven days, plates were kept in incubator at 28±2°C with dark and light alternated cycles. The fungal colony diameter was measured to determine the MIC.

3.7.6 Statistical Analysis

Using the data analysis tool in Excel, data was averaged and the means were analyzed with the help of Minitab. DMRT (Duncan's Multiple Range Test) at the 5% probability level was used to calculate pair wise comparisons between means

3.8 Identification and quantification of allelochemicals in top plants (screened through dish pack method) using HPLC Analysis

Plants screened out on the basis of allelopathic potential through dish pack method as discussed in the previous section were then further analysed for nonvolatile compounds through HPLC (for identification and quantification) and LCMS (for identification only). The details of these procedures are described in this section and the subsequent section.

3.8.1 Plant Sample Preparation

Plant leaves were grounded into fine powder with coffee bean grinder and placed in aluminum foil to avoid light.

3.8.2 Preparation of Methanolic Leaf extract and Standard Solutions

The finely grounded homogenized sample (5 g) was drawn in a 50 mL amber-colored polypropylene centrifuge tubes by adding a quality control standard (Forchlorfenuron, 1 mg/L). It was instantly extracted with methanol (20 mL, 1% formic acid) by vortexing at 2000 revolutions per minute (rpm) for 2 minutes in the dark conditions. Then it was followed by centrifugation (10000 rpm for 10 minutes) at 10°C. Dilution of the supernatant was done with water (1:2, v/v) and injected (10 µL) into the HPLC for further analysis.

Preparation of standard solutions: Stock solution was prepared by mixing one gram of standard (St 1-St 8) in one liter of methanol and 20 % diluted with DI water and then further dilutions were prepared.

3.8.3 HPLC Analysis

The leaf extracts were analyzed by reversed phase HPLC on Shimadzu HPLC 2020 (Kinetex ®□ 2.6 µm XB-C18 column, 100 A, LC 100 x 2.1mm) with UV detection. The mobile phase consisted of eluent A (0.1% aq. HCOOH) and eluent B (acetonitrile containing 0.1% HCOOH). The metabolites were separated on a gradient elution of 5% to 95% of B in deionized water over 7 min, followed by an isocratic elution using 95% of B for 5 min. The flow rate was 0.5 mL/min and the temperature of the column was maintained at 25°C with an injection volume ("loop") of 10 µL. The identity of each compound was verified by comparing UV-VIS spectra of samples and the authentic standards.

3.8.4 Identification of Compounds by HPLC

By comparing the HPLC retention time (Rt) and matching the UV-VIS spectra of the compounds present in various extracts with the authentic external standards, the task of

identification of allelochemicals was performed

Allelochemicals present in experimental plants were identified using the following steps:

- Comparison of retention time at which aromatic compound eluted peak with the standard used in the study.
- By the comparison of chromatograms of authentic standard and the sample (leaf extract).
- By Matching the UV-VIS spectra of the compounds present in various extracts with the authentic external standards.
- Quantification of allelochemicals by using the regression equation prepared by different concentrations of the standard compounds used and plotting calibration curve for each standard.

3.8.5 Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) of authentic standards of phenolic compounds for the quantification of these compounds in the leaf extracts of samples

The total compound content is expressed as µg/mL of methanolic leaf extract of experimental plants calculated through calibration curves of authentic standards. To quantify compounds, standard solutions containing 6 µg/mL, 8 µg/mL and 11 µg/mL of standard were prepared and analyzed on HPLC. Standard curve was plotted using three concentrations of authentic standard solutions against peak areas of standard using its characteristic UV-VIS absorption. The data obtained was subject to regression analysis and the coefficient of determination along with the regression equation $\mathbf{y = ax - b}$ was obtained, where '*y*' represents peak area and '*x*' denotes concentration. The LOD is the lowest analyte concentration that can be reliably detected by an analytical method and LOQ is the lowest analyte concentration that can be reliably determined by an analytical method.

3.9 Algicidal Effect of Leaf Extracts of *Selected* in Aquatic Environment

3.9.1 Cell Culture

Chlamydomonas reinhardtii CC-5119 21 gr mt-wild type was provided by Dr. Robert Jinkerson Lab (University of California, Riverside USA) and was grown in TAP Tris-Acetate-Phosphate medium (Gorman and Levine, 1965). The Chlamydomonas was grown under 24-hour light at room temperature. The cell density was determined with the help of automated cell counter Bio-Rad TC20 Automated.

3.9.2 TAP medium (Tris-Acetate-Phosphate medium)

Use of TAP: TAP is a freshwater medium (Gorman *et al.*, 1965) and is used for the culturing of *Chlamydomonas*. Acetate in the TAP medium is used as a fix carbon source which lets *Chlamydomonas* strains to grow fast.

Preparation: Media bottle was filled with DI water up to 80% of the final volume. Tris base was added to each stock solution. Glacial acid was added to make sure that all the acid was transferred by washing the pipette. The medium pH was adjusted to 7.20 by using HCL. Normally, the pH should be adjacent to 7.20 or marginally higher (~7.3-7.4). The medium was subsequently diluted to final volume (1000 mL) and then it was autoclaved.

Table 3.2. Composition of 1000mL of TAP medium

Components of media	**Amount Needed**	**Media Conc (g/l)**	**Molarity of media (m mole/l)**
Tris base	2.42 g	2.42	19.98
TAP salts	25 mL		
Phosphate Solution	0.375 mL		
Hutner's Trace Elements	1 mL		
Glacial Acetic Acid	1 mL	1	16.65
Distilled water	972.625 mL		

Table 3.3. TAP stock solutions

TAP Stock Solutions	**grams needed**	**Unit**	**Stock Conc (g/L)**	**Media Conc (g/L)**	**Media Molarity (m mole/L)**
TAP Salts	1000	mL			
NH_4Cl	15	g	15	0.375	7.01
$MgSO_4 \cdot 7H_2O$	4	g	4	0.1	0.41
$CaCl_2 \cdot 2H_2O$	2	g	2	0.05	0.34
Phosphate Solution	100	mL			
K_2HPO_4	28.8	g	288	0.108	0.62
KH_2PO_4	14.4	g	144	0.054	0.40
Hutner's trace elements	1000	mL			
$ZnSO_4 \cdot 7H_2O$	22	g in 250	22	0.022	0.0765

		mL H_2O			
H_3BO_3	11.4	g in 100 mL H_2O	11.4	0.0114	0.1844
$MnCl_2 \cdot 4H_2O$	5.06	g in 200 mL H_2O	5.06	0.00506	0.0256
$CoCl_2 \cdot 6H_2O$	1.61	g in 50 mL H_2O	1.61	0.00161	0.0068
$CuSO_4 \cdot 5H_2O$	1.57	g in 50 mL H_2O	1.57	0.00157	0.0063
$(NH4)_6Mo_7O_{24} \cdot 4H_2O$	1.1	g in 50 mL H_2O	1.1	0.0011	0.00089
$FeSO_4 \cdot 7H_2O$	4.99	g in 50 mL H_2O	4.99	0.00499	0.0179
Na_2 EDTA	50	g in 50 mL H_2O	50	0.05	0.1487

3.9.3 Bioassays

Methanolic leaf extracts of *Mentha royleana, Melia azedarach L, Malatus philpensis* and *Raphanus sativus L.* dried leaves were prepared. The leaves of test plants were dried in shade and grounded. Five g grounded dried leaves sample were added in 50 ml amber colored polypropylene centrifuge tube. Then 20 mL methanol and 1 % formic acid was added. Vortexed at 1000 rpm for 2 min in dark and then centrifuged at 10000 rpm for 10 min at 10 °C. Then it was filtered and supernatant was used in bioassays.

Fifteen sterilized flasks (3-flasks for each treatment) were taken and 20 ml of Tris-Acetate-Phosphate medium was poured into each flask and inoculated with 1 ml inoculum of C. reinhardtii (Total count 1.32 × 106 cells/ml). Then 1 ml leaf extracts of Donor experimental plants (*Melia azedarach L., Mentha royleana* Wall. ex Benth, *Mallotus philippensis* (Lam.) Muell Arg, *Raphanus sativus* L.) were added in each flask as per treatments. One ml methanol + 1 % formic acid solution was used as control. The number of cells were counted by placing 1 ml of sample on the slide (3 slides for each sample) and counted with automated cell counter Bio-Rad TC20 after 3, 8, 15, 30 days.

3.9.4 Statistical Analysis and Calculations

By following the method of Bruce William and Richardson (1988), the response index for leaf extracts of selected aromatic plants was calculated by using the following equation. The positive value for RI is stimulation and negative value is inhibition.

$$RI = \begin{cases} 1 - \frac{T}{C} & when\ T \geq C \\ \frac{T}{C} - 1 & when T < C \end{cases}$$ Equation (II)

Where, C = control response and T = treatment response

Statistical analysis (one-way ANOVA) with p value 0.05 using SPSS version 21

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Screening of selected aromatic plants for allelopathic potential

In this chapter, experimental results and discussion of current study are described. Leaves of 121 aromatic plants were collected from different regions of Pakistan and screened for allelopathic potential through dish pack method (Fujii et al., 2005). Volatile compounds from the leaves of aromatic plants generally diffuse and it is hard to determine enough compounds for an effective inhibitory activity to the neighboring plants in the field, so dish pack method (section 3.4.3) is used in this study for assessment of allelopathic potential. Experimental results of allelopathic potential of selected plants are shown in Table 4.1 (% age of elongation of radical and hypocotyl of *Lettuce sativa*). The criteria is denoted by *, **, ***, **** indicate hypocotyl and radicle elongation which is less than the mean value minus standard deviation, mean–1 STD, mean-1.5 STD, mean-2 STD and mean-2.5 STD i.e., 62.32, 44.91, 27.50 and 10.09 respectively.

By statistical analysis and compilation of results, between 80% to 100% lettuce hypocotyl growth inhibition was observed in 2 species viz., *Raphanus sativus* and *Cymbopogan Citraus,* and 4 species shows hypocotyl growth inhibition between 60-79% viz., *Mentha royleana, Justicia adhatoda, Melia azedarch,* and *Rubus fruiticosus* (Rosaceae). Fourteen plant species showed hypocotyls inhibition between 40-59%. Between 20-39% hypocotyl growth inhibition was shown in 23 plant species, 32 species showed hypocotyl inhibition below 19% and 48 species showed stimulatory effect to hypocotyl growth.

It was found that inhibition potential between 80% to 100% against the lettuce radicle growth was found in 2 species viz., *Raphanus sativus* (Brassicaceae) and *Mentha royleana* (Lamiaceae). Between 60-79% radicle growth inhibition observed in 7 species viz. *Justicia adhatoda* (Acanthaceae), *Cymbopogan Citratus* (Poaceae), *Melia azedarach* (Meliaceae), *Menthapiperita* (Lamiaceae), *Mallotus philipensis* (Euphorbiaceae), *Albizia lebbeck* (Fabaceae) and *Mentha longifolia* (Lamiaceae). Radicle growth was observed between 40-59% in 14 species, between 20-39% in 17 species, 23 species showed level of radicle growth inhibition below 19% and remaining plant species showed stimulatory effect to lettuce root growth.

Table 4.1. Allelopathic potential screening of 121 aromatic plants of Pakistan

Sr	Plant species (Scientific Name)	Family	Extension (%) *		Criterion**	
			Radicle	Hypocotyl	Radicle	Hypocotyl
1.	*Raphanus sativus* L.	Brassicaceae	13.60	11.10	***	***
2.	*Cymbopogon citrates* (DC.) Stapf.)	Poaceae	30.82	14.10	**	***
3.	*Mentha royleana* Wall. ex Benth	Lamiaceae	17.60	24.90	***	**
4.	*Justicia adhatoda* L.	Acanthaceae	29.90	30.40	**	**
5.	*Melia azedarach* L.	Meliaceae	31.25	37.68	**	**
6.	*Mallotus philippensis* (Lam.) Muell Arg.	Euphorbiaceae	37.60	45.50	**	*
7.	*Lantana camara* L.	Verbenaceae	41.70	41.90	**	*
8.	*Rubus fruiticosus* L.	Rosaceae	43.20	38.20	**	*
9.	*Punica granatum* Linn., Sp.	Lythraceae	45.90	41.40	*	*
10.	*Carissa opaca* Stapf ex Haines.	Apocynaceae	55.40	40.20	*	*
11.	*Morus alba* L.	Moraceae	58.30	51.50	*	*
12.	*Acacia catechu* (Linnaeus f.) Willdenow	Fabaceae	73.03	54.28		*
13.	*Elaeagnus angustifolia* Linn., Sp.	Elaeagnaceae	77.61	45.57		*
14.	*Piper nigrum* Linnaeus, Sp.	Piperaceae	83.99	51.83		*
15.	*Buddleja asiatica* Loureiro, Fl. Cochinch.	Scrophulariaceae	103.36	46.24		*
16.	*Setcreasea purpurea* (Schau.) Boom	Commelinaceae	107.27	46.52		*
17.	*Geranium wallichianum* D. Don ex Sweet	Geraniaceae	113.64	48.51		*
18.	*Ficus carica* L.	Moraceae	132.90	54.80		*
19.	*Tagetes minuta* L.	Asteraceae	138.64	49.75		*
20.	*Mentha piperita* L.	Lamiaceae	32.18	67.31	**	
21.	*Albizia lebbeck* (Linnaeus) Bentham.	Fabaceae	38.80	76.70	**	
22.	*Mentha longifolia* (L.) L., Fl. Monspel.	Lamiaceae	39.61	64.74	**	
23.	*Prunus domestica* Linnaeus.	Rosaceae	40.10	70.20	**	
24.	*Bougainvillea spectabilis* var.*glabra* (Choisy) W. J.Hooker.	Nyctaginaceae	41.50	70.50	**	
25.	*Medicago sativa* L.	Fabaceae	43.80	82.80	**	
26.	*Broussonetia papyrifera* (L.) L'Herit. ex Vent.	Moraceae	44.71	191.14	**	
27.	*Lavatera cachemiriana* Cambess.	Malvaceae	46.15	200.24	*	
28.	*Acacia nilotica Linn.*	Mimosaceae	53.67	86.39	*	
29.	*Juglans regia* L.	Juglandaceae	54.30	69.30	*	
30.	*Bambusa arundinacea* (Retz.) Willd.	Poaceae	55.90	66.01	*	
31.	*Ocimum basilicum* L.	Lamiaceae	60.80	87.50	*	
32.	*Zanthoxylum armatum* DC.	Rutaceae	69.50	86.80		
33.	*Cassia fistula* L.	Fabaceae	70.41	80.71		

34.	*Pinus roxburghii* Sargent, Silva N. Amer.	Pinaceae	71.54	68.82		
35.	*Citrus reticulate* Blanco, Fl.Filip.	Rutaceae	71.73	101.55		
36.	*Ziziphus jujuba* Miller, Gard.	Rhamnaceae	73.06	96.97		
37.	*Eucalyptus globulus* Labill.	Myrtaceae	74.51	54.46		*
38.	*Viola odorata* L.	Violaceae	74.70	70.20		
39.	*Mangifera indica* L.	Anacardiaceae	75.19	103.67		
40.	*Bauhinia variegata* Linn.	Caesalpiniaceae	76.97	90.22		
41.	*Ficus virgata* Reinw. Ex Blume.	Moraceae	76.90	104.40		
42.	*Pongamia pinnata* (Linn.) Pierre.	Fabaceae	76.62	92.84		
43.	*Citrus paradisi* Macfadyen in Hook.	Rutaceae	78.88	75.84		
44.	*Hibiscus mutabilis* Linn.	Malvaceae	79.75	74.84		
45.	*Psidium guajava* Linnaeus	Myrtaceae	80.51	68.57		
46.	*Murraya exotica* Linnaeus, Mant.	Rutaceae	82.26	92.74		
47.	*Ficus religiosa* Linnaeus, Sp.	Moraceae	82.89	67.42		
48.	*Anaphalis nepalensis* (Sprengel)	Asteraceae	83.52	123.58		
49.	*Populus ciliata* Wall. Ex Royle,III.	Salicaceae	83.00	98.90		
50.	*Cyamopsis tetragonoloba* (L.) *Taubert*	Fabaceae	85.83	87.08		
51.	*Equisetum arvense* Linnaeus, Sp.	Equisetaceae	86.01	113.19		
52.	*Citrus limon* (Linnaeus) Osbeck, Reis	Rutaceae	86.61	78.98		
53.	*Eucalyptus camaldulensis* Dehnh.	Myrtaceae	88.28	65.79		
54.	*Verbena tenuisecta* Briq. in Ann.	Verbenaceae	88.70	111.66		
55.	*Ocimum tenuiflorum* L.	Lamiaceae	88.74	82.86		
56.	*Berberis lycium* Royle.	Berberidaceae	88.94	100.72		
57.	*Rosa indica* Lindl, Ros. Monogr.	Rosaceae	89.00	75.81		
58.	*Ipomoea nil* (Linn.) Roth.	Convolvulaceae	89.02	76.07		
59.	*Syzygium cumini* L.	Myrtaceae	89.25	104.66		
60.	*Thevetia peruviana* (Pers.) Schum.	Apocynaceae	89.39	119.57		
61.	*Zanthoxylum armatum* DC., Prodr.	Rutaceae	89.90	91.01		
62.	*Robinia pseudo-acacia* L.	Papilionaceae	95.10	122.60		
63.	*Lactuca serriola* L.	Asteraceae	97.50	128.60		
64.	*Vinca rosea* L.	Apocynaceae	98.53	87.89		
65.	*Fragaria indica* Andrews, Bot.	Rosaceae	98.80	128.30		
66.	*Adiantum capillus-veneris* L.	Pteridaceae	99.03	180.46		
67.	*Aesculus indica* (Wall.ex Camb) Hook	Sapindaceae	100.60	111.80		
68.	*Dodonaea viscosa* (Linn.) Jacq., Enum	Sapindaceae	100.77	76.97		
69.	*Acacia modesta* Wall.	Mimosaceae	100.80	112.00		
70.	*Carissa opaca* Stapf ex Haines	Apocynaceae	101.60	81.46		

71.	*Dryopteris ramosa* (C.Hope) C.Chr.	Dryopteridaceae	101.23	143.31		
72.	*Tagetes erecta* L.	Asteraceae	101.41	161.36		
73.	*Sonchus asper* (Linnaeus) Hill.	Asteraceae	101.70	138.30		
74.	*Pyrus pashia* Buchanan-Hamilton ex D. Don.	Rosaceae	102.10	99.70		
75.	*Pinus wallichiana A. B. Jackson.*	Pinaceae	103.30	121.40		
76.	*Xanthium strumarium* Linnaeus, Sp.	Asteraceae	103.36	84.95		
77.	*Jatropha integerrima* Jacq., Enum.	Euphorbiaceae	103.43	98.36		
78.	*Tecoma stans* (*L.*) *H. B. & K.*	Bignoniaceae	103.63	96.92		
79.	*Foeniculum vulgare* mill.	Apiaceae	103.76	98.15		
80.	*Cestrum nocturnum* L.	Solanaceae	104.13	98.02		
81.	*Pterospermum acerifolium* (Linn.) Willd.	Sterculiaceae	106.71	95.14		
82.	*Bauhinia variegata* Linn., Sp.	Fabaceae	110.00	128.60		
83.	*Amaranthus hybridus* L.	Amaranthaceae	111.11	97.72		
84.	*Lavatera* trimestris Linn.	Malvaceae	114.61	76.07		
85.	*Skimmia laureola* (DC.) Decne.	Rutaceae	116.05	103.76		
86.	*Convolvulus arvensis* Linn., Sp.	Convolvulaceae	117.58	83.06		
87.	*Platanus orientalis* Linn., Sp.	Platanaceae	118.69	78.13		
88.	*Otostegia limbata* (Benth.) Boiss.	Labiatae	119.00	108.10		
89.	*Cyperus rotundus* Linnaeus, Sp.	Cyperaceae	119.50	163.90		
90.	*Helianthus annuus* L.	Asteraceae	119.70	106.80		
91.	*Artemisia maritima* L.Engl.	Asteraceae	120.96	148.30		
92.	*Morus nigra* L.	Moraceae	121.60	140.50		
93.	*Solanum incanum* L.	Solanaceae	123.78	92.36		
94.	*Cupressus sempervirens* L., Sp.	Cupressaceae	125.00	130.64		
95.	*Myrtus communis* L.	Myrtaceae	126.10	133.60		
96.	*Citrus limetta* Risso.	Rutaceae	126.50	110.00		
97.	*Crataegus oxyacantha* L.	Rosaceae	126.80	93.50		
98.	*Litchi chinensis* Sonnerat,Voy	Sapindaceae	126.72	83.84		
99.	*Schefflera bengalensis* Gamble	Araliaceae	130.56	110.20		
100.	*Olea ferruginea* Royle.	Oleaceae	132.21	230.58		
101.	*Dillenia indica* Linn.	Dilleniaceae	132.90	176.30		
102.	*Melaleuca armillaris* (Sol. ex Gaertn.) Sm.	Myrtaceae	133.90	108.55		
103.	*Nerium oleander* L.	Apocynaceae	140.91	70.90		
104.	*Mahonia borealis* Takeda in Notes Roy.	Berberidaceae	141.00	58.50		
105.	*Verbascum thapsus* (Linn.)	Scrophulariaceae	146.23	118.22		
106.	*Datura stramonium* L.	Solanaceae	146.42	159.64		

107.	*Canna indica* L.	Cannaceae	150.29	98.70		
108.	*Datura metel* L.	Solanaceae	151.20	**183.80**		
109.	*Lagerstroemia indica* Linn.	Lythraceae	154.55	62.19		
110.	*Salix alba* Linnaeus.	Salicaceae	155.22	91.63		
111.	*Melanthera biflora* (L.) Wild.	Asteraceae	155.50	75.50		
112.	*Woodfordia fruticose* (Linn.) S. Kurz	Lythraceae	157.30	168.00		
113.	*Murraya koenigii* (Linn.) Spreng.	Rutaceae	158.64	100.30		
114.	*Celosia argentea Linn.*	Amaranthaceae	160.29	108.38		
115.	*Citrus sinensis* (Linn.) Osbeck, Reise Ostind.	Rutaceae	162.25	132.51		
116.	*Bryophyllum pinnatumn*(Lam.) Oken.	Crassulaceae	167.49	98.70		
117.	*Youngia japonica.* (L.) DC.	Asteraceae	173.80	156.50		
118.	*Myrsine africana* Linnaeus, Sp.	Primulaceae	175.60	139.10		
119.	*Elettaria cardamomum* (L.) Maton	Zingiberaceae	182.22	93.37		
120.	*Olea europaea* Linn.	Oleaceae	196.47	209.68		
121.	*Berberis vulgaris* L	Berberidaceae	241.60	153.80		

Mean	97.14	95.47
SD	34.82	38.24
Mean-1SD	62.32	57.23
Mean-1.5SD	44.91	38.11
Mean-2SD	27.50	18.98
Mean-2.5SD	10.09	-0.14

% age growth rate of L.stiva, compared to that of the control; criteria shows stronger inhibition in the radicle and hypocotyl: *M–1(σ), ** M – 1.5(σ), ***M– 2(σ), and ****M – 2.5(σ)

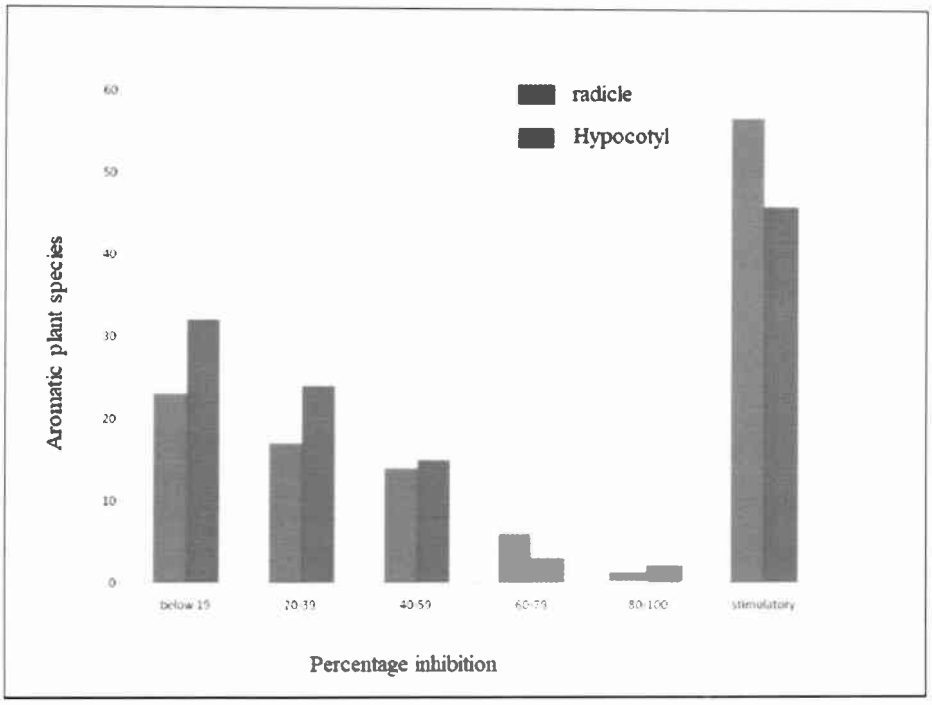


Fig 4.1. Frequency distribution of % inhibition among aromatic plants through dish pack method.

4.1.1 Discussion

This study describes screening of allelopathic activity of selected aromatic plants from different regions of Pakistan by using Dish Pack Method (Table 4.1). In this study, 21 species showed 40% or more growth inhibition to lettuce hypocotyl and 23 species showed more than 40% radical growth inhibition. It has been reported by researchers that allelopathic effect is more on root growth as compared to shoot growth (Munir & Tawaha, 2002; Rawat et al., 2012). Allelopathic effect is mentioned in the form of hypocotyl and radicle growth inhibition and stimulation. Negative values are mentioned as growth promotion as compared with the controls (as shown in Table1). 121 plant species were screened and analyzed for allelopathic potential using dish pack method. *Raphanus sativus* (Brassicaceae) and *Mentha royleana* (Lamiaceae) showed high allelopathic potential against test plant lettuce, which fully inhibited the seed germination of lettuce. Other 11 species of plants belonging to families Asteraceae, Anacardiaceae, Fabaceae and Solanaceae confirmed strong allelopathic potential which caused growth inhibition of test plant (Morikawa et al., 2012).

The experimental results showed maximum (more than 80%) inhibition by *Raphanus sativus* (Brassicaceae) and *Cymbopogan* (Poaceae), followed by *Mentha royleana* (Lamiaceae),

Justicia adhatoda (Acanthaceae), *Melia azedarach* (Meliaceae) and *Mallotus philipensis* (Euphorbiaceae) (see Table 4.1).

Most of the ecological zones are under threat of food insecurity due to climate change, rise in temperature and changing rain patterns which can decrease the agricultural yield in future (Lobell et al., 2011). However, it is important to go for sustainable food production by using the natural resources. Use of allelopathy is a sustainable strategy that can help to control the food insecurity problem. Secondary metabolites secreted from the plants have positive and negative effects on the plant growth and neighboring microorganisms. Plants related to the brassica family have allelopathic potential, as most of brassica species produce strong allelochemicals, for example, brassinosteroids, glucosinolates and allyl isothiocyanates. These secondary metabolites are extremely phytotoxic to other plants which effect their growth and further development. Allelopathic potential of Brassicaceae can be used for weed management and sustainable food production by using them as intercrops, cover crops and companion crops as well as for residue incorporation, mulching and simply using them in crop rotations. Allelochemical of brassica species can also be used for the management of abiotic stresses. These allelochemicals can act as crop growth promoters when used at lower concentrations (Rehman et al., 2019).

The results of this study comply with previous studies on allelopathy where these species were found to have allelopathic effects on growth of plants and germination of seeds of test species. The phytotoxicity i.e., toxic effect of plants caused by variety of chemical compounds varied with mode of its release. Some of these effects are concentration dependent. In the present study, *Raphanus raphanistrum subsp. Sativus* appeared to have an inhibitory effect, which matches with the finding of study of Uremis et al. (2009) where inhibitory effect of six Brassica species was determined.

In the present study, *Raphanus Sativus* showed 80-100% hypocotyl and radicle growth inhibition of test plant *L. sativa* which is supporting by the research conducted in a field study during 2004 and 2005 near Blackville and Tifton GA for the evaluation of allelopathic effects of wild radish and rye (cover crop) on sweet corn yield and it was observed that Wild radish and rye without herbicides reduced the density of weed *Florida pusley* 65%, while total density of weed was decreased up to 50% (Malik et al., 2008). Wild radish can be used as a natural cover crop. It was investigated by Norsworthy (2003), who conducted study under controlled conditions, that weed emergence and growth could be controlled by wild radish residues and extracts. Wild radish has detrimental effect on broad leaf weeds and likely alter the competition between crops and weeds, providing a competitive benefit to monocotyledonous crops over

dicotyledonous weeds (Norsworthy, 2003). All of the abovementioned studies are supporting results of present study.

A study conducted by Suwitchayanon et al., (2013) showed that roots of test plants in their study showed more sensitivity to the extract as compared to their shoots. They also reported allelopathic activity of methanolic extract of *Cymbopogon nardus* (Poaceae) against eight test plant species including lettuce (*Lactuca sativa* L.). The inhibitory effect of extract was shown on shoot and growth of four plants namely lettuce, cress, Italian rye grass and rapseed (*Brassica napus*). Their results indicate the presence of allelochemicals in *Cymbopogon nardus*, which can be selected as a candidate for further isolation and identification of allelochemicals for the development alternative weed control option for sustainable crop production. Begum *et al.* (2020) concluded that allelopathic species due to presence of volatile allelochemicals can be used for sustainable management of weeds in agricultural systems.

In the present study, *Mentha piperata* shows radicle growth inhibition (60-79%) which is more than the hypocotyl growth inhibition (20-39%). These results are supported by the study of Skrzypek et al. (2015) in which it was investigated that active ingredients present in *Mentha piperita* consists of phenolic acids, menthol and many other compounds, having different quantitative and qualitative chemical characteristics. Radicle growth inhibition of the present study is supported by the studies conducted by Rawat et al. (2012) on plant extract of different weed species; their studies proved that the toxicity of compounds presents in the extract delayed the seed germination and seedlings growth and also caused abnormalities in root apices anatomy and morphology. Further, in the study conducted by Shinwari et al. (2013) using dish pack method, the maximum radical growth inhibition was observed in *Tagetes minuta* followed by *Lantana camara* and *Prosopis juliflora*.

In present study, *Justicia adhatoda* inhibited the radicle and hypocotyl growth (60-79%) which are in conformance with the results of Devkota and Sharma (2014) who showed that leaf extract of *Justicia adhatoda* Linn strongly inhibited the seed germination and growth of root and hypocotyl of wheat. Leaf extract of *Justicia adhatoda* and extract of *Cheilocostus speciosus* rhizome showed inhibitory effects on seed germination of wheat and pea. The degree of inhibition was increased with the increase in the concentration of extract. The present study is supported by the study of Devkota & Sharma (2014) who reported that the use of highest extracts concentration significantly reduced the root and shoot lengths when compared with control.

In the present study, *Melia azedarach L* showed the growth inhibition of Lettuce between 60-79% hypocotyl and radicle growth inhibition. This growth inhibition is supported by the

evidence provided by Tur et al., (2012) who analyzed aqueous extract of dried and fresh leaves along with fruits of *Melia azedarach L* for seed germination and growth inhibition of tomato, and found that the effect of dried leaf extracts of *M. azedarach* was more as compared to the extract of fresh leaves and dried and fresh fruits. Secondary metabolites presence was observed in the leaves of *M. azedarach* that are cause of inhibition due to their effect on α-amylase activity during the process of seed germination e.g., *Echinochloa crus-galli*, as they are water soluble and absorbed by plants with water uptake (Phuwiwat et al., 2012).

The present study induces the presence of various allelochemicals in leaves of *Mallotus philipinensis* which caused inhibitory stress against the radicle and hypocotyl of *Lettuce sativa;* and these results are comparable with findings of study of Sher et al. (2014) in which used Aqueous extracts from all parts of *M. philipinensis* significantly inhibited the germination, radicle and plumule growth of all test species due to allelopathic effects.

The present research findings also exhibited minimum growth inhibition and maximum growth stimulation by *Berberis vulgaris,* which is followed by *Olea europaea* and *Elettaria cardamomum* as shown in Table. 4.1.1. It was also proved in the recent research study of Isah (2019) that secondary metabolites presence induced the tolerance in plants against abiotic stresses like increase in temperature. So, the use of these allelochemicals to the plants at different phonological stages can supplement the stress resistance and can act as natural measures to minimize the climate change impacts. The results of this study can be helpful for the development of new bioactive chemicals from natural products that can be used to formulate sustainable agricultural practices and a natural algicide to control algal blooms.

Justicia adhatoda was further analyzed through GC-MS to identify the volatile allelochemicals which can be a cause of hypocotyl and radical growth inhibition by using the dish-pack method. Lab experiments were performed to analyze its antifungal effects. Furthermore, the effect of allelochemicals on *Chlamydomonas reinhardtii* by application of top plants leaf extracts to prove their antialgal properties. The results of GC-MS analysis of *Justicia adhatoda* are described in the subsequent sections.

4.2 GC-MS Analysis of *Justicia adhatoda* Dried leaves for Identification of Volatile Compounds

Through GC-MS analysis, *Justicia adhatoda* was selected for the identification of allelochemicals which could be the cause of hypocotyl and radical growth inhibition. *Justicia adhatoda* was particularly focused; though *Raphanus sativus* and *Mentha royleana* were identified as the top most screened plants followed by *Justicia adhatoda* among all the studied

plants, but the former plants are the cultivated species whereas *Justicia adhatoda* is indigenous specie and is top most among the indigenous plants in this study. Hence, *Justicia adhatoda* was further analyzed for identification of allelochemicals through GC-MS analysis. Previously, research focus on *Justicia adhatoda* was for medicinal uses only. Therefore, another reason to select this plant is to determine its effectiveness for weed control and antifungal properties to treat plant infections by analyzing allelochemicals present in it.

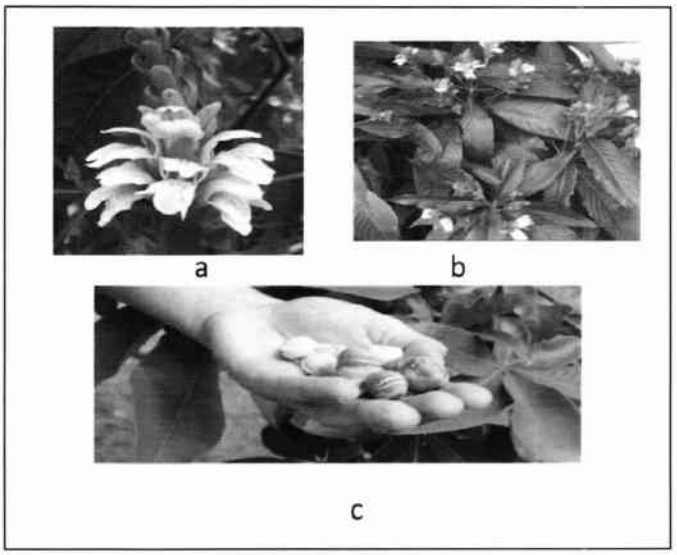


Fig. 4.2. *Justicia adhatoda*, a. Justicia flower, b. Justicia leaves, c. Justicia fruit.

Method proposed (section 3.5.1) by Fuji et al. (2005) was used for GC-MS analysis of volatile compounds.

GC–MS chromatogram of *Justicia adhatoda* dried leaves indicated the presence of different compounds (Fig.4.2.). The spectral fingerprints of compounds identified by using the data library, molecular weight and retention time in minutes are shown in Table. 4.3.

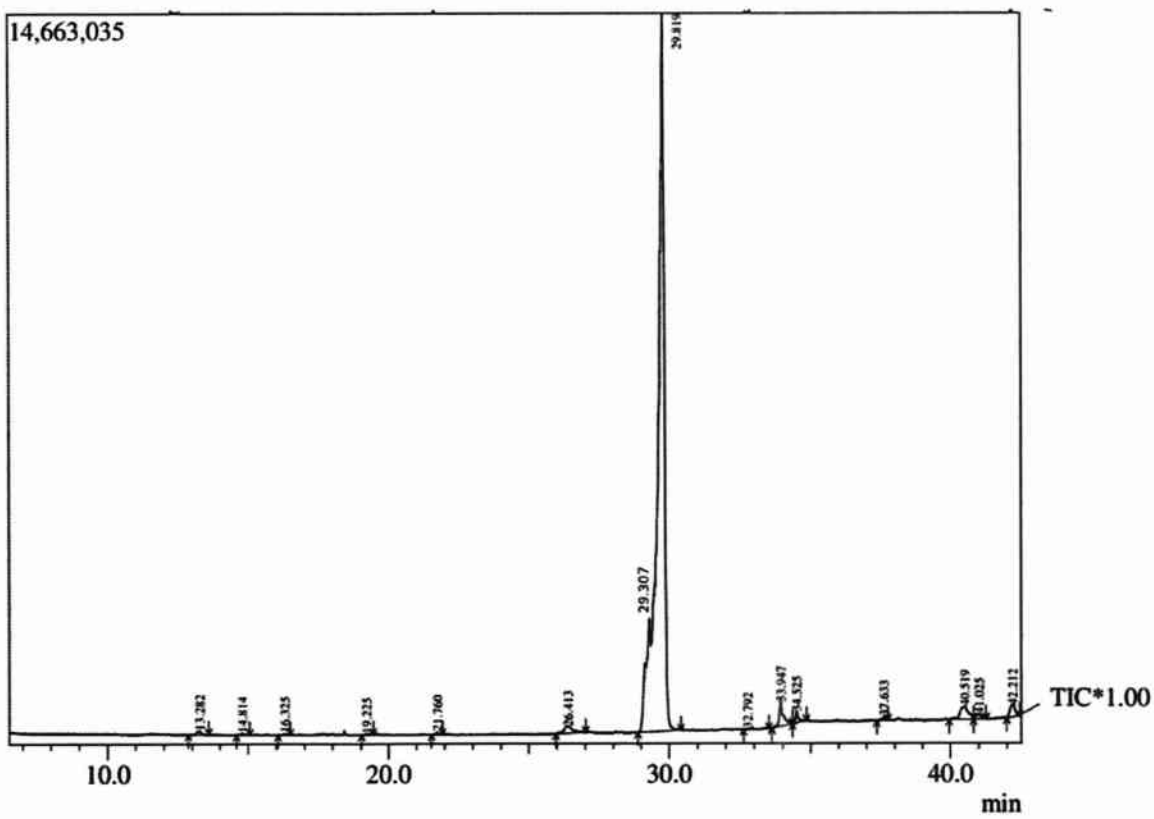
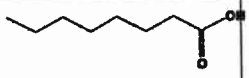
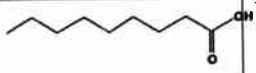
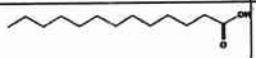
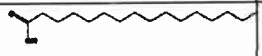
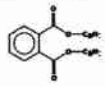
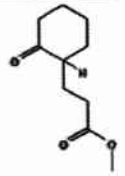
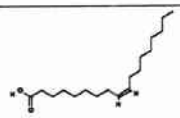
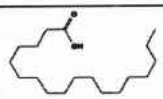
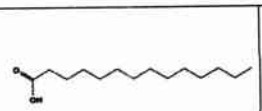
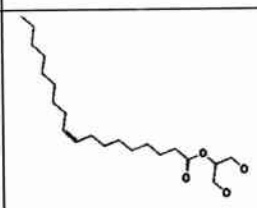
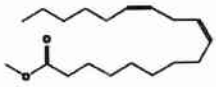
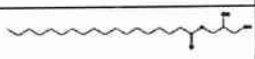


Fig 4.3 Chromatogram of *Justicia adhatoda* dried leaves

Table 4.2. Volatile Compounds Identified in the Dried Leaves of *Justicia Adhatoda* through GCMS Analysis

R_t	Molecular Weight	Molecular Formula	Main Fragments	Area %	Chemical structure	Compound Name
13.282	144	$C_8H_{16}O_2$	31, 41, 44, 60, 73, 101, 115	0.35		Octanoic acid
14.814	256	$C_9H_{18}O_2$	31, 41, 43, 60, 73, 84, 98, 129, 158	0.12		Nonanoic acid
16.325	126	$C_8H_{14}O$	30, 31, 39, 41, 42, 43, 55, 57, 69, 70, 83, 85, 98	0.08		2-Octenal, (E)-
19.225	214	$C_{13}H_{26}O_2$	41, 43, 60, 73, 129, 171	0.10		Tridecanoic acid
26.413	330	$C_{19}H_{38}O_4$	41, 43, 57, 71, 84, 98, 134, 239, 330	1.18		Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
29.307	256	$C_{16}H_{32}O_2$	41, 43, 60, 73, 85, 98, 115, 129, 213, 256	11.31		*n*-Hexadeconoic acid
29.819	390	$C_{24}H_{38}O_4$	41,57,71,84,104,113,132,149, 167,168,279	80.93		1,2-Benzenedicarboxylic acid, diisooctyl ester
32.792	184	$C_{10}H_{16}O_3$	32, 41,43, 55, 59, 69, 74, 77, 97, 110, 184	0.10		Cyclohexanepropanoic acid, 2-oxo-, methyl ester
33.947	282	$C_{18}H_{34}O_2$	41, 43, 55, 69, 83, 97, 264	1.99		Oleic Acid
34.525	284	$C_{18}H_{36}O_2$	41, 43, 55, 57, 60, 73, 97, 129, 185, 241, 284	0.69		Octadecanoic acid

37.633	228	$C_{14}H_{28}O_2$	41, 43, 55, 57, 71 98, 129, 228	0.21		Tetradecanoic acid
40.519	356	$C_{21}H_{40}O_4$	41, 43, 55, 69, 83, 97, 98, 112, 129, 285, 264, 356	2.12		9-Octadecenoic acid *(Z)-*, 2,3-dihydroxypropyl ester
41.025	294	$C_{19}H_{34}O_2$	41, 43, 54, 55, 68, 83, 96, 130, 281	0.59		9,12-Octadecadienoic acid *(Z,Z)-*, methyl ester
42.212	358	$C_{21}H_{42}O_4$	43, 41, 55, 57, 98, 84, 74, 134, 267, 284, 358	1.20		Octadecanoic acid, 2,3-dihydroxypropyl ester

In the present study, phytochemical analysis of dried leaves of *Justicia adhatoda* is performed through GC-MS analysis. Phytochemical analysis revealed the presence of 14 allelochemicals in the dried leaves of *Justicia adhatoda* namely 1,2-Benzenedicarboxylic acid, diisooctyl ester (80.93%) at retention time 29.819 minutes and *n*- hexadeconoic acid (11.31%) at retention time 29.307 minutes were the prominent followed by 9-Octadecenoic acid *(Z)-*, 2,3-dihydroxypropyl ester (2.12%) at 40.519 minutes, oleic acid (1.99%) at retention time 33.947 minutes and Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (1.18%) at retention time 26.413 minutes.

4.2.1 Discussion

Allelopathy analysis through bioassay is imperative for the identification of new allelochemicals and the dish pack method is a novel technique developed for the analysis of volatile compounds and allelochemicals (Fuji et al., 2005). As volatile compounds diffuse into the air due to wind, therefore, their enough concentrations do not reach or applied to the neighboring plants to exhibit considerable growth inhibitory effect. However, through cover plants, fallen leaves and leachate, allelochemicals can inhibit the growth of other plants.

Compounds mentioned in Table.4.2. are naturally occurring volatile compounds present in the dried leaves of *Justicia adhatoda* and are also reported for their bioactive properties in different research studies. Different bioactive compounds present in this plant showed allelopathic potential and their allelopathic potential is due to the effect of the compounds on different biochemical pathways. Li et al. (2021) reported that the chlorophyll synthesis inhibition of recipient plants was one of the main mechanisms for plant growth inhibition.

The 1, 2-Benzenedicarboxylic acid, diisooctyl ester is the major compound identified in the dried leaves of *Justicia adhatoda* (Rt 29.819 min) through GC-MS analysis. Its antifungal, antibacterial and cytotoxic activities are reported in contemporary literature (Haoui et al., 2016). Aforesaid compound has been considered as the main cause of hypocotyl and radical growth inhibition of *Lactuca sativa* in this research study. The results reported by Haoui et al. (2016) show that the essential oil extracted from *Inula viscosa* contains phytochemicals including 1,2-Benzenedicarboxylic acid, diisooctyl ester which is useful for green plant protection and can be used in pharmaceutical and food industries. 1,2-Benzenedicarboxylic acid, diisooctyl ester was also identified in the leaf extract of *Sisymbrium irio* which showed antifungal activity (Akhtar et al., 2020). In another study, Rahman et al. (2006) determined the cytotoxic activity of the1,2-benzenedicarboxylic acid, diisooctyl ester on newborn brine shrimp (*Artemia salina*) and found it effective against newborn brine shrimp and also screened for antifungal activities against three fungi species. The results of the present study are in conformance with the abovementioned studies and showed that the cytotoxic activity of 1,2-benzenedicarboxylic acid, diisooctyl ester is the cause of hypocotyl and radical growth inhibition of *Lactuca sativa* (test plant).

Benzenedicarboxylic acid, diisooctyl ester showed an excellent binding affinity to peptide deformylase and inhibited the growth of fungi and bacteria by inhibiting the protein synthesis (Joshi et al., 2021). Originally peptide deformylase thought to be restricted to prokaryotic organisms, this enzyme was also discovered in numerous plant species e.g., *Arabidopsis thaliana* as a result of genome sequencing efforts (Giglione *et al.*, 2000). The essentialness and widespread conservation of plant peptide deformylase in plants makes it an important molecular target for the new class of broad-spectrum herbicides. Hou et al. (2007) suggest plant peptide deformylase as a potential broad-spectrum herbicide target. Although the agricultural use of peptide deformylase inhibitors has yet to be recognized, thus, the potential effectiveness of this system needs further investigation.

Hexadecanoic acid is the second major compound present in the leaves of *Justicia adhatoda* and its peak was revealed at retention time (Rt 29.307 min) through GC-MS analysis. It was reported by Yuan et al. (2020) that Hexadecanoic acid can destroy the cell membrane and interferes with photosynthesis to inhibit growth; thus, these findings support the results of our study.

Oleic acid is also a volatile compound identified in the dried leaves of *Justicia adhatoda* (Rt 33.947 min). The fatty acids, particularly oleic acid, seem to be the key intermediate metabolites in the biosynthesis of certain hydrocarbons and are more important than other fatty acids for phytoplankton growth inhibition (Chiang et al., 2004). Oleic acid is inhibitory to hypocotyl growth and shows synergetic activity with the other fatty acids. Further, lettuce roots are more sensitive to the inhibitory activity of oleic acid (Gomes et al., 2016. Apparently, the higher lipophilicity of oleic acid also contributes to its stronger phytotoxic activity (Macías et al., 2006); thus, the characteristics of oleic acid became the cause of hypocotyl and radical growth inhibition of test plant in the present study. Another study by Ma et al. (2011) provides evidence in support of growth inhibition of lettuce in the present study. Ma et al. (2011) used aqueous extracts of *Jatropha curcas* leaves and roots which contain oleic acid and other compounds that inhibited the growth of corn (*Zea mays* L.) and tobacco (*Nicotiana tabacum* L.). Augmentation of the rate of inhibition of corn and tobacco by increasing the concentration of extracts suggests that the extracts may have an inhibitory substance that possesses allelopathic potential. It can, therefore, be deduced that in the present study, oleic acid played a significant role in the growth inhibition of hypocotyl and radical.

Nonanoic acid (pelargonic) is identified in dried leaves powder of *Justicia adhatoda* (Rt 14.814 min) in the present study and the growth inhibition of hypocotyl and radical may be due to the toxicity of this volatile compound and the same is supported by Sahin *et al.* (2006) who reported antibacterial properties of nonanoic acid against streptomyces. It was also reported by Lahmadi et al. (2021) that nonanoic acid which was originally acquired from the leaves of Pelargonium, though it is also prepared synthetically. The cytotoxic properties of nonanoic acid can be the cause of growth inhibition of lettuce and support the results of the present study.

2-Octenal, *(E)*- was also identified through GC-MS analysis in the dried leaves of *Justicia adhatoda* (Rt 16.325 min) and it could have played role in the growth inhibition of hypocotyl and radical of lettuce, and its herbicidal activity is in conformance with the studies of Merad et al. (2021) in which the essential oils of *Eryngium triquetrum* showed herbicidal activity against watercress and also identified 2-Octenal, *(E)*- and other compounds through GC-MS analysis.

Octadecanoic acid was also found in dried leaves of *Justicia adhatoda* (Rt 34.525 min). Tahir et al. (2020) reported in their study that octadecanoic acid showed allelopathic activity in the germination and seedlings growth of wild mustard (a weed) which is in agreement with the present study in which *Justicia adhatoda* inhibited the growth of hypocotyl and radical of lettuce.

Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester is a fatty acid identified in the dried leaves of *Justicia adhatoda* (Rt 26.413 min). This compound is responsible for the antibacterial activity of the *Piliostigma reticulatum* with other fatty acids present in this plant (Daniels et al., 2021). In another study by Khan et al. (2022), it was investigated that the leaf extract of *Chenopodium quinoa* contains Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester with other compounds and can be used as antimicrobial. The antimicrobial activity is due to the interference with the synthesis of vital components of the cell and it supports Lettuce growth inhibition observed in the present study.

Tridecanoic acid was identified at retention time (Rt 19.225 min) in the dried leaves of *Justicia adhatoda* and can be a marginal contributor to the allelopathic properties of *Justicia adhatoda*. Bhuiyan et al. (2009) studied the composition of essential oils of *Coriandrum sativum L.* which has medicinal properties and found that tridecanoic acid was among the major constituents. Tridecanoic acid carries allelopathic potential and contributed to the growth inhibition of hypocotyl and radical in this study.

The 9-Octadecenoic acid *(Z)-*, 2,3-dihydroxypropyl ester is also present in the leaves of *Justicia adhatoda* and revealed a peak at retention time (Rt 40.519 min). This allelochemical compound contributed to allelopathic potential in hypocotyl and radical growth inhibition. The allelopathic activity of this compound is supported by the study of Zahid et al. (2018) in which 9-Octadecenoic acid *(Z)-*, 2,3-dihydroxypropyl ester was identified in the n-hexane fraction of leaf extract of *Annona squamosa* L which has antimicrobial properties and is used in treatment of different ailments.

9,12-Octadecadienoic acid *(Z,Z)-*, methyl ester is present in the leaves of *Justicia adhatoda* showed a peak at (Rt 41.025 min). This allelochemical possesses allelopathic potential and contributed its role in the growth inhibition of hypocotyl and radical. This is supplemented by the study conducted by Bashir et al. (2019) who revealed the presence of 9,12-Octadecadienoic acid *(Z, Z)-*, methyl ester in the oil of *Azadirachta indica, Gerwia tenax, Gerwia tenax* through GC-MS analysis for their antimicrobial activities.

Octadecanoic acid, 2,3-dihydroxypropyl showed a peak at retention time (Rt 42.212) through GC-MS analysis of the gas collected from the air-tight bottle in which dried leaves of *Justicia*

adhatoda were placed. The inhibitory potential of *Justicia adhatoda* is supported by the study of Arora et al. (2018) in which the presence of Octadecanoic acid, 2,3-dihydroxypropyl ester was revealed through GC-MS analysis in the leaf extract of *Cenchrus biflorus* by various solvents having the herbicidal effect.

Octanoic acid revealed a peak at retention time (Rt 13.282) in the leaves *of Justicia adhatoda* in the present study. In a research study conducted by Razavi et al. (2010), it was reported that allelopathic potentials of the essential oil of *Zosima absinthifolia* were due to its chemical composition which contains octanoic acid with other compounds.

It is concluded that 1, 2-benzenedicarboxylic acid diisooctyl ester ($C_{24}H_{38}O_4$) was found to be the most abundant compound followed by Hexadeconoic acid in the dried leaves of *Justicia adhatoda* and these two compounds are probably the major bioactive compounds responsible for the growth inhibition of the radical and hypocotyl of lettuce and therefore can be good candidates for natural herbicide preparation. The findings of this research study show that leaves of *Justicia adhatoda* can be used for the preparation of natural herbicides for weed control.

4.3 GC-MS Analysis of methanolic leaf extract of *Justicia adhatoda* for identification of allelochemicals and antifungal effects of extract

In this study, methanolic leaf extract was analyzed for the identification of phytochemicals present in the *Justicia adhatoda* which can instigate inhibition of fungal growth in a sustainable way. For GC-MS analysis, methanolic leaf extract was prepared by using the protocol mentioned in Section 3.5.1, GC–MS chromatogram of *Justicia adhatoda* methanolic leaf extract indicated the presence of different compounds (Fig.4.2.). The spectral fingerprints of compounds identified by using the data library, molecular weight and retention time in minutes are shown in Table. 4.3.

The methanolic leaf extract of *Justicia adhatoda* were analyzed against three fungal species of Aspergillus i.e., *Aspergillus niger, A. flavus* and *A. terreus.* The methanolic leaf extract were most effective against *Aspergillus terreus* (24mm), followed by *Aspergillus niger* (22mm) and *Aspergillus flavus* (16mm) at the concentration of 200 mg/ml of the extract. By decreasing concentration of the extract, the antifungal activity was also decreased.

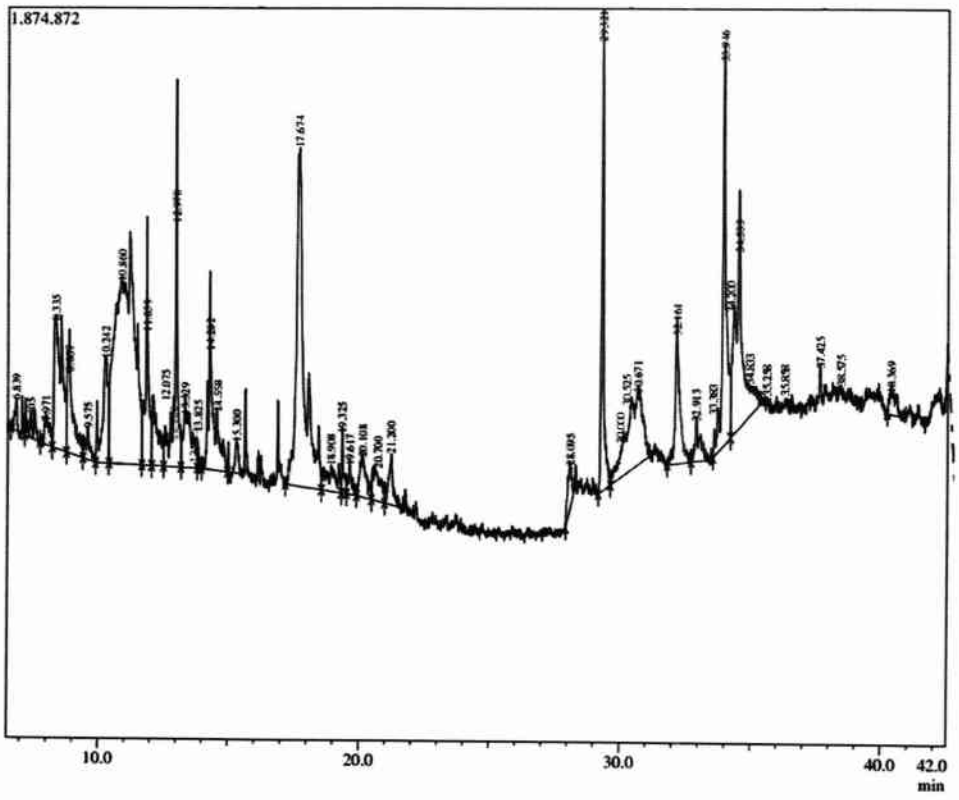
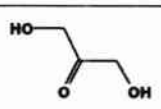
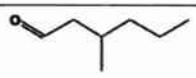
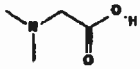
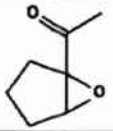
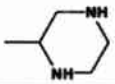
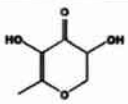
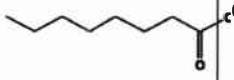
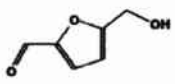
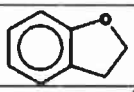
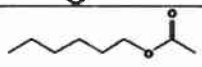
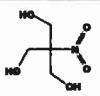
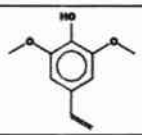
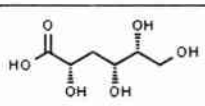
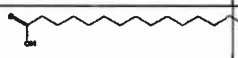
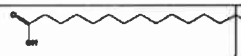
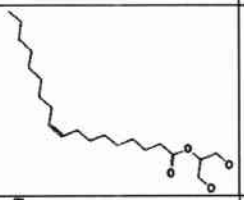
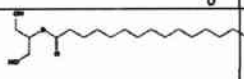
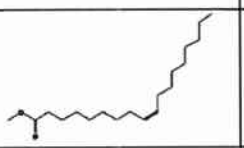
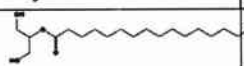
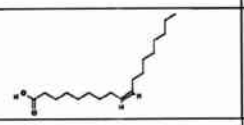
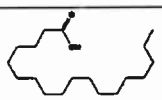
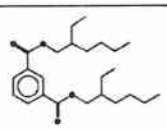
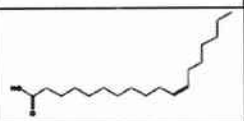


Fig 4.4. Chromatogram of Methanolic Leaf Extract of *Justicia adhatoda*

Table 4.3 Phytoconstituents Identified by GC-MS in the Methanol Leaf Extract of *Justicia Adhatoda*

R_t	Molecular Weight	Molecular Formula	Area %	Main Fragments	Chemical structure	Compound Name
8.335	90	$C_3H_6O_3$	4.98	31, 42, 43, 72, 90		Dihydroxyacetone
8.792	114	$C_7H_{14}O$	1.33	42, 43, 69, 70, 71, 114		Hexanal, 3-methyl-
8.867	98	$C_6H_{10}O$	2.77	41, 42, 55, 69, 98		Cyclopentanone, 2-methyl-
10.242	142	$C_{10}H_{22}$	2.90	41, 43, 57, 75, 81, 99, 112, 142		Decane
10.860	103	$C_4H_9NO_2$	19.82	30,41, 42, 58, 103		N,N-Dimethylglycine
11.859	126	$C_7H_{10}O_2$	3.36	43, 55, 83, 126		Cyclopentane, 1-acetyl-1,2-epoxy
12.075	100	$C_5H_{12}N_2$	1.87	44, 57, 71, 85, 100		Piperazine, 2-methyl-
12.978	144	$C_6H_8O_4$	4.60	43, 73, 101, 144		3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one
13.625	144	$C_8H_{16}O_2$	0.51	31, 41, 44, 60, 73, 101, 115		Octanoic acid
14.282	126	$C_6H_6O_3$	4.23	41, 69, 97, 126		5-Hydroxymethylfurfural
14.558	120	C_8H_8O	0.24	39, 63, 91, 120		Benzofuran, 2,3-dihydro
15.300	144	$C_8H_{16}O_2$	0.49	43, 55, 56, 61, 144		Acetic acid, hexyl ester
17.674	151	$C_4H_9NO_5$	12.67	43, 57, 73, 103, 151		1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-
19.325	180	$C_{10}H_{12}O_3$	0.61	32, 66, 91, 105, 122, 137, 151, 165, 180		4-vinylsyringol
19.617	202	$C_{10}H_{18}O_4$	0.51	41, 43, 55, 74, 152, 171, 184		Nonanedioic acid, monomethyl ester
20.108	$C_6H_{12}O$	180	1.31	44, 57, 73, 57, 73, 84, 102, 117, 131		3-Deoxy-D-arabino-hexonic acid
29.321	256	$C_{16}H_{32}O_2$	5.09	41, 43, 60, 73, 85, 98, 115, 129, 213, 256		Hexadeconoic acid

30.525	242	$C_{15}H_{30}O_2$	1.45	41, 43, 60, 73, 85, 98, 115, 129, 213, 256		Pentadecanoic acid
30.675	356	$C_{21}H_{40}O_4$	5.84	41, 43, 55, 69, 83, 97, 98, 112, 129, 285, 264, 356		9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester
32.161	330	$C_{19}H_{38}O_4$	3.10	41, 43, 57, 71, 84, 98, 134, 239, 330		Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
32.913	296	$C_{19}H_{36}O_2$	1.05	41, 55, 69, 83, 97, 180, 222, 264, 296		9-Octadecenoic acid (Z)-, methyl ester
33.383	330	$C_{19}H_{38}O_4$	6.98	41, 43, 57, 71, 84, 98, 134, 239, 330		Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
33.946	282	$C_{18}H_{34}O_2$	6.74	41, 43, 55, 69, 83, 97, 264		Oleic Acid
34.200	296	$C_{19}H_{36}O_2$	2.50	41, 55, 69, 83, 97, 180, 222, 264, 296		9-Octadecenoic acid, methyl ester, (E)-
34.533	284	$C_{18}H_{36}O_2$	7.45	41, 43, 55, 57, 60, 73, 97, 129, 185, 241, 284		Octadecanoic acid
34.833	390	$C_{24}H_{38}O_4$	0.24	57, 70,12, 149, 167, 161, 279, 361		1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
35.258	282	$C_{18}H_{34}O_2$	0.22	41, 55, 69, 83,97, 111, 264, 282		11-Octadecenoic acid (Z)

This study aims to reduce usage of synthetically derived fungicides by determining antifungal activities of the leaves extract of *Justicia adhatoda* against *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus flavus*. In this study, the efficacy of methanol extracts of *Justicia adhatoda* was tested against in vitro fungal inhibition. GC-MS technique was used in this study to identify the phytochemical components in *Justica adhatoda.*

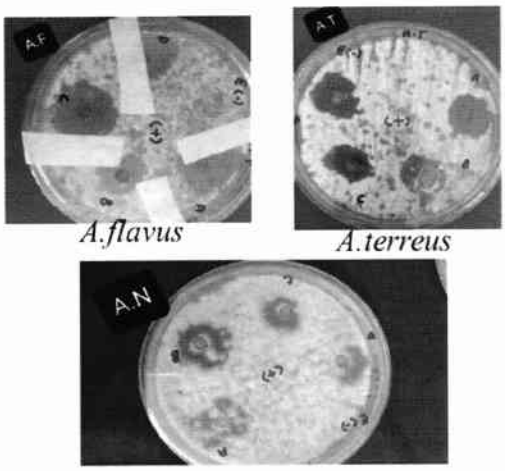


Fig.4.5 Growth inhibition of three species of Aspergillus

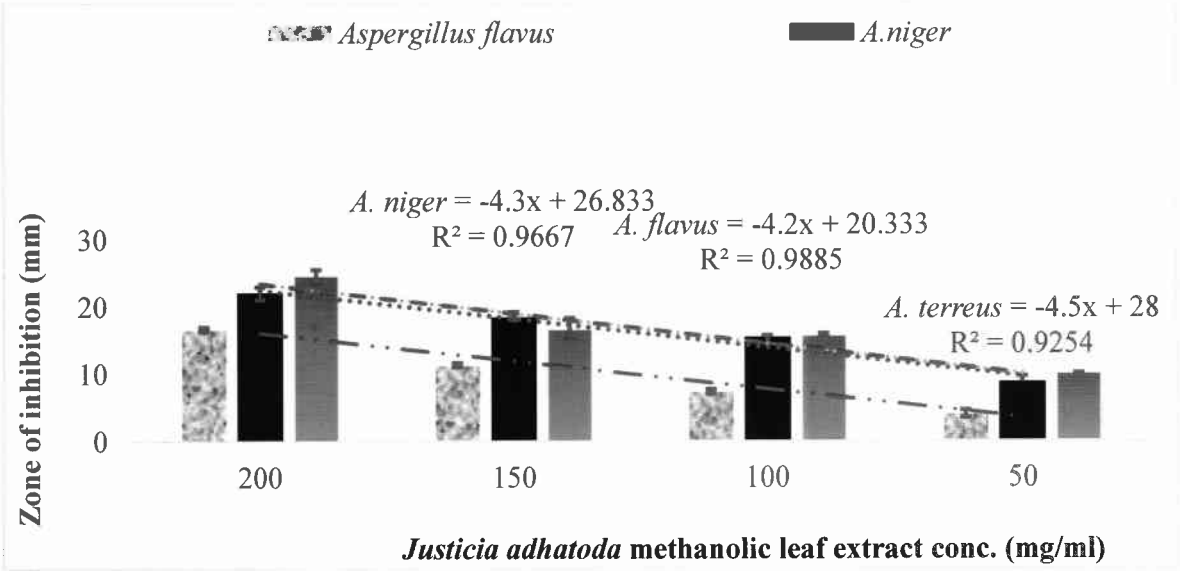


Fig.4.6 Antifungal Activity of *Justicia adhatoda.*

In the present study, phytochemical screening is carried out with methanolic leaf extract of *Justicia adhatoda* through GC-MS analysis. Phytochemical analysis revealed the presence of 26 allelochemicals in the methanolic leaf extract of *Justicia adhatoda* of which *N, N*-Dimethylglycine (19.82%) at retention time10.860 minutes, 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro- (12.67%) at retention time 17.674 minutes, Octadecanoic acid (7.45%) at retention time 34.533 minutes, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (6.98%) at retention time 33.383 minutes, Oleic Acid (6.745) at retention time 33.946 minutes, 9-Octadecenoic acid *(Z)*-, 2,3-dihydroxypropyl ester (5.84%) at

30.675 minutes, Hexadeconoic acid (5.09%) at retention time 29.321 minutes, Dihydroxyacetone (4.98%) at 8.335 minutes, 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (4.60%) at retention time 12.978 minutes, 5-Hydroxymethylfurfural (4.23%) at retention time 14. 14.282 minutes, Cyclopentane, 1-acetyl-1,2-epoxy (3.36%) at retention time 11.859 minutes, Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (3.10%) at retention time 32.161 minutes, Decane (2.90%) at retention time 10.242, Cyclopentanone, 2-methyl- (2.77%) at retention time 8.867 minutes, were the prominent compound. Each compound from the above mentioned allelochemicals have shown antifungal properties reported in previous literature (Walters et al., 2004; Abubacker et al., 2013; Stopiglia et al., 2011; Ramasubramaniaraja. 2011; Shin et al., 2005; Kumar et., 2010; Elsherbiny et al., 2015; Kumar et al., 2012).

4.3.1 Discussion

Ramasubramaniaraja (2011) reported in their study that *Abutilon indicum* have antifungal activities and through GC-MS analysis, Flavonoids, Terpenes, Amino acids (*N, N*-Dimethylglycine), Aldehyde, Hydrocarbon, Ketone, Fatty acids and esters were identified. Nandagopalan et al. (2015) determined the presence of *N, N*-Dimethylglycine in the methanol leaf extract of *Hibiscus tiliaceus* and provided the scientific evidence for the antifungal properties of N, N-Dimethylglycine with other compounds; which support the antifungal properties of *Justicia adhatoda*. The *N, N*-Dimethylglycine was identified in the methanolic leaf extract of *Justicia adhatoda* in a considerable amount.

Kandeepan et al. (2022) reported similar compounds from the methanolic leaf extract of *Moringa oleifera* i.e., Dihydroxyacetone; 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl Hexadecanoic acid, Methyl palmitate; *n*-Hexadecanoic acid (Palmitic acid); 9-Octadecenoic acid, methyl ester and these compounds have antimicrobial activities, which support present study results.

Secondary metabolites of plants can affect the growth of phytopathogenic fungi by interfering with molecular targets in the fungi cells, tissues and organs. The main target sites include plasma membranes, nucleic acids and proteins (Engelmeier and Hadacek, 2006) by altering the membrane permeability (Cotoras et al., 2004) and losing the membrane integrity (Ito et al., 2005). Plant metabolites effects the spore germination by penetrating the cell wall followed by damaging the cell membrane and DNA (Luo et al., 2002).

Eid et al. (2014) reported in their study that 1, 2-benzenedicarboxylic acid diisooctyl ester and hexadeconoic acid were isolated from the buds and flowers of *Spathodea campanulate* and the

allelochemicals constituents in these extracts could be responsible of their high cytotoxic potential. Due to the use to this cytotoxic potential, these compounds inhibited the growth of fungi.

Rahman et al. (2006) reported in their study that 1,2-Benzenedicarboxylic acid, diisooctyl ester isolated from roots of Plumbago zeylanica showed antifungal activity against phytopathogenic fungi which in conformance with the present study results in which methanolic extract of *Justicia adhatoda* inhibited the growth of *Aspergillus niger, A. flavus* and *A. terreus.* The major constituent isolated through GC-MS analysis from the leaves of *Justicia adhatoda* is 1,2-Benzenedicarboxylic acid, diisooctyl ester which inhibited fungal growth due to its cytotoxic activity.

Holanda et al. (2020) studied that octanoic acid and hexanoic acids were major short chain fatty acid in the essential oils of *Morinda citrifolia* fruits and used for the inhibition of fungi and bacterial growth which support the present study results in which octanoic acid was also identified in the dried leaves of *Justicia adhatoda* and these leaves extract inhibited the fungal growth.

Accordingly, it can be concluded from the present study results and previous literature that 1, 2-benzenedicarboxylic acid diisooctyl ester and hexadeconoic acid, oleic acid and other bioactive compounds present in the leaves of *Justicia adhatoda* substantiates the use of its leaves and leaves litter for various antifungal activities to control the fungal infections and get quality yield of vegetables and crops through traditional agriculture practices. Many of the phytochemicals serve as defense compounds against pathogens and could serve as biopesticides or as guides for the synthesis of new pesticides by analyzing their chemistry. The presence of some of the constituents in the *Justicia adhatoda* extract provides the scientific evidences for the antifungal properties of the plant.

4.4 HPLC analysis of Selected Plants of Pakistan and Their Algicidal Effects in Aquatic Environment

As discussed earlier, top five aromatic plants (*Raphanus sativus* L, *Cymbopogon citrates, Mentha royleana, Melia azedarach* and *Mallotus philippensis*) out of 121 test plants screened on the basis of allelopathic potential using dish pack method. High Performance Liquid Chromatography (HPLC) was used to identify and quantify the allelochemicals present in these plants to ascertain their efficacy for growth inhibition of algae. The methanolic leaf extracts of these plants were applied on *Chlamydomonas reinhardtii* Dangeard to find their antialgal properties.

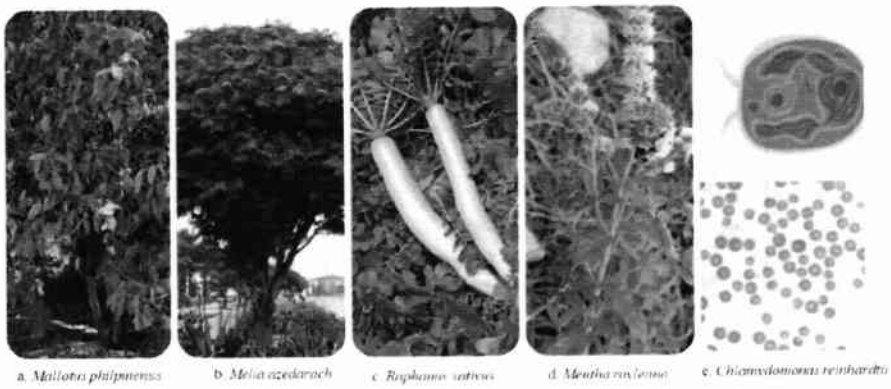


Fig 4.7 Donor plant species (a) *Mallotus philpinensis* (Lam.) Muell Arg, (b) *Melia azedarach* L. (c) *Raphanus sativus* L., (d) *Mentha royleana* Wall. ex Benth. Recipient algal species (e) *Chlamydomonas reinhardtii* Dangeard

4.4.1 Identification of Allelochemicals through HPLC

The identification of each compound was verified by comparing UV-VIS spectra of samples and the authentic standards is shown in Table. 4.4. The amount of each metabolite was quantified in the methanolic leaf extract by using the highest characteristic UV-VIS absorption followed by drawing calibration curve (peak areas vs three different concentrations) of the authentic standards. The amount of each compound in the experimental plants is presented in Table 4.6.

Table 4.4 Phenolic compounds retention time and characteristic UV-VIS.

	Plant name	**Retention time (minutes)**	**Wave length λ (nm)**
Rutin	*Raphanus sativus*	2.8	347
	Mentha royleana	2.8	350
	Melia azedarach	2.8	355
trans-Ferulic acid	*Mallotous philipinensis*	2.9	330
p-Coumaric	*Mallotous philipinensis*	2.7	300
Caffeic acid	*Mentha royleana*	2.3	301
	Mallotous philipinensis	2.3	300
Naringin	*Mentha royleana*	3.0	303

4.4.2 Identification of rutin in *Raphanus sativus, Mentha royleana* and *Melia azedarach* through HPLC

The identification of rutin in the leaf extracts of *Raphanus sativus, Mentha royleana* and *Melia azedarach* and authentic standards are shown in figures provided in the subsequent sections. The identification of rutin was based on a combination of retention time, chromatogram comparison and spectral matching.

(i) *Raphanus sativus*

The presence of rutin was confirmed and quantified in the leaf extract of *Raphanus sativus* (Rt= 2.8 minutes). Diode array response spectrum displayed three bands 266, 284 and 345nm in *Raphanus sativus*. By comparing λ max= 347 value and relative intensity with the value observed in literature (Mabry et al., 2012) indicated the presence of rutin in the leaf extract of *Raphanus sativus*. Further identification of consistent standard rutin was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard has the same Rt as of the identified extract. Furthermore, UV spectrum of the standard was very much alike in term of λ max value and intensity of bands with the phytoconstituent anticipated (Fig. 4.8). This study results commensurate with research findings of M'rabet et al. (2017) who identified 26 phenolic compounds in their study and the main phenolic compound was rutin which has antialgal effects. The amount of rutin in the leaf extract of *Raphanus sativus* is 3.74 ± 0.02 µg/mL (Table 4.6).

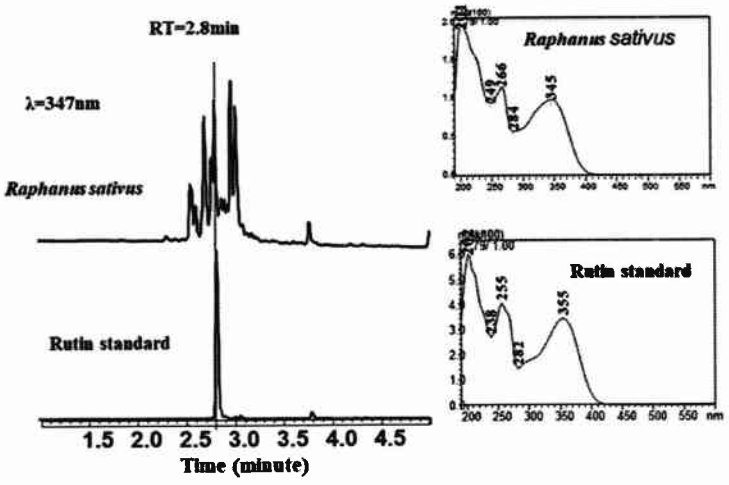


Fig. 4.8 HPLC chromatograms of *Raphanus sativus* along with the (rutin) standard.

(i) *Mentha royleana*

The presence of rutin was confirmed in the leaf extract of *Mentha royleana* by comparing with the authentic standard. The presence of rutin was confirmed and quantified in the leaf extract

of *Mentha royleana* at Rt= 2.8 minutes. The diode array response spectrum displayed three bands 255, 282 and 354nm in *Mentha royleana*. By comparing λ max= 350 value and relative intensity with the value reported in literature (Mabry et al., 2012) indicated the presence of rutin in the leaf extracts of *Mentha royleana*. The further identification of consistent standard rutin was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard has the same Rt as of the identified extract. Furthermore, UV spectrum of the standard was very much alike in term of λ max value and intensity of bands with the phytoconstituent anticipated (Fig. 4.9). The amount of rutin in the leaves extract of *Mentha royleana* is 4.22± 0.31 µg/mL (Table 4.6).

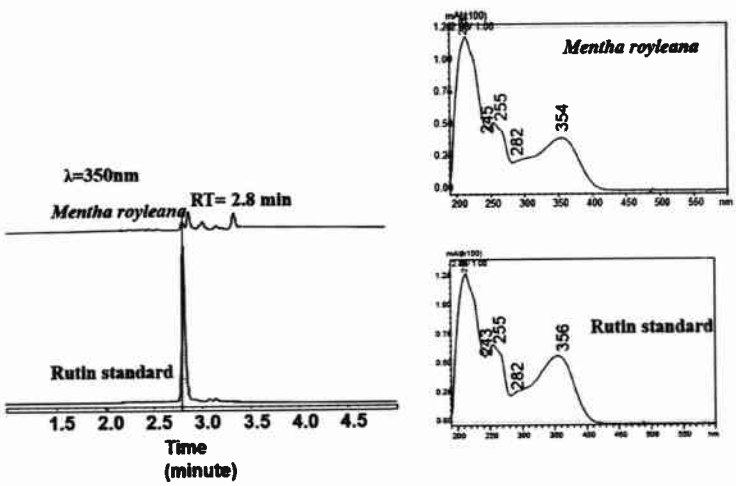


Fig. 4.9 HPLC chromatograms of *Mentha royleana* along with the (rutin) standard.

(ii) *Melia azedarach*

The presence of rutin was confirmed and quantified in the leaf extracts of *Melia azedarach* (Rt= 2.8 minutes). Diode array response spectrum displayed three bands 255, 282 and 354 in *Melia azedarach* (Fig. 4.10). By comparing λ max value and relative intensity with the value reported in previous literature (Mabry et al., 2012) indicated the presence of rutin in the leaf extracts of *Melia azedarach*. Further identification of consistent standard rutin was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard has the same Rt as of the identified extract. Furthermore, UV spectrum of the standard was very much alike in term of λ max value and intensity of bands with the phytoconstituent anticipated. These results are in agreement with the study of Rao & Ahmed (2013) who identified rutin which is

a biologically active flavonoidal compound found in the ethanolic leaf extracts of *Melia azedarach*. However, the results of this study showed rutin presence when methanolic leaf extracts were analyzed instead of the ethanolic leaf extract used by Rao & Ahmed (2013). The purpose of using methanol for extraction was due to its effectiveness for extraction of bioactive compounds. It was found that methanol is the best solvent and most effective for bioactive compounds extraction ensuing maximum yield and Truong et al (2019) reported the similar results. The amount of rutin present in the leaf extract of *Melia azedarach* calculated in this study is 4.21 ± 0.34 µg/mL.

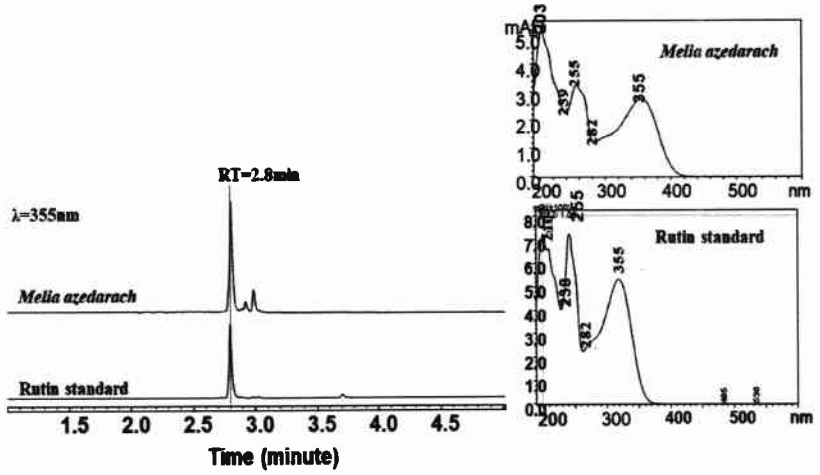


Fig. 4.10 HPLC chromatograms of *Melia azedarach* along with the (rutin) standard.

4.4.3 Identification of *trans*-Ferulic acid in *Mallotous philpinensis* through HPLC

HPLC chromatogram of *Mallotous philipinensis* and standard *trans*-ferulic acid is illustrated in Fig.4.11. Diode array response spectrum displayed three bands i.e., 218, 264 and 331 nm; the λ max value and relative intensity with the value reported in literature (Mabry et al., 2012) indicated the presence of *trans*-ferulic acid in the leaf extracts of *Mallotous philipinensis.* The extended identification of consistent standard *trans*-ferulic acid was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard has the same Rt (2.9 min) as of the identified extract. Furthermore, UV spectrum of the standard was similar in term of λ max value and intensity of bands with the compound. Similar study conducted by Kumar (2017) who used the methanolic plant extract identified ferulic acid along with other phenolic compounds through HPLC analysis at λ=190-650 nm. The presence of *trans*-Ferulic acid was confirmed in the leaf extract of *Mallotous philipinensis* at retention time 2.9 minutes, UV-VIS

absorption at λ= 330 nm and its concentration is 3.78 ± 0.12 µg/mL.

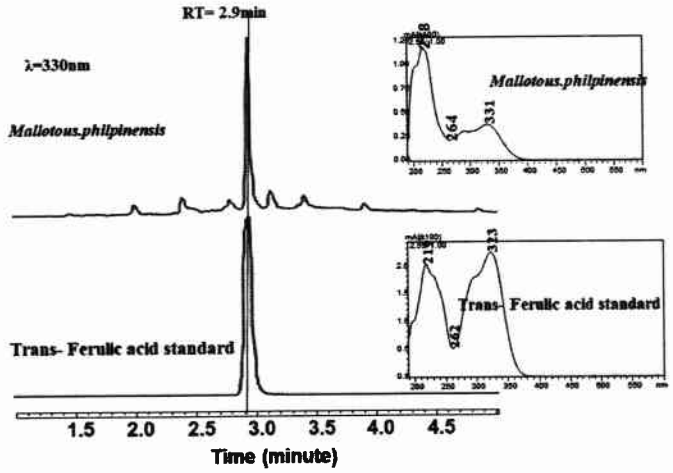


Fig. 4.11 HPLC chromatograms of *Mallotous philipinensis* along with the (*trans*-Ferulic acid) standard.

4.4.4 Identification caffeic acid in *Mentha royleana* and *Mallotous philipinensis* through HPLC

The identification of caffeic acid in the leaf extracts of *Mentha royleana* and *Mallotous philipinensi,* along with standards are shown in figures provided in the following sections. The identification of Caffeic acid was based on a combination of retention time, chromatogram comparison and spectral matching.

(i) *Mentha royleana*

The existence of caffeic acid was confirmed in the leaf extract of *Mentha royleana* at retention time 2.3 minutes. The diode array response spectrum displayed bands 218, 266 and 321 nm in *Mentha royleana.* By comparing λ max value and relative intensity with the value reported by Mabry et al. (2012) indicated the presence of caffeic acid in the leaf extracts of *Mentha royleana.* Further identification of standard caffeic acid was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard has the same retention time as of the identified extract. Moreover, UV spectrum of the standard was alike in term of λ max=301 value and intensity of bands with the phytoconstituent anticipated. HPLC chromatogram of the plants and standard caffeic acid is illustrated in Fig. 4.12. The concentration of trans-ferulic acid in the *Mentha royleana* is mentioned in Table 4.6. The presence of caffeic acid in *Mentha royleana* is supported by the study conducted by Brahmi et

al. (2017) in which it was shown that mentha species are rich source of caffeic acid. In the present study, caffeic acid concentration in the methanolic leaf extract of *Mentha. royleana* was calculated as 4.67±0.03 µg/mL.

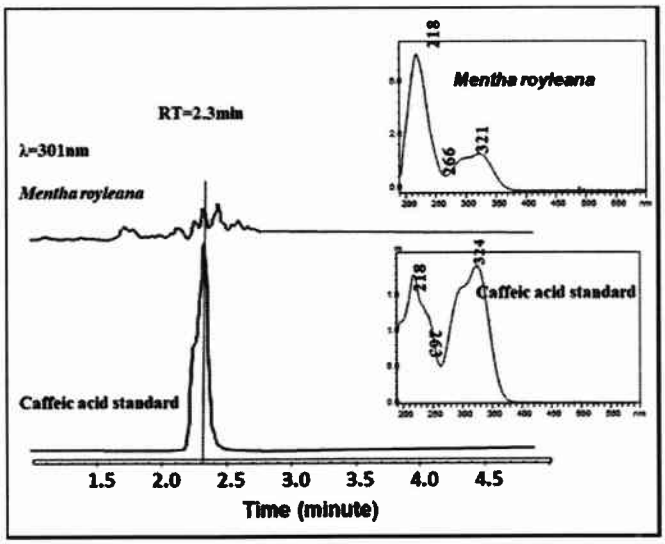


Fig. 4.12 HPLC chromatograms of *Mentha royleana* along with the (Caffeic acid) standard.

(ii) *Mallotous philipinensis*

The presence of caffeic acid was confirmed in the leaf extract by comparing with the authentic standard. HPLC chromatogram of *Mallotous philipinensis* and standard caffeic acid is illustrated in Fig. 4.13. The retention time is 2.3 minutes and UV-VIS absorption at λ= 300 nm. The diode array response spectrum displayed bands 263 and 323 nm in *Mallotous philipinensis*. By comparing λ max value and relative intensity with the value presented in the results of Mabry et al. (2012) indicated the presence of caffeic acid in the leaf extracts of *Mallotous philipinensis*. Further identification of standard caffeic acid was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard has the same retention time as of the identified extract. Moreover, UV spectrum of the standard was alike in term of λ max value and intensity of bands with the phytoconstituent anticipated. The concentration of caffeic acid in the *Mallotous philipinensis* is mentioned in Table 4.6.

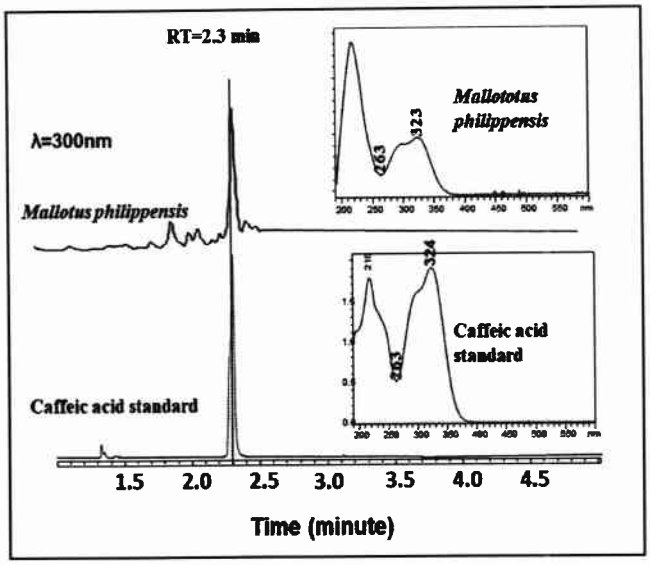


Fig. 4.13 HPLC chromatograms of *Mallotous philipinensis* along with the (Caffeic acid) standard.

4.4.5 Identification of naringin in the leaf extract of *Mentha royleana* through HPLC

The identification of naringin in the leaf extracts of *Mentha royleana* along with standards are shown in figures provided in the following sections. The identification of naringin was based on a combination of retention time, chromatogram comparison and spectral matching.

The presence of naringin was confirmed in the leaf extract by comparing with the authentic standard purchased from Fisher Scientific. HPLC chromatogram of *Mentha royleana* and standard naringin is illustrated in Fig. 4.14. The identification of naringin was based on a combination of retention time and spectral matching of chromatogram of leaf extract of *Mentha royleana* and naringin standard. The retention time is 3.0 minutes and UV-VIS absorption is at λ= 301 nm. Diode array response spectrum showed three bands i.e., 262, 283 and 331 nm, λ max value and relative intensity with the value given in literature (Mabry et al., 2012) indicated the presence of naringin in the leaf extracts of *Mentha royleana.* Further identification of standard naringin was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard has the same Rt (3.0 minutes) as of the identified extract. Furthermore, UV spectrum of the standard was similar in term of λ max value and intensity of bands with the compound. The presence of naringin is supported by the study results of Johnson and Jorge (2005) who reported the presence of naringin at λ= 284, 334 nm in the leaves of

rosemary plants. The amount of naringin in the leaf extract of *Mentha royleana* is 4.77±0.48 µg/mL.

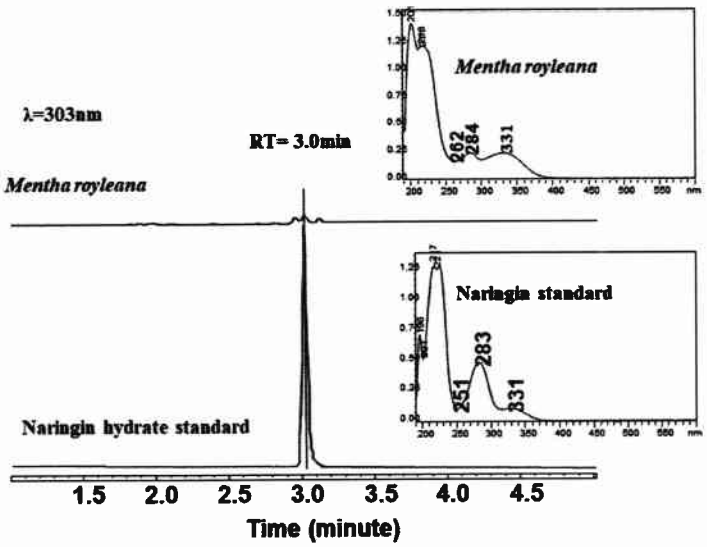


Fig. 4.14 HPLC chromatograms of *Mentha royleana* along with the (naringin) standard.

4.4.6 Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) of rutin, caffeic acid, naringin and trans- ferulic acid

All the compounds were identified through HPLC analysis and quantified using the calibration curves. These curves were plotted with three concentrations 6 µg/mL, 8 µg/mL and 11 µg/mL against peak areas using their characteristic UV-VIS absorption for each standard i.e., rutin, caffeic acid, naringin and *trans*-Ferulic acid. The total compound content is expressed as µg/mL of methanolic leaf extract of experimental plants. The data was subjected to regression analysis and LOD and LOQ were determined. The LOD is the lowest analyte concentration that can be reliably detected by an analytical method and LOQ is the lowest analyte concentration that can be reliably determined by an analytical method.

Rutin: The characteristic UV-VIS absorption of rutin is λ=254-355 nm. The coefficient of determination using regression analysis is 0.9921. The regression equation $\mathbf{y = 964991\ x - 4 \times 10^6}$ is obtained, where *y* represents peak area and *x* denotes concentration. LOD and LOQ values for rutin are 1.05 µg/mL and 3.18 µg/mL respectively as shown in Table 4.5. The rutin concentrations in the leaf extracts was determined using the calibration curve of the standard. The results are presented in Table 4.6. The rutin (standard) calibration curve is illustrated in

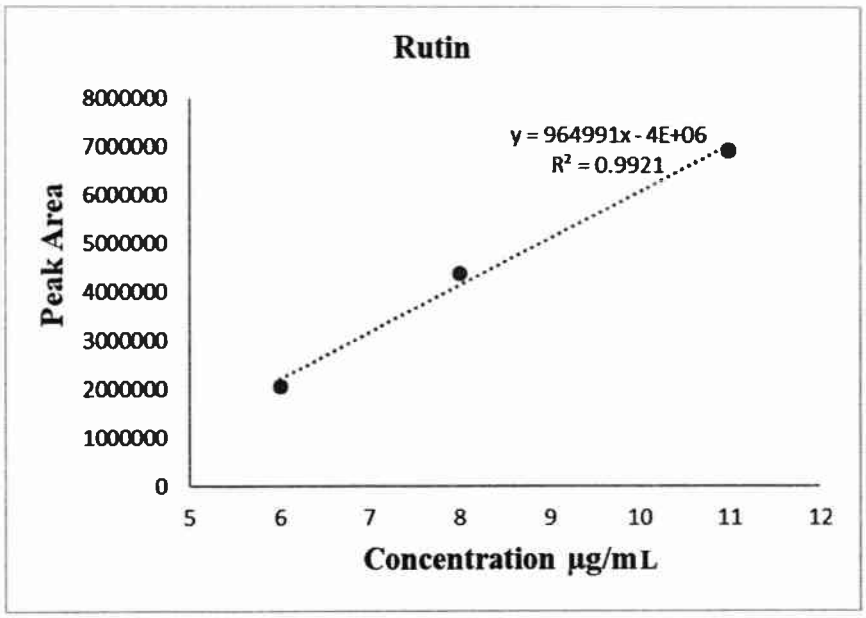


Fig. 4.15 Calibration curve for rutin.

Table 4.5 Data for calibration curve for rutin, caffeic acid, naringin, and *trans*-Ferulic acid.

Compound	Linear Range (µg/mL)	Coefficient of determination (r^2)	LOD (µg/mL)	LOQ (µg/mL)	Regression equation
Rutin	6-11	0.9921	1.05	3.18	$y = 964991\ x - 4 \times 10^6$
Caffeic acid	6-11	0.9907	1.14	3.46	$y = 2 \times 10^6\ x - 7 \times 10^6$
Naringin	6-11	0.9930	0.98	2.98	$y = 205385\ x - 820192$
Trans-Ferulic acid	6-11	0.9361	3.06	9.29	$y = 1 \times 10^6\ x - 5 \times 10^6$

Table 4.6 Phenolic compounds (µg/ml) in methanolic leaf extracts of plants

Compound	*Raphanus sativus L.*		*Mentha royleana* Wall. ex Benth		*Mallotous philpinensis* (Lam.) Muell Arg		*Melia azedarach L*	
	RT	Conc	RT	Conc	RT	Conc	RT	Conc
Rutin	2.82	3.74±0.02	2.84	4.22±0.31	-	-	2.81	4.21±0.34
Trans-ferulic acid	-	-	-	-	2.90	3.78±0.12	-	-
Caffeic acid	-	-	2.3	4.67±0.03	2.33	4.65±0.02	-	-
Naringin	-	-	3.0	4.77±048	-	-	-	-

RT: Retention Time (min), Conc: Concentration (leaf extract µg/ml)

***trans*-Ferulic acid**: The characteristic UV-VIS absorption of *trans*-Ferulic is λ=254-330 nm. The coefficient of determination using regression analysis was 0.9361. Further, the regression equation $y = 1 \times 10^6 x - 5 \times 10^6$ was obtained, where *y* represents peak area and *x* denotes concentration. LOD and LOQ values for *trans*-Ferulic acid were 3.06 µg/mL and 9.29 µg/mL. The *trans*-Ferulic acid concentrations in the leaf extracts were determined using the calibration curve of the trans-Ferulic standard. The *trans*-Ferulic acid (standard) calibration curve is illustrated in Fig 4.17.

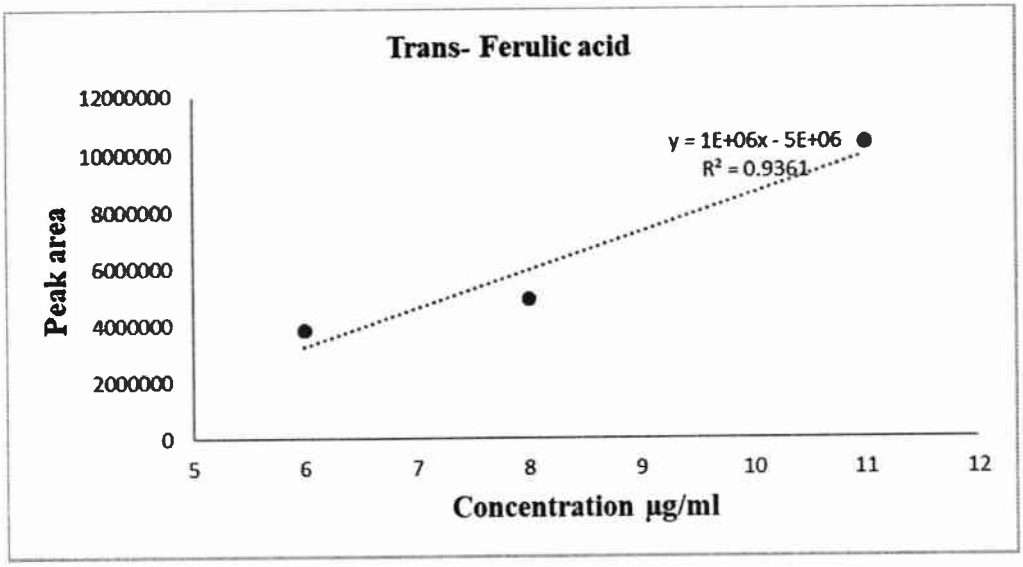


Fig. 4.16 Calibration curve for *trans*-Ferulic acid.

Caffeic acid: The characteristic UV-VIS absorption of caffeic acid is λ=254-330 nm. The coefficient of determination using regression analysis was $R^2 = 0.9907$. Further, the regression equation $y = 964991 x - 4 \times 10^6$ was obtained, where *y* represents peak area and *x* denotes

concentration. LOD and LOQ values for caffeic acid were 1.14 µg/mL and 3.46 µg/mL respectively. The caffeic acid concentrations in the leaf extracts was determined using the calibration curve of the standard. The caffeic acid (standard) calibration curve is illustrated in Fig 4.18.

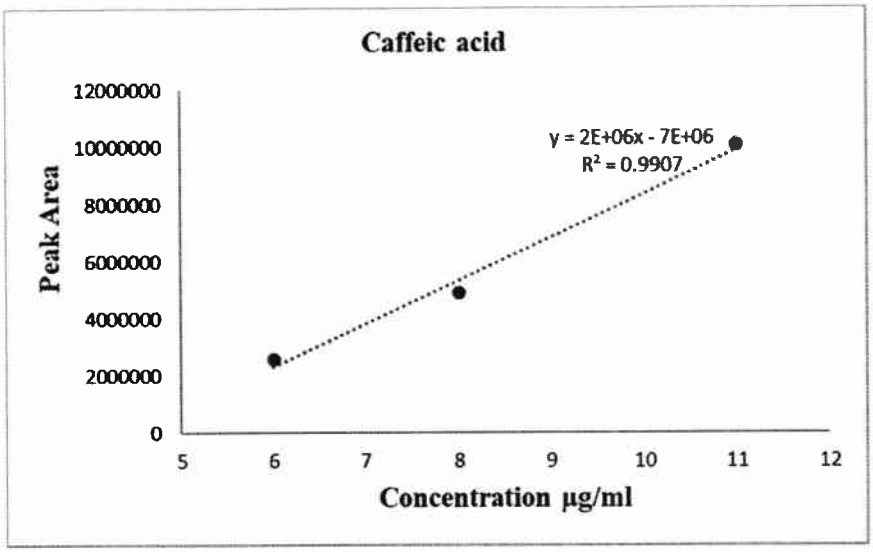


Fig. 4.17 Calibration curve for caffeic acid.

Naringin: The characteristic UV-VIS absorption of naringin is λ=254-303 nm. The coefficient of determination using regression analysis was $R^2 = 0.993$. Further, the regression equation y = 205385 x - 820192 was obtained, where *y* represents peak area and *x* denotes concentration. LOD and LOQ values for naringin were 0.98 µg/mL and 2.98 µg/mL respectively. The naringin concentrations in the leaf extracts was determined using the calibration curve of the standard. The naringin (standard) calibration curve is illustrated in Fig 4.19.

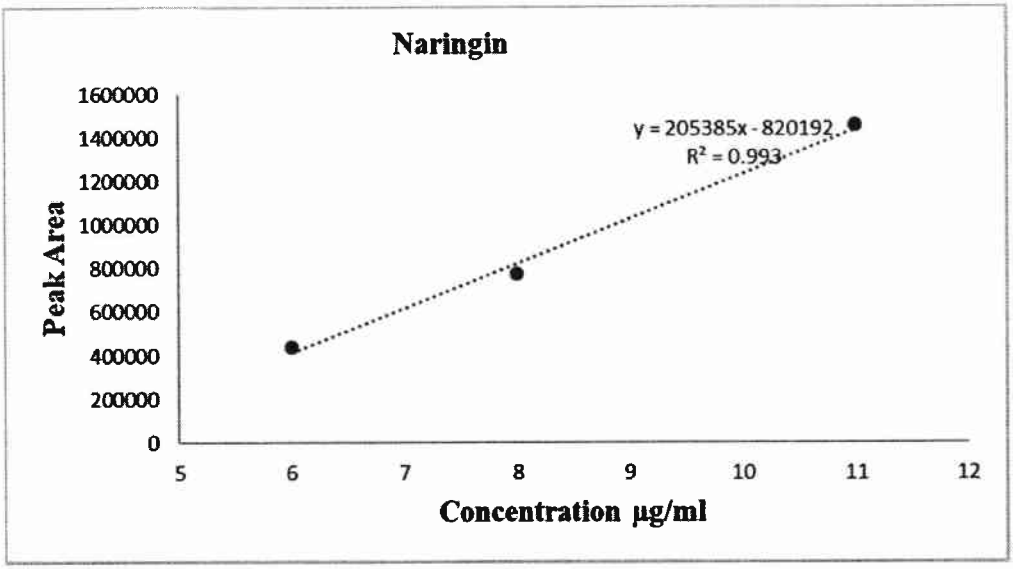


Fig. 4.18 Calibration curve for naringin.

4.4.7 Discussion

To get an insight into the chemical composition of top six plants namely *Raphanus sativus, Cymbopogan citratus, Mentha royleana, Mallotous philppinensis and Melia azedarach* on the basis of allelopathic activity, determined by dish pack method. Then HPLC analysis of their leaf extracts was performed. Phenolic compounds analytical resolution was quite difficult because of close retention time of allelochemicals. UV investigation confirmed the phenolic nature of the main constituents.

Rutin has been identified in the *Raphanus sativus*, *Mentha royleana* and *Melia azedarach.* According to investigation of Rao & Ahmed (2013), quercetin and rutin which are biologically active flavonoidal compounds were present in the ethanolic leaf extract of *Melia azedarach* by using high-performance thin-layer chromatography (HPTLC). The results of this study indicate rutin presence when methanolic leaf extracts were analyzed rather than ethanolic leaf extract. The purpose of using methanol for extraction was due to its effectiveness for extraction of bioactive compounds. According to the research findings of Truong et al. (2019), it was found that the methanol is the best solvent and most effectual for the bioactive compounds' extraction, ensuing in the maximum yield.

In the present study, HPLC analysis results for *Raphanus sativus* showed the presence of rutin. By comparing the results with the findings of Niranjan et al. (2011) in which HPLC analysis for *Raphanus sativus* identified the presence of rutin in leaves. Jani and Goswami (2019) showed the presence of rutin in hydroalcoholic extract of *Raphanus sativus* leaves, whereas in

the present study rutin presence was indicated in methanolic extract of *raphanus sativus* leaves. Naringin is identified in *Mentha royleana.* Mentha species are good source of phenolic compounds, mainly rich quantity of phenolic acids and flavonoids are present in mint (Ghoulami et al., 2001). The biologic activities of mint are usually due to phenolic compounds and components of essential oils (Orhan et al., 2012; Elansary et al., 2016).

The present study results show that methanolic leaf extract of *Mallotous philipinensis* have maximum response for caffeic acid at λ= 300 nm and for *Mentha royleana* at λ= 350 and proved the presence of caffeic acid in the leaves of the abovementioned plants. Caffeic acid showed maximum response at λ= 320 nm in the leaf extract of grapes in a study conducted by Zeb (2015). The presence of caffeic acid and its derivatives in various organs i.e., roots, leaves and fruits of plants is different and also varies in growth stages of plants and by analyzing the distribution pattern of different metabolites in the plant organs it was found that leaves have more metabolites as compared to roots (Scognamiglio et al., 2012); so, the leaves were selected for the identification of different phenolic compounds in this study. In another study conducted by Johnson and Jorge (2005) indicate the presence of naringin at λ= 284, 334 nm and caffeic acid at λ= 296, 324 nm in the leaves of rosemary plants. The Mentha (genus) specifically contains good amount of caffeic acid and according to the information provided by Brahmi et al., (2017) about phenolic acids, genus Mentha is the rich source of caffeic acid. In the present study, caffeic acid concentration in the leaf extract was calculated and it was 4.67±0.02 µg/mL of leaf extract.

In the present study, *Mallotous philipinensis* showed presence of *trans*-Ferulic acid and caffeic acid. Akhtar and Mirza (2018) who used HPLC coupled with diode array detector for the analysis of bioactive compounds present in *Mallotous philipinensis* and indicated the presence of gallic acid in methanol/ chloroform extract and aqueous extract; however, rutin was found in methanol/ chloroform extract but not in the aqueous extract and caffeic acid was present in aqueous extract. In the present study rutin, caffeic acid, *trans*-Ferulic acid and naringin were identified and quantified from the plants with maximum allelopathic potential (section 4.1) which are useful for the identification of plants with allelopathic potential on the basis of compounds present in them.

4.5 Algicidal effects of selected plants of Pakistan on *Chlamydomonas reinhardtii* Dangeard

There is need to develop a sustainable approach to control the algal blooms. Therefore, this study assessed the allelopathic potential of 5-test plants screened out through dish pack method (*Raphanus sativus, Cymbopogan citratus, Mentha royleana, Melia azedarach and Mallotus*

philippensis to explored their efficacy to control algal blooms. The cell multiplication was tested after 3rd, 6th, 15th and 30th day. The results are shown in Fig.4.20

By following the method of William and Richardson (1988), the response index for leaf extracts of selected aromatic plants was calculated by using the following equation; the positive and negative value of RI represent stimulation and inhibition respectively.

$$RI = \begin{cases} 1 - \frac{T}{C} \ when\ T \geq C \\ \frac{T}{C} - 1 \ when T < C \end{cases}$$ Equation (II)

C = control response and T = treatment response

The growth of C. reinhardtii was significantly inhibited by methanolic leaf extracts of test plants of *Raphanus sativus* L. ($p < 0.01$), *Cymbopogon citratus* ($p<0.05$), *Mentha royleana* ($p <0.05$), *Melia azedarach* ($p <0.05$) and *Mallotus philippensis* ($p <0.01$ respectively as per Tukey's test on 3, 6, 15 and 30 days.

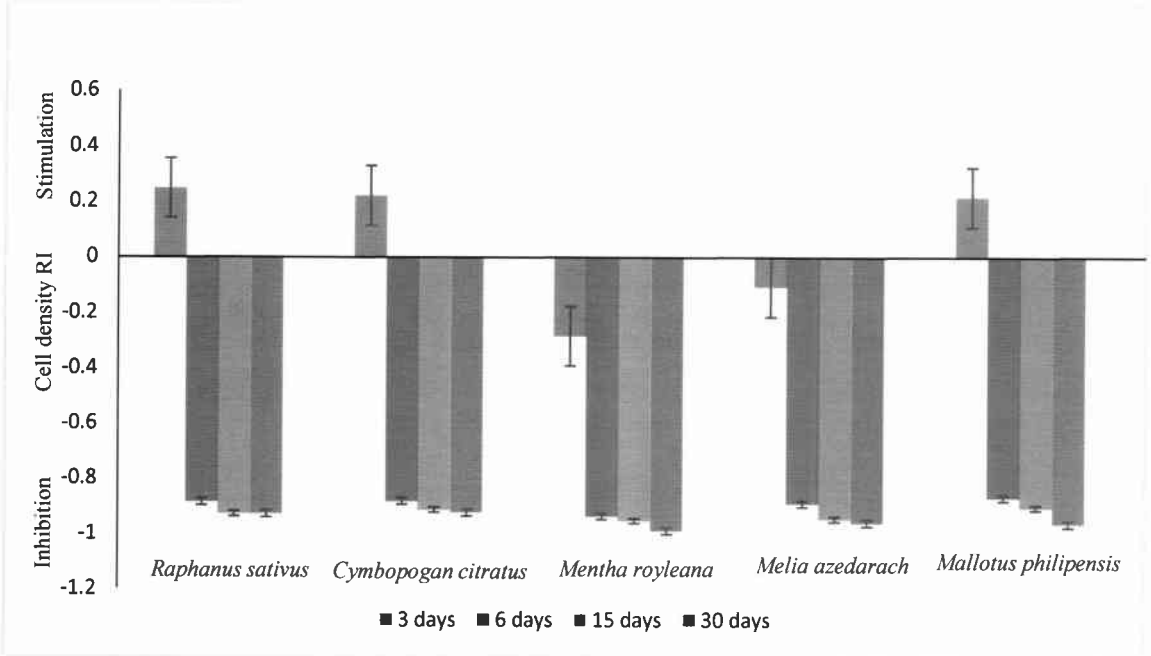


Fig. 4.19 Algicidal effects of leaf extracts of *Raphanus sativus, Mentha royleana, Melia azedarach, Mallotus philippensis* and *Cymbopogon citratus* on *C. reinhardtii* cell multiplication. Data values are mean of three replicates of each sample ± SE.

(i) *Raphanus sativus*

When *Chlamydomonas reinhardtii* was treated with methanolic leaf extract of *Raphanus sativus* and cell multiplication was tested after 3rd, 6th, 15th and 30th day. *Raphanus sativus* extract significantly inhibited the growth of *Chlamydomonas* and reached the minimum value of Response Index -0.93 on 30th day. A significant inhibition was detected by Tukey's test on

3rd, 6th, 15th and 30th day ($p < 0.01$). Rutin was identified in methanolic leaf extract of *Raphanus sativus L.,* which has antialgal properties. Present study results commensurate with research findings of M'rabet et al. (2017) who identified 26 phenolic compounds in their study and the main phenolic compound was rutin, which had antialgal effects. Benyelles et al. (2014) also detected rutin in addition to other allelochemicals in the olive extracts and observed their antifungal effects against test fungi and their results support the present study results due to their antialgal properties and presence of allelochemicals.

(ii) *Cymbopogon citratus*

When *Chlamydomonas reinhardtii* were treated with methanolic leaf extract of *Cymbopogon citratus* and cell multiplication was tested after 3rd, 6th, 15th and 30th day. *Cymbopogon citratus* extract significantly inhibited the growth of *Chlamydomonas* and reached the minimum value of Response Index -0.91 and -0.92 on day 15th and 30th respectively. A significant inhibition ($p < 0.05$) was detected by Tukey's test on 3rd, 6th, 15th and 30th day; thus, proving significance of the leaf extract. Zeng & Luo (1996) reported antialgal properties of *Cymbopogon citratus* and Yangui et al., (2010) detected hydroxytyrosol in olive mill waste water which can be used as antifungal.

(iii) *Mentha royleana*

Chlamydomonas reinhardtii was treated with methanolic leaf extract of *Mentha royleana* and cell multiplication was observed after 3rd, 6th, 15th and 30th. *Mentha royleana* extract significantly inhibited growth of *Chlamydomonas* and reached the minimum value of Response Index -0.95 and-0.99 on 15th and 30th day respectively. A significant inhibition ($p < 0.05$) was detected by Tukey's test on 3rd, 6th, 15th and 30th day. These results indicate that *Mentha royleana* leaf extract is very effective against *Chlamydomonas reinhardtii.* The previous literature reported that main compounds identified in *Mentha royleana* were rutin, caffeic acid and naringin, the mentha species are rich in phenolic compounds, especially phenolic acids and flavonoids (Ghoulami et al., 2001). Its methanolic leaf extract significantly inhibited the *C. reinhardtii* cell multiplication and its inhibitory activity could be due to presence of allelochemicals. Wang et al. (2013) reported that allelochemicals present in submerged macrophytes inhibited the esterase activity, chlorophyll a fluorescence and cell volume in M. aeruginosa. This study results pertaining to the growth inhibition of *Chlamydomonas reinhardtii* are consistent with Nakai et al. (2001) who evaluated inhibitory effects of phenols

produced by plants including caffeic acid on blue-green algae. The study of Ateyyat et al., (2012) showed that Naringin has phytochemical properties which can act as herbal insecticides and supports the inhibitory activity of *Mentha royleana* in present study. The biological activities of mint are usually due to phenolic compounds and components of essential oils (Elansary et al., 2016) which is also supporting the antialgal properties of *Mentha royleana.*

(iv) *Melia azedarach*

Chlamydomonas reinhardtii was treated with methanolic leaf extract of *Melia azedarach* and cell multiplication was tested after 3rd, 6th, 15th and 30th day. *Melia azedarach* extract significantly inhibited growth of *Chlamydomonas* and reached the minimum value of Response Index -0.95 and -0.96 on 15th and 30th day respectively. A significant inhibition ($p < 0.05$) was detected by Tukey's test on 3rd, 6th, 15th and 30th day. The inhibitory effect of *M. azedarach* due to presence of rutin in this plant which was identified by HPLC analysis in present study. Present study results commensurate with research findings of M'rabet et al. (2017) who identified 26 phenolic compounds and the main phenolic compound was rutin, which had antialgal effects. Leaf extract of Melia azedarach L. exhibited allelopathic activity as per study results of Ateyyat et al. (2012), in which rutin hydrate, quercetin dehydrate were botanical herbicides and recommended for integrated management for weeds control.

(v) *Mallotus philippensis*

Chlamydomonas reinhardtii was treated with methanolic leaf extract of *Mallotus philippensis* and cell multiplication was experienced after 3rd, 6th, 15th and 30th day. *Mallotus philippensis* extract significantly inhibited the growth of Chlamydomonas and reachedthe minimum value of Response Index -0.90 and -0.96 on 15th and 30th day respectively. A significant difference ($p < 0.01$) was detected by Tukey's test on 3rd, 6th, 15th and 30th day. *Mallotus philippensis* showed growth extension initially when data was recorded on 3rd day while Response Index as compared to control was 0.22. The growth inhibition may be due to *trans*-ferulic acid which was identified in the methanolic leaf extract of *Mallotus philippensis*. Our research study was supported by Singh and Dean (2014) in which the methanolic leaf extract of *Mallotus philippensis* exhibited phytotoxic effects on tomato seedlings growth. Caffeic acid was also identified in *Mallotus philippensis* leaf extract in the present study through HPLC analysis.

In the present study, leaves extract of, *Mallotus philippensis, Cymbopogon citratus* and *Raphanus sativus* showed increase in the cell counts recorded on 3rd day of application on *C.*

reinhardtii. This phenomenon may be due to the resistance against environmental stress. Belz et al. (2005) reported the response of an organism which it shows to an active compound is not a simple process and is basically based on the mode of action of compound (allelochemical). However, the dose-response can be altered by various parameters, with the range of different concentrations, way of application and the length of exposure. It is concluded that leaves of aromatic plants can be used to control algal blooms developed in the aquatic ecosystem.

Leaf extracts of experimental plants exhibited allelopathic activity which was supported by the results of Ateyyat et al. (2012). This study results pertaining to the growth inhibition of *Chlamydomonas reinhardtii* were consistent with study of Nakai et al. (2001) who evaluated inhibitory effects of phenols produced by plants including caffeic acid on blue-green algae. Benyelles et al. (2014) detected caffeic acid and rutin with other allelochemicals in the olive extracts and observed their antifungal effects against test fungi and their results support the present study results due to their antialgal properties and presence of caffeic acid and rutin in the methanolic leaf extracts of our test plants. *Mallotus philippensis* and *Raphanus sativus* L. showed growth stimulation initially on 3rd day, while Response Index than control was 0.14 and 0.24, respectively. The results showed significant allelopathic effects. Hence, it can be concluded that allelopathic potential of plants can be used to control algal blooms developed in the aquatic ecosystem.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

Allelopathic studies are as important as the chemical and ecological studies and are integrated approach to control the environmental problems. A number of plants with allelopathic potential have been identified through dish pack method in this study which offers a sound contribution in the existing research. The top six plants identified in this study include *Raphanus sativus, Mentha royleana, Cymobopogan citratus, Justicia adhatoda, Melia azedarach and Mallotus philipensis*. These plants were screened out due to their maximum allelopathic potential. *Raphanus sativus* and *Cymbopogan Citraus* showed 80% to 100% lettuce hypocotyl growth inhibition. *Mentha royleana, Justicia adhatoda* and *Melia azedarach* showed hypocotyl growth inhibition between 60-79%.

The inhibition potential between 80% to 100% against the lettuce radicle growth was found in *Raphanus sativus* and *Mentha royleana.* 60-79% radicle growth inhibition was observed in *Justicia adhatoda, Cymbopogan Citratus, Melia azedarach* and *Mallotus philippensis.* The growth inhibition was due the presence of volatile compounds in the top screened out plants. *Justicia adhatoda* was particularly focused; though *Raphanus sativus, Cymbopogon citratus* and *Mentha royleana* were identified as the top most screened plants followed by *Justicia adhatoda* among all the studied plants, but the former plants are the cultivated species and also invasive whereas *Justicia adhatoda* is indigenous species and is top most among the indigenous plants in this study. Hence, *Justicia adhatoda* was further analyzed for identification of allelochemicals through GC-MS analysis. The effects of compounds present in *Justicia adhatoda* for crop protection was not a focused area in the past; however, it has been investigated extensively for cure of ailments in the humans. In this study, Phytochemicals analysis (GC-MS) of *Justicia adhatoda* dried leaves was performed to determine presence of volatile compounds in it. Fourteen compounds were identified from the dried leaves of *Justicia adhatoda*. The prominent compounds include 1, 2-Benzenedicarboxylic acid, diisooctyl ester (80.93 %) and n-hexadeconoic acid (11.31 %) were the prominent followed by 9-Octadecenoic acid (*Z*)-, 2,3-dihydroxypropyl ester (2.12 %), oleic acid (1.99 %) and Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (1.18%). 1, 2-Benzenedicarboxylic acid, diisooctyl ester and *n*-hexadeconoic acid Phytochemical have cytotoxic potential and caused growth inhibition of hypocotyl and radical of lettuce.

GC-MS analysis revealed the presence of 26 allelochemicals in the methanolic leaf extract of *Justicia adhatoda* of which *N, N*-Dimethylglycine (19.82%), 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro- (12.67%), Octadecanoic acid (7.45%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (6.98%), Oleic Acid (6.745), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (5.84%), Hexadeconoic acid (5.09%) were the foremost compounds.

This study revealed that *J. adhatoda* has broad spectrum of antimicrobial activity and a potential source of antimicrobial agents that could be useful for control of infectious diseases in the crops. In another bioassay in which methanolic leaf extract of *Justicia adhatoda* was applied on different fungal strains and significantly inhibited the fungal growth. The methanolic leaf extract of *Justicia adhatoda* were analyzed against three fungal species of Aspergillus, i.e., *Aspergillus niger, Aspergillus niger* and *Aspergillus flavus*. The methanolic leaf extract was effective against *Aspergillus terreus* (24mm), followed by *Aspergillus niger* (22mm) and *Aspergillus flavus* (16mm) at the 200 mg/ml concentration of the extract. With the decreased concentration of the extract, the antifungal activity was also decreased.

As resistance develop in different fungi and weeds against synthetic fungicides/ herbicides and this is a great challenge in agricultural. The results of this study may provide a natural fungi growth and weed growth inhibitor against wide range of fungi and weeds to improve the quality of crops.

The identification of above mentioned allelochemicals from dried leaves and from the methanolic leaf extract of *Justicia adhatoda* is the major contribution of this research study which can be used in the preparation of herbicide and fungicide.

In addition to the above cited results, another experiment was carried out to identified allelochemicals present in the remaining top plants and their effects in aquatic environment. Through HPLC analysis, rutin was identified in methanolic leaf extracts of *Raphanus sativus*, *Mentha royleana* and *Melia azedarach. Trans*-Ferulic acid was found in *Mallotous philpinensis.* Caffeic acid was found in *Mallotous philpinensis* and *Mentha royleana.* The methanolic leaf extracts of *Raphanus sativus, Cymbopogan citratus, Mentha royleana, Melia azedarach* and *Mallotus philippensis* were applied on *Chlamydomonas reinhardtii* to check their allelopathic inhibitory potential again.st *C. reinhardtii.* The experimental results showed strong inhibition against *C. reinhardtii* (a major cause of algal blooms). Methanolic leaf extract of *M. royleana* showed significant effect on the growth of Chlamydomonas and reached the minimum value of Response Index followed by *M. azedarach, C. citratus* and *M. philippensis.* It is also ascertained that selected experimental plants in this study carry strong allelochemical

potential that can be helpful for extraction of bioactive compounds from plant species. These bioactive compounds carry potential to be used as natural herbicides, control of weeds and algal blooms. Finally, algal blooms caused by *Chlamydomonas reinhardtii* can be controlled in a sustainable way by allelochemicals present in experimental plants. This study would be effective by replacing the synthetic and non-ecofriendly algicides with ecofriendly substances from natural resources. Findings of this study establish that allelopathic potential in majority of the experimental plants is due to the presence of allelochemicals (rutin, caffeic acid, *trans*-ferulic acid and naringin) which can be helpful in controlling the algal blooms.

The results of this study may provide a natural fungi growth, algal growth and weed growth inhibitor against wide range of fungi, algae and weeds to improve the quality of crops. The identification of 1,2-benzenedicarboxylic acid, diisooctyl ester in the dried leaves of *Justicia adhatoda* is the major contribution of this research, which can be used in the preparation of herbicide and fungicide. The investigation of herbicidal and fungicidal properties for crop protection of *Justicia adhatoda* could be beneficial for future research in this area.

Recommendations

- This study results may be useful for further research for the identification of allelochemical in the aromatic plants.
- An in-extenso study of the purified phytoconstituents of *Justicia adhatoda* may pave a way to identify some highly promising allelochemicals from this species.
- Plants having allelopathic compounds can be brought into use as effective bio-herbicides, fungicides, algicides and plants growth enhancers in future.
- Allelopathic compounds may be used to develop an alternative weed, fungi and algae control strategy in a sustainable way in the future.
- Results of this research study can be used as reference data for further research on aromatic plants.

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APPENDIX I

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Algicidal effects of selected plants of Pakistan on *Chlamydomonas reinhardtii* Dangeard

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ABSTRACT

This study deals with the antialgal properties of allelochemicals present in 4-plants (*Mallotus philippensis* (Lam.) Muell Arg., *Melia azedarach* L., *Mentha royleana* Wall. ex Benth and *Raphanus sativus* L.). The methanolic leaf extracts of these plants were applied on *Chlamydomonas reinhardtii* Dangeard to find their antialgal properties. High Performance Liquid Chromatography (HPLC) was used to identifiy and quantify the allelochemicals present in these plants to ascertain their efficacy for growth inhibition of algae. Leaf extracts of plants significantly inhibited the growth of *Chlamydomonas reinhardtii* Dangeard ($p < 0.05$). The p value for these plant species were: *Mentha royleana* Wall. ex Benth ($p < 0.05$), *Melia azedarach* L. ($p < 0.05$), *Mallotus philippensis* (Lam.) Muell Arg ($p < 0.01$) and *Raphanus sativus* L. ($p < 0.01$); indicating that number of cells of *Chlamydomonas reinhardtii* Dangeard were significantly inhibited. The most abundant allelochemicals identified by HPLC in *M. royleana* were caffeic acid (4.67 µg /ml) followed by rutin, *trans*-ferulic acid and naringin. The *trans*-ferulic acid was detected only in *Mallotus philippensis* (Lam.) Muell Arg but rutin was found in most plants. Most of the allelochemicals were found in *Mentha royleana*, hence, its extract was comparatively more inhibitory to algal growth. The inhibitory effects of methanolic leaf extracts on growth of *C. reinhardtii* strongly supports the hypothesis that these test plants have ample potential for use as algaecides due to presence of allelochemicals.

Key words : Allelochemicals, allelopathic potential, *Chlamydomonas reinhardtii*, growth inhibition, HPLC, *Mallotus philippensis*, *Melia azedarach*, *Mentha royleana*, *Raphanus sativus* and rutin

INTRODUCTION

Controlling eutrophication caused by excessive nutrients input into the aquatic ecosystem is a big challenge. Eutrophication enhances the growth of green algae and cyanobacteria resulting in formation of blooms. *Chlamydomonas reinhardtii* Dangeard is a unicellular green alga found in temperate regions in pelagic zone lakes and occasionally develops the blooms (15). The *C. reinhardtii* is photosynthetic algae but in stressed conditions, due to K-strategy, its growth is increased which causes Harmful Algal Blooms (HABs) (16). Increase in algal blooms is a serious threat to ecosystem and water quality (8). Algal bloom control is an important concern to protect the aquatic ecosystem, because floating algal blooms suffocate plants and thus animals present in the aquatic environment and reduce aquatic biodiversity (29). Globally, the aquatic ecosystem are badly affected by devastating impact of algal blooms. HABs can cause serious threat to the aquatic ecosystem and use of allelochemicals obtained from the plants is an environment friendly approach to overcome this problem.

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The leaves of plants used in this study have been used to control pests and disease-causing microorganisms (Figure 1). *Mallotous philpinensis* parts are used to prepare dyes, pesticide and religious rituals, etc. and also have medicinal uses as vermifuge, purgative, antibacterial, anticancer and wound healing (6). *Melia azedarach* L. timber has multiple-uses [construction, furniture, farm tools, boats and musical instruments (17)]. Besides, it has high potential to control pests and medicinal properties (14). *Mentha royleana* Wall. ex Benth is food ingredient with medicinal properties (19). Radish is part of food and has medicinal properties (32).

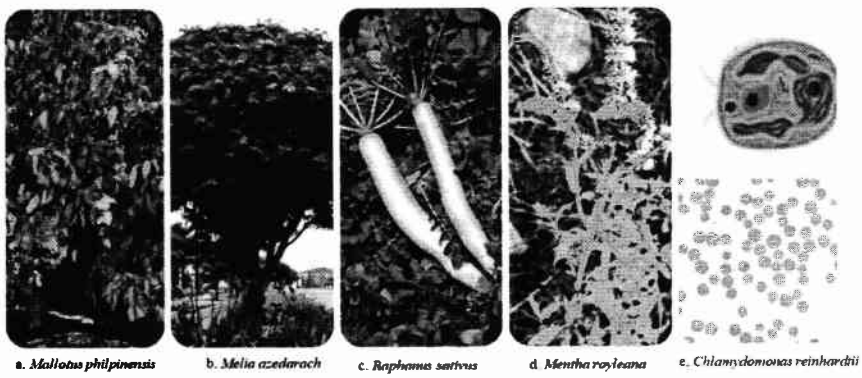


Figure 1. Donor plant species (a) *Mallotus philpinensis* (Lam.) Muell Arg, (b) *Melia azedarach* L. (c) *Raphanus sativus* L., (d) *Mentha royleana* Wall. ex Benth. Recipient algal species (e) *Chlamydomonas reinhardtii* Dangeard

To control HABs, we need sustainable methods. Allelopathy is an evolving field with its sustainable approach to control weeds and algae. Allelochemicals inhibits the growth of algae by impairing structure of its cells, inhibiting enzymes activity and affecting the rate of photosynthesis (27). Zuo *et al.* (35) investigated allelopathic potential of aquatic plant species and inferred that these plants could play an important role to control harmful algal bloom. Allelochemicals are naturally present in the plants and are effective to inhibit the growth of neighbouring plants and act as algaecides (22,34). Zuo *et al.* (36) reported that various plants showed inhibitory effects on the algal growth e.g., *Chlamydomonas reinhardtii* Dangeard growth was inhibited by application of grape extracts and he also studied effects of garlic juice on harmful algal species e.g., *Alexandrium tamarense, A. catenella, A. satoanum* and *Scrippsiella trochoidea* and reported that garlic has inhibited the growth of algae. Aragão *et al.* (2) found that allelochemicals from eucalyptus may be developed as ecofriendly herbicide for sustainable ecosystem.

There is need to develop a sustainable approach to control the algal blooms. Therefore, we assessed the allelopathic potential of 4-test plants (*Malatus philpensis* (Lam.) Muell Arg, *Melia azedrach* L, *Menthe royleana* and *Raphanus sativus* L) and explored their efficacy to control algal blooms. This study aimed to identify and quantify the allelochemicals present in these test plants through HPLC and explore their allelopathic effects on *Chlamydomonas reinhardtii* Dangeard to find alternatives of synthetic algicides used to control HABs.

MATERIALS AND METHODS

Samples of test plants (*Menthe royleana, Melia azedarach* L., *Mallotus philippensis* (Lam.) Muell Arg and *Raphanus sativus* L.) were collected in June 2018 from Margalla hills, Islamabad (Latitude: 33° 44' 23.99" N, Longitude: 73° 02' 18.00" E, Weather conditions: humid subtropical climate). These samples were brought to Lab., Department of Environmental Science, International Islamic University, Islamabad, Pakistan and washed in Tap Water to remove dirt if any.

Chlamydomonas reinhardtii CC-5119 21 gr mt-wild type was provided by Dr. Robert Jinkerson Lab (University of California, Riverside USA) and was grown in TAP Tris-Acetate-Phosphate medium (11). The initial Chlamydomonas culture was grown in light under control conditions having total count 1.32 × 106 cells/ml (counted with automated cell counter Bio-Rad TC20).

I. Bioassays

Methanolic leaf extracts of *Menthe royleana, Melia azedarach* L, *Malatus philpensis* and *Raphanus sativus* L. dried leaves were prepared. The leaves of test plants were dried in shade and grounded. Five g grounded dried leaves sample were added in 50 ml amber colored polypropylene centrifuge tube. Then 20 mL methanol and 1 % formic acid was added. Vortexed at 1000 rpm for 2 min in dark and then centrifuged at 10000 rpm for 10 min at 10 °C. Then it was filtered and supernatant was used in bioassays.

Fifteen sterilized flasks (3-flasks for each treatment) were taken and 20 ml of Tris-Acetate-Phosphate medium was poured into each flask and inoculated with 1 ml inoculum of *C. reinhardtii* (Total count 1.32 × 106 cells/ml). Then 1 ml leaf extracts of Donor experimental plants (*Melia azedarach* L., *Mentha royleana* Wall. ex Benth, *Mallotus philippensis* (Lam.) Muell Arg, *Raphanus sativus* L.) were added in each flask as per treatments. One ml methanol + 1 % formic acid solution was used as control. The number of cells were counted by placing 1 ml of sample on the slide (3 slides for each sample) and counted with automated cell counter Bio-Rad TC20 after 3, 8, 15, 30 days.

Response Index: The response index for leaf extracts of test plants was calculated as under (1). The positive value for RI was stimulation and negative value was inhibition (31).

$$RI = \begin{cases} 1 - \frac{T}{C} & When\ T \geq C \\ \frac{T}{C} - 1 & When\ T < C \end{cases} \qquad Equation(1)$$

Where, C = Control response and T = Treatment response.

Statistical Analysis: The mean and standard errors were obtained for each treatment and significance of the differences was determined by one-way analysis of variance (ANOVA) with p value 0.05 using SPSS version 21.

II. Identification of Allelochemicals

HPLC Analysis

The filtrate (prepared for bioassay) was diluted with water (1:2, v/v). Five µL of each sample was injected into the HPLC for analysis. The leaves extracts were analyzed by reversed phase HPLC on Shimadzu HPLC 2020 (Kinetex ® 2.6 µm XB-C18 column, 100

A, LC 100 x 2.1mm) with UV detection at 254, 300 and 350 nm. The mobile phase consisted of eluent A (0.1 % aq. HCOOH) and eluent B (acetonitrile containing 0.1 % HCOOH). The metabolites were separated on a gradient elution of 5 % to 95 % of B in deionized water over 7 min, followed by an isocratic elution using 95 % of B for 5 min. The flow rate was 0.5 ml min. and the temperature of the column was maintained at 25 °C with an injection volume ("loop") of 10 µL.

By comparing the HPLC retention time (Rt) and matching the UV-VIS spectra of the compounds present in various extracts with the authentic external standards, the allelochemicals were identified and quantified. To prepare calibration curve the standard solutions containing 6 µg/mL, 8 µg/ml and 11 µg/ml of standard were prepared and analyzed on HPLC. Calibration curve was plotted using the 3-concentrations of authentic standard solutions against peak areas of standard using its characteristic UV-VIS absorption. The quantitative estimation of allelochemical was expressed as µg/ml of methanolic leaf extract of experimental plants calculated through calibration curves of authentic standards. The data obtained was subject to regression analysis and the coefficient of determination along with the regression equation "y = ax - b" was obtained, where 'y' represents peak area and 'x' denotes concentration. The LOD is the lowest analyte concentration that can be reliably detected by an analytical method and LOQ is the lowest analyte concentration that can be reliably determined by an analytical method were calculated.

Standards Solution preparation

Standards (rutin, caffeic acid, naringin, *trans*-ferulic acid) were bought from Sigma Aldrich and Fisher. All standards were prepared as stock solutions in methanol 1 mg/1 mL Working standards were made by diluting stock solutions in 20 % methanol with deionized water.

RESULTS AND DISCUSSION

Bioassays

Some allelochemicals present in plants have allelopathic effects on the growth of other plants and algae. The antialgal potential of 4-test plant species *Raphanus sativus* (Radish), *Mentha royleana* (Mint), *Mallotous philpinensis* (Kamala) and *Melia azedarach* (Chinaberry) was assessed to control algal blooms. The allelochemicals in donor test plants were determined using HPLC. The compounds content in plants was expressed as µg/ml of methanolic leaf extracts, calculated by calibration curves of authentic standards. The Methanolic leaf extracts of *Mentha royleana, Melia azedarach, Mallotus philippensis and Raphanus sativus* were applied to *Chlamydomonas reinhardtii* and the cell multiplication was determined at 3, 8, 15 days and 30 days after application. The results are shown in Figure 2.

The growth of *C. reinhardtii* was significantly inhibited by methanolic leaf extracts of test plants of *Mallotus philippensis* ($p < 0.01$), *Melia azedarach* ($p < 0.05$), *Mentha royleana* ($p < 0.05$) and *Raphanus sativus* L. ($p < 0.01$), respectively, as per Tukey's test on 3, 8, 15 and 30 days ($p < 0.05$).

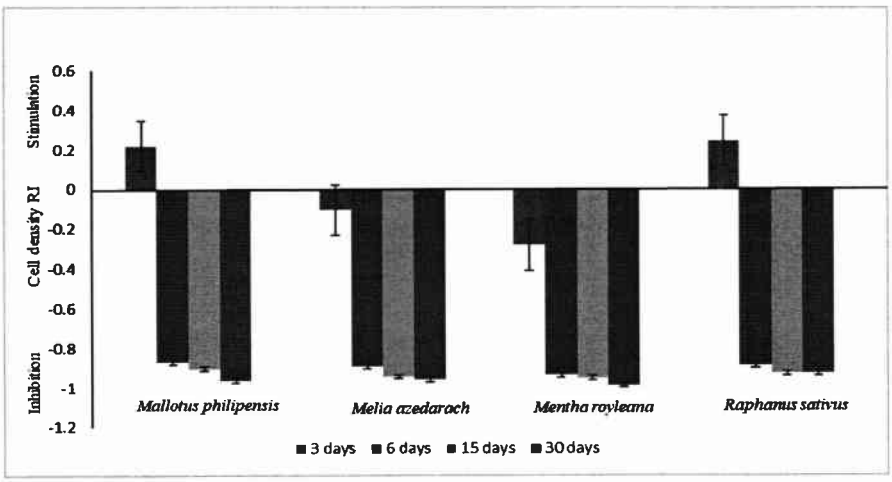


Figure 2. Effects of methanolic leaf extracts of *M. royleana, M. azedarach* L., *M.s philippensis and R. sativus* L. on *Chlamydomonas reinhardtii* Dangeard cell multiplication. Data values are mean of three replicates of each sample ± SE

(i). *Mentha royleana*: Its extract significantly inhibited the growth of Chlamydomonas and reached the minimum value of Response Index -0.95 and-0.99 on 15 and 30 days, respectively. The main compounds identified in *Mentha royleana* were rutin, caffeic acid and naringin, the previous literature reported that mentha species are rich in phenolic compounds, especially phenolic acids and flavonoids (10). Its methanolic leaf extract significantly inhibited the *C. reinhardtii* cell multiplication and its inhibitory activity could be due to presence of allelochemicals. Wang *et al.* (30) reported that caffeic acid present in submerged macrophytes inhibited the esterase activity, chlorophyll a fluorescence and cell volume in *M. aeruginosa*. This study results pertaining to the growth inhibition of *Chlamydomonas reinhardtii* are consistent with Nakai *et al.* (25) who evaluated inhibitory effects of phenols produced by plants including caffeic acid on blue-green algae. The study of Ateyyat *et al.* showed that Naringin has phytochemical properties which can act as herbal insecticides (3) and supports the inhibitory activity of *M. royleana* in present study. The biologic activities of mint are usually due to phenolic compounds and components of essential oils (9) which is also supporting the antialgal properties of *M. royleana.*

(ii). *Melia azedarach* L.: Its extract significantly inhibited the growth of Chlamydomonas and reached the minimum value of Response Index -0.95 and -0.96 on days 15 and 30 respectively. The inhibitory effect of *M. azedarach* may be due to presence of rutin in this plant, which was identified by HPLC analysis in present study. Present study results commensurate with research findings of M'rabet *et al.* (20) who identified 26 phenolic compounds and the main phenolic compound was rutin, which had antialgal effects. Leaf extract of *M. azedarach* L. exhibited allelopathic activity as

per study results of Ateyyat *et al.* (3), in which rutin hydrate, quercetin dehydrate were botanical herbicides and recommended for integrated management for weeds control.

(iii). *Mallotus philippensis*: Its extract significantly inhibited the growth of Chlamydomonas and reached the minimum value of Response Index -0.90 and -0.96 on days 15 and 30, respectively. The growth inhibition may be due to *trans*-ferulic acid which was identified in the methanolic leaf extract of *M. philippensis*. Our research study was supported by Singh and Dean (26) in which ferulic acid exhibited phytotoxic effects on tomato seedlings growth. Caffeic acid was also identified in *M. philippensis*.

(iv). *Raphanus sativus* L: Its extract significantly inhibited the growth of Chlamydomonas and reached the minimum value of Response Index -0.93 on day 30. Rutin was identified in methanolic leaf extract of *R. sativus* L., which has antialgal properties. Present study results commensurate with research findings of M'rabet *et al.* (20) who identified 26 phenolic compounds in their study and the main phenolic compound was rutin, which had antialgal effects. Benyelles *et al.* (4) who detected rutin in addition to other allelochemicals in the olive extracts and observed their antifungal effects against test fungi and their results support the present study results due to their antialgal properties and presence of rutin.

Leaf extracts of experimental plants exhibited allelopathic activity which was supported by the results of Ateyyat *et al.* (3). This study results pertaining to the growth inhibition of *Chlamydomonas reinhardtii* were consistent with study of Nakai *et al.* (21) who evaluated inhibitory effects of phenols produced by plants including caffeic acid on blue-green algae. Benyelles *et al.* (4) detected caffeic acid and rutin with other allelochemicals in the olive extracts and observed their antifungal effects against test fungi and their results support the present study results due to their antialgal properties and presence of caffeic acid and rutin in the methanolic leaf extracts of our test plants. *Mallotus philippensis* and *Raphanus sativus* L. showed growth stimulation initially on 3rd day, while Response Index than control was 0.14 and 0.24, respectively. The results showed significant allelopathic effects. Hence, it can be concluded that allelopathic potential of plants can be used to control algal blooms developed in the aquatic ecosystem.

Identification of Allelochemicals

The compounds contents were expressed as µg/ml of methanolic leaf extracts of experimental plants and were calculated by calibration curves of authentic standards.

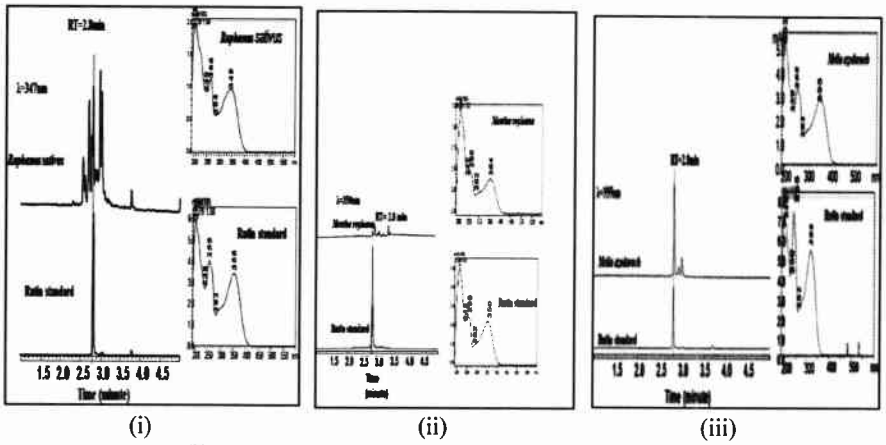
(i). Rutin: Its presence was confirmed and quantified in the leaf extracts of *Raphanus sativus, Mentaha royleana* and *Melia azedarach* (Rt= 2.8 minutes) diode array response spectrum displayed three bands 266, 284 and 345nm in *Raphanus sativus,* 255, 282 and 354 in *Mentaha royleana*, 255, 282 and 355 in *Melia azedarach,* so comparing λ max value and relative intensity with the value presented in literature (18) indicated the presence of rutin in the leaf extracts of *Raphanus sativus* , *Mentaha royleana* and *Melia azedarach.* The further identification of consistent standard rutin was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard have the same Rt as the identified extract. Furthermore, UV spectrum of the standard was similar in term of λ max value and intensity of bands with the phytoconstituent anticipated. HPLC chromatograms of these test donor plants and standard rutin were presented in Figure 3. The concentration of rutin in these plants are mentioned in Table 1. These

results are in agreement with the study of Rao and Ahmed (24) who identified rutin, a biologically active flavonoidal compound found in the ethanolic leaf extracts of *Melia azedarach*. However, the results of this study showed rutin presence when methanolic leaf extracts were analyzed instead of the ethanolic leaf extract which was used by Rao and Ahmed (24). The purpose of using methanol for extraction was due to its effectiveness for extraction of bioactive compounds. It was found that the methanol is the best solvent and most effective for bioactive compounds extraction ensuing maximum yield and Truong *et al.* (28) reported the similar results. In the present study, the presence of rutin in *Raphanus sativus* identified through HPLC, is also comparable with the results reported by Niranjan *et al.* (23). A study conducted by Jani and Goswami (12) showed the presence of rutin in hydroalcoholic extract of *Raphanus sativus* leaf which support the results of the present study. *Mallotus philipinensis* was also analysed for bioactive compounds by Akhtar and Mirza (1) through HPLC coupled with diode array detector and indicated the presence of rutin in methanol/chloroform extract and these results are in conformity with the present study.

Table 1. Phenolic compounds (µg/ml) in methanolic leaf extracts of last plants

Compound	*Raphanus sativus L.*		*Mentha royleana* Wall. ex Benth		*Mallotous philpinensis* (Lam.) Muell Arg		*Melia azedrach* L.	
	RT	Conc	RT	Conc	RT	Conc	RT	Conc
Rutin	2.82	3.74±0.02	2.84	4.22±0.31	-	-	2.81	4.21±0.34
Trans-ferulic acid	-	-	-	-	2.90	3.78±0.12	-	-
Caffeic acid	-	-	2.3	4.67±0.03	2.33	4.65±0.02	-	-
Naringin	-	-	3.0	4.77±048	-	-	-	-

RT: Retention Time (min), Conc: Concentration (leaf extract µg/ml)



(i) (ii) (iii)

Figure 3. UV-VIS spectra corresponding to the rutin (i), (ii) and (iii)

(ii). *trans*-ferulic acid : HPLC chromatogram of *Mallotus* and standard *trans*-ferulic acid was illustrated in Figure 4. Diode array response spectrum displayed three bands i.e. 218, 264 and 331nm, λ max value and relative intensity with the value presented in literature (18) indicated the presence of *trans*-ferulic acid in the leaf extracts of *Mallotus philipinensis*. The extended identification of consistent standard *trans*-ferulic acid was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard have the same Rt (2.9 min) as the identified extract. Furthermore, UV spectrum of the standard was similar in term of λ max value and intensity of bands with the compound. Similar studies conducted by Kumar (17) using the methanolic plant extract and identified ferulic acid along with other phenolic compounds through HPLC analysis at λ=190-650 nm. The *trans*-ferulic was identified in methanolic leaf extract of *Mallotus philipinensis* in this study.

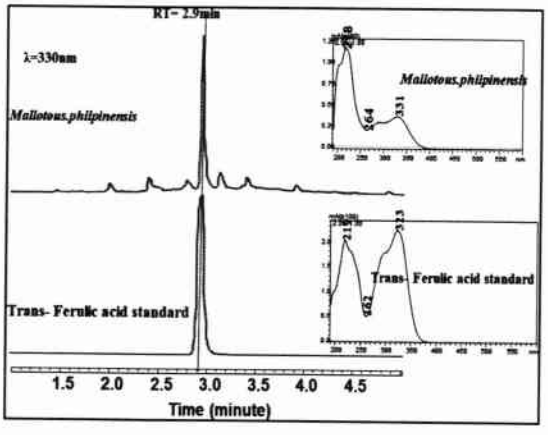
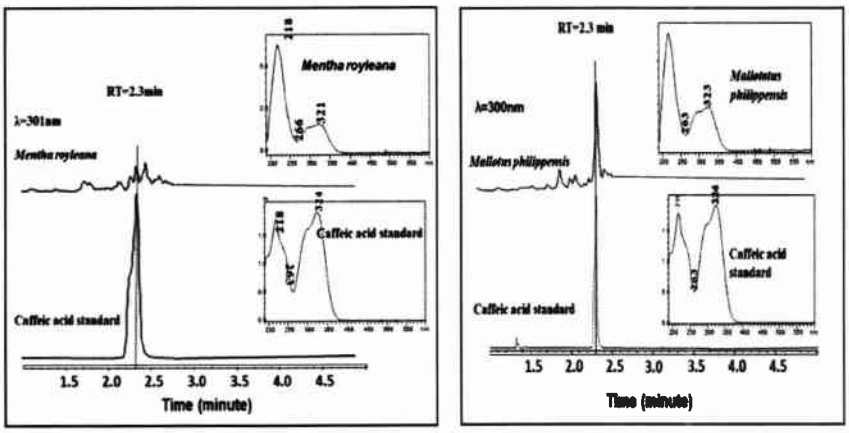


Figure 4. UV-VIS spectra corresponding to the *trans*-ferulic acid

(iii). Caffeic acid: The presence of caffeic acid was confirmed in the leaf extracts of *Mentha royleana* and *Mallotus philipinensis* at retention time 2.3 minutes. The diode array response spectrum displayed bands 218, 266 and 321 nm in *Mentha royleana* and 263 and 323 and in *Mallotus philipinensis*. By comparing λ max value and relative intensity with the value presented in literature (18) indicated the presence of caffeic acid in the leaf extracts of M*entha royleana* and *Mallotus philipinensis*. The further identification of consistent standard caffeic acid was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard had the same retention time as the identified extract. Moreover, UV spectrum of the standard was alike in term of λ max value and intensity of bands with the phytoconstituent anticipated. HPLC chromatogram of the plants and standard caffeic acid was illustrated in Figure 5. The concentration of trans-ferulic acid in the aforesaid plant is mentioned in Table 2. The presence of caffeic acid in *Mentha royleana* is supported by the study conducted by Brahmi *et al.* (5) he showed that mentha species are rich source of caffeic acid. *Mallotus philipinensis* was also analysed for bioactive compounds by (1) HPLC coupled with diode array detector and indicated the presence of caffeic acid in aqueous extracts of leaves. In the present study, caffeic acid concentration in the methonalic leaf extract of *Mentha. royleana* was calculated as 4.67±0.02 µg/ml. Correspondingly, caffeic acid

showed maximum response at λ= 320 nm in the leaf extract of grapes in a study conducted by Zeb (33) which back results of the present study.



(i) (ii)

Figure 5. UV-VIS spectra corresponding to the Caffeic acid (i) and (ii)

(iv). Naringin: The HPLC chromatogram of *Mentha royleana* and standard naringin was illustrated in Fig 6. Diode array response spectrum showed three bands i.e. 262, 283 and 331nm, λ max value and relative intensity with the value given in literature (18) indicated the presence of naringin in the leaf extracts of *Mentha royleana.* The further identification of consistent standard naringin was analyzed under the same analytical conditions. The resulting HPLC chromatogram of the standard have the same Rt (3.0 minutes) as the identified extract. Furthermore, UV spectrum of the standard was similar in term of λ max value and intensity of bands with the compound. The presence of naringin is supported by the study conducted by Johnson and Jorge (13) who indicated the presence of naringin at λ= 284, 334 nm in the leaves of rosemary plants.

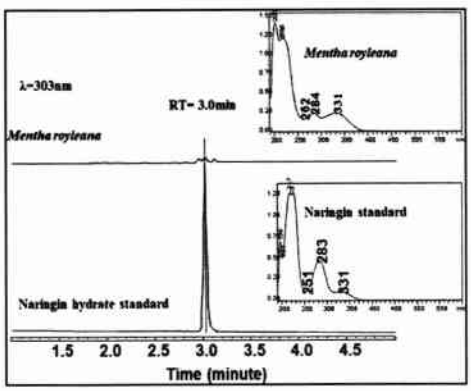


Figure 6. UV-VIS spectra corresponding to the Naringin

Leaf extracts of experimental plants were selected as a standard for the identification of different phenolic compounds, because presence of caffeic acid and its derivatives in various organs i.e., roots, leaves and fruits of plants is different and also varies in different growth stages of plants. By analyzing the distribution pattern of different metabolites in the plant organs, it was found that leaves have more metabolites as compared to the roots (25) and in the present study methanolic leaf extract was used because methanol is the best solvent and most effective for bioactive compounds extraction ensuing maximum yield (34).

CONCLUSIONS

The test plant species [*Raphanus sati*vus L. (Radish), *Mentha royleana* (Mint), *Mallotous philpinensis* (Kamala) and *Melia azedarach* L. (Chinaberry)] strongly allelopathically inhibited the growth of *Chlamydomonas reinhardtii* major cause of algal blooms. The methanolic leaf extract of *M. royleana* significantly inhibited the growth of Chlamydomonas and reached the minimum value of Response Index followed by *M. azedarach* L. and *M. philippensis.*

The rutin and caffeic acid were the most common allelopathic compounds followed by *trans*-ferulic acid and naringin detected in test plants. Hence, the allelopathic potential of these test plants was mostly due to the presence of these compounds (rutin, caffeic acid, *trans*-ferulic acid and naringin) in their leaves. The strong allelopathic potential against *C. reinhardtii* may help in selection of bioactive compounds from these test plant species and to develop natural herbicides and algicide for ecologically control of aquatic weeds and algal blooms. Thus, algal blooms caused by *C. reinhardtii* may be sustainably controlled by allelochemicals present in plants, thereby, replacing the synthetic and non-ecofriendly algicides by ecofriendly algicides.

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DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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