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4. Letters to the editor: These include opinions, comments relating to the publishing policy of the International Journal of Secondary Metabolite, news, and suggestions. Letters are not to exceed one journal page.

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Research Article

The Effect of some secondary metabolites on food consumption and pupal parameters of male and female *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae) larvae

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Abstract: Plants produce secondary metabolites for defense against herbivorous insects. In this study, the effects of different concentrations of quinine, nicotine, and tannic acid on food consumption and pupal parameters of the larva of a severe pest, *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae), were investigated in 2007. Artificial diets containing 0.125%, 0.25%, and 0.5% concentrations of quinine or nicotine were prepared. In addition, diets containing 1.25%, 2.5%, and 5% tannin and a control diet without secondary metabolite were prepared. The feeding experiments revealed that the food consumption and pupal parameters of male and female larvae were different, and all parameters of female individuals were higher than those of male larvae. It has been determined that tannins cause more reductions in food consumption and pupal parameters than alkaloids in male and female individuals. We observed an increase in male larvae's pupal mass with increased concentration of nicotine to 0.5%. Nicotine did not cause a decrease in pupal parameters of larvae compared to quinine. In addition, all parameters of female individuals decreased with the addition of secondary metabolite. Although food consumption in alkaloid-containing diets was less than in the control group, there was a positive relationship between food consumption and pupal lipid and protein content. However, quinine had a more significant effect on triggering protein storage than nicotine. In diets containing tannins, more lipids were stored. A decrease was observed in the food consumption and pupal parameters of all individuals in the tannin-containing diets compared to the other diets.

1. INTRODUCTION

Most insects are herbivores and have co-evolved with plants for about 350 million years. During long-term coevolution, plants have also developed morphological, chemical, and biochemical defense mechanisms to prevent insect feeding (Belete, 2018; Yuan *et al*., 2020). Compounds that play a role in chemical defense are secondary metabolites. Secondary metabolites are small molecular-weight organic compounds. They mainly contain phenols, terpenes, and nitrogenous organic compounds and play an essential role in plant resistance against phytophage insects

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(Jiang *et al*., 2021). In addition to being a feeding deterrent, they may have inhibitory properties on insect development and growth (Yuan *et al*., 2020). They are thus used as potential insecticides (Divekar *et al*., 2022; Gajger & Dar, 2021).

Tannins, the second most abundant polyphenol, are found in all vascular plants and mainly function as defense compounds that protect plants against pests and other abiotic stresses. They are protease inhibitors; they can form complexes with digestive enzymes. They can cause the precipitation of proteins and reduce digestion by reducing the use of proteins by herbivorous insects (Pizzi *et al*., 2009). In addition, they are oxidized in the insect gut in a highly acidic environment and bind to various enzymes in the gut. They cause significant nutrient loss as they bind to lipids and reduce their digestibility. They also inhibit insect growth by causing midgut lesions. Because of their bitter taste, they also act as nutritional deterrents (Price *et al*., 2019).

Another group of secondary metabolites is nitrogen-containing alkaloids. Nicotine and quinine are derived from amino acids. They are highly reactive and have biological potential (Divekar *et al*., 2022). Due to its bitter taste, alkaloids are nutritional deterrents after ingestion or consumption (Munoz *et al*., 2020). Nicotine is a pyridine alkaloid that affects the central nervous system of insects, acting as an agonist of the post-synaptic nicotinic acetylcholine receptors. Thus, they are used as insecticides (Saremba *et al*., 2018). Moreover, sublethal concentrations cause an increase in energy investment, antioxidant functions, and heat-shock responses in bees (Randt *et al*., 2015). Quinine also reduces food preferences due to their bitter taste. Gustatory receptor neurons (GRNs) are activated by noxious compounds and mediate deterrent/aversive responses in many insect species (Munoz *et al*., 2020).

Insects may tolerate or have acquired the ability to detoxify secondary plant metabolites during coevolution (Yuan *et al*., 2020). Phytophage insects have developed several behavioral and physiological adaptive mechanisms as a defensive shield to protect themselves from the harmful effects of secondary metabolites and maintain their population development under selective pressures (Jiang *et al*., 2021).

Malacosoma neustria is a widespread species inhabiting the whole territory of Europe and a large part of Asia (i.e., China, Iran, Japan, Mongolia, Russia, and Türkiye) and the countries of northern Africa (Zikic *et al*., 2017). It is an essential defoliator of fruit trees, ornamental trees, and shrubs (Özbek & Çoruh, 2010). *M. neustria* is univoltine and overwinters in the form of egg masses on twigs of the host plants. Caterpillars are gregarious, eat together, and make a silken group tent as a shelter when resting. They feed on young shoots and leaves, often resulting in the defoliation of solitary trees or even entire forests (Zikic *et al*., 2017). Secondary metabolites are one of the defense mechanisms of plants against herbivores. Tannins and alkaloids are also among plants' most common secondary metabolites (Pizzi *et al*., 2009; Divekar *et al*., 2022). During coevolution, herbivores can also adapt to secondary metabolites. *M. neustria* is a species with melanic dimorphism (Dhillon *et al*., 2022). It is unknown whether males and females respond physiologically to secondary metabolites differently. The present study investigated the effects of tannins, nicotine, and quinine on the food consumption and pupal parameters of *M. neustria* larvae, a critical forest pest. Pupal mass, pupal total lipid amount, and pupal protein amount were investigated as pupal parameters.

2. MATERIAL and METHODS

2.1. Collection of Larval Samples

M. neustria larvae were collected from the Kızılırmak Delta (N 41° 30′ E 36° 05′) in Samsun directly on feeding plants (*Hippophae rhamnoides* L. *subsp. caucasica* Rousi) while they were feeding. They were placed into transparent labelled containers and brought to the laboratory, where they were fed artificial diets until the emergence of the adults. Adults laid eggs, and the

larvae hatched from these eggs were used in feeding experiments. Eggs and larvae were taken to the air-conditioning room at 24 °C, 70 ± 5% RH, at 16:8 h light/dark period.

2.2. Sex Separation in Larval Stage

The feeding experiment groups were repeated collectively. In the related feeding experiment, the sex determinations of the individuals emerging from the pupa were made. Larger pupae were considered females. These data were used to determine nutritional indices.

2.3. Artificial Diets

In order to reveal the food preference of *M. neustria* larvae, an artificial diet developed and modified by Yamamoto (1969) was used. The content of Yamamoto's artificial diet is wheat germ, casein as the protein, saccharose or arabinose as the carbohydrate, torula yeast, vitamin mixture, salt mixture, cholesterol, sorbic acid, methylparaben, linseed oil, agar, and water. Diets containing secondary metabolites were prepared by adding different concentrations of tannic acid, nicotine, and quinine to the Yamamoto's nutritional content. The concentration of alkaloids in plants is not as high as in tannins, so the concentrations of tannins and alkaloids in the artificial diet were also prepared differently. A diet containing tannic acid in 3 different concentrations was prepared by containing 1.25%, 2.5%, and 5% percent tannic acid. Nicotine and quinine were added at 0.125, 0.25%, and 0.5%. The control diet did not contain any secondary metabolite. A total of 10 different artificial diets were prepared.

2.4. Feeding Experiment

The larvae of the *M. neustria* were collectively fed on each food group tested. The larvae that reached the last larval stage were taken into plastic containers one by one, with ten larvae in each diet, and the daily feeding experiment was started. The food was given (2 cm2) by weighing on a balance with a precision of 0.001 grams. The food left unconsumed is packaged by giving new food every other day. The remaining food was dried in the oven until it reached constant weight. Thus, the amount of consumption was calculated. Thus, the food consumption amount of the larvae was determined. Weight changes in the larvae each day were also noted. This process continued until all larvae reached the pupal stage (Lee et al., 2002). A total of 100 larvae were used for feeding experiments.

2.5. Pupal Parameters

The pupa mass, lipid, and protein content of the pupa were determined for growth performance. Pupae from each experimental group were dried in an oven at 50 °C and weighed after they reached the constant weight. The pupal mass was determined by weighing the dry weight. The total lipid amount was calculated using the methods modified from Loveridge (1973) (Simpson & Raubenheimer, 2001). The total amount of lipids stored in each pupa was determined with chloroform extraction (Simpson & Raubenheimer, 2001). After this application, the pupae were dried again at 50 ^{0}C, and the lipid-free pupal weight was determined. The difference gives the pupal total lipid amount.

The nitrogen content of the pupae was measured by the semi-micro Kjeldahl method with a Kjeltec Auto 1030 analyzer (Tecator model, Sweden) (Bergvinson *et al*., 1997). Lipid-free pupa samples were taken and wet burned in a mixture of concentrated sulfuric acid and potassium sulfate-copper sulfate (95-5), and then distilled by adding 40% NaOH. The resulting nitrogenous substances were kept in 4% boric acid. The boric acid solution was then back-titrated with 0.1 N HCl (Allen *et al*., 1986). The % N (Nitrogen) amounts found at the end of this process were multiplied by the constant 6.25, and the % protein amounts were found (Oonincx *et al*., 2015).

2.6. Statistical Analyses

The food consumption and pupal performances of *M. neustria* female and male larvae in each food group were analyzed for normality. In order to determine the differences between the groups, One-Way ANOVA and TUKEY tests were performed on the data with normal distribution. Correlation analysis revealed the relationships between pupal mass, pupal protein, lipid content, and larvae's food consumption according to the quinine, nicotine, and tannic acid concentration. Regression analysis was performed after the relationship was determined. SPSS version 23.0 was used for statistical analysis.

3. RESULTS

The food consumption and development parameters of male and female individuals of *M. neustria* larvae differed from each other. The food consumption of females was higher than that of males (Figure 1, Figure 2). The highest food consumption in female individuals was determined in the control diet and the diet containing 0.125% quinine. It was determined that the food consumption of diets containing alkaloids at 0.25% and 0.5% concentrations was the same between male and female individuals. Minor food consumption is in diets containing tannins. However, there is no difference in food consumption at 2.5% and 5% concentrations in these diets (Figure 1). In male individuals, the highest food consumption occurred in the diet containing 0.125% nicotine. While food consumption decreased in the diet containing 0.25% quinine, it increased in the diet containing quinine at 0.5% concentration. However, with the increase in nicotine concentration, another alkaloid, food consumption decreased (Figure 2).

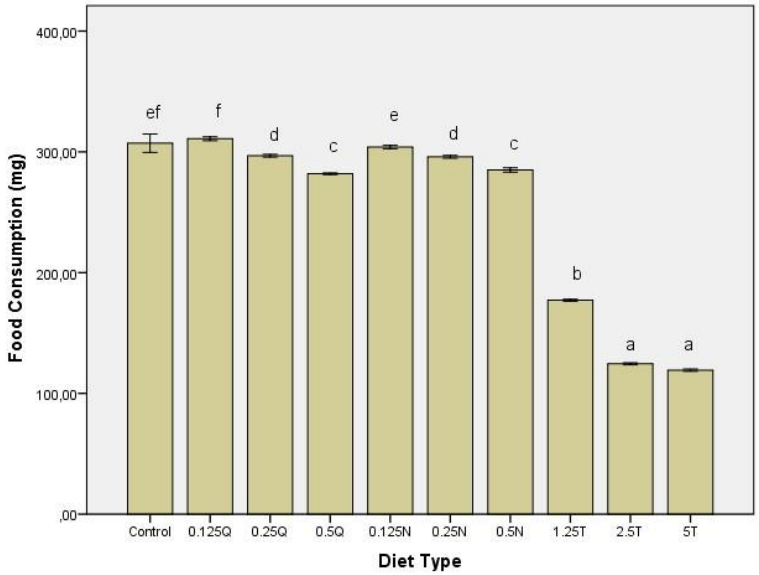

Figure 1. Amount of food consumption of female larvae in different diets (mg). Diets with the same letter are not significantly different.

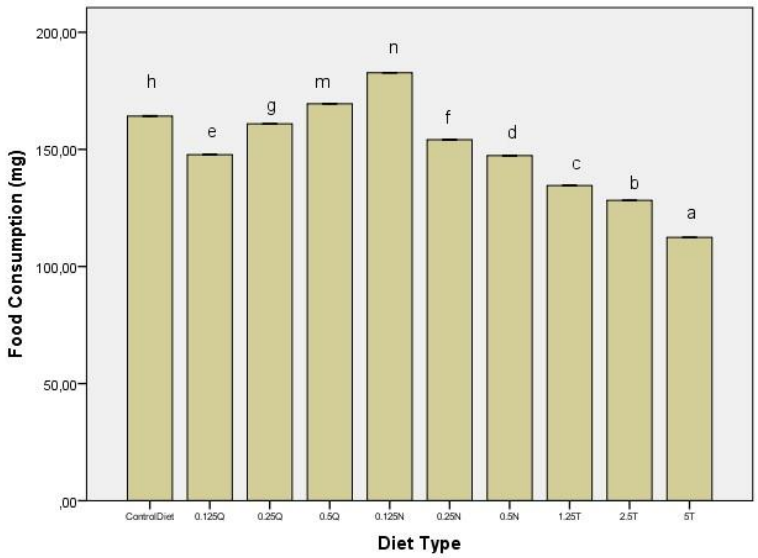

Figure 2. Amount of food consumption of male larvae in different diets (mg). Diets with the same letter are not significantly different.

Pupal mass also differed between male and female larvae. The female individuals' pupal masses are higher than those of male individuals (Figure 3, Figure 4). In females, pupal mass decreased with increasing concentration in all diets containing secondary metabolite. While the highest pupal mass was detected in the control group, the minor pupal mass was detected in the diet containing a 5% concentration of tannin (Figure 3). When the alkaloids were compared, the increase in quinine concentration resulted in a more significant reduction in pupal mass than nicotine (Figure 3). There was a positive relationship between food consumption and pupal mass in all diets containing secondary metabolite (quinine; r=0.98, p<0.01; nicotine; r=0.93, p<0.01; tannin; r=0.82, p<0.01). In male individuals, the highest pupal mass was detected in the control diet and the diet containing 0.5% nicotine. The lowest pupal mass was determined in diets containing tannin at 2.5% and 5% concentrations (Figure 4). A decrease in pupal mass was detected in diets containing both quinine and nicotine. However, there was no difference in pupal mass with increasing quinine concentration. The pupal mass of larvae fed on diets containing 0.125% and 0.25% nicotine did not differ from those with quinine (Figure 4). In nicotine-containing diets, the pupal mass of the larvae fed on the diet containing 0.5% nicotine was not different from the control group. The pupal mass of the diets containing 0.125% and 0.25% nicotine was decreased compared to the control group (Figure 4). In males, there was a positive correlation between food consumption and pupal mass in diets containing quinine and tannin (respectively; r=0.51, p<0.01; r=0.87, p<0.01). A negative correlation was found between food consumption and pupal mass in diets containing nicotine (r= -0.69, p<0.01).

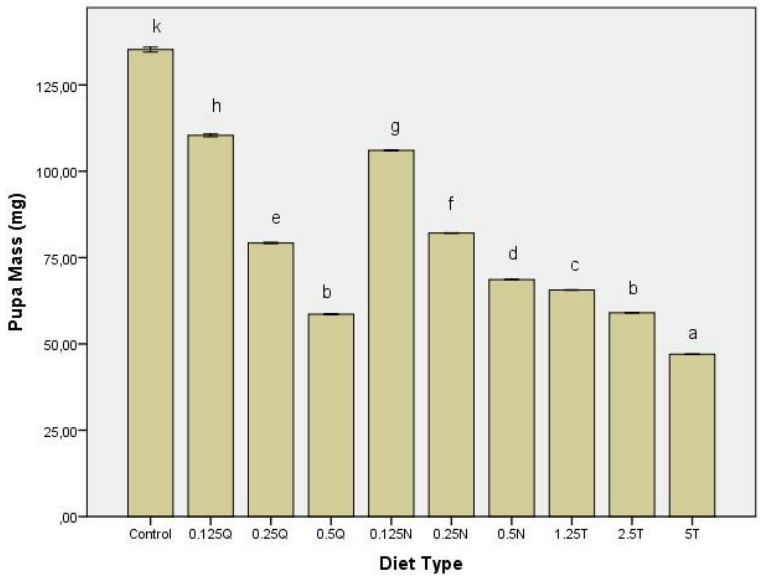

Figure 3. Pupal masses of females fed on different diets (mg). Diets with the same letter are not significantly different.

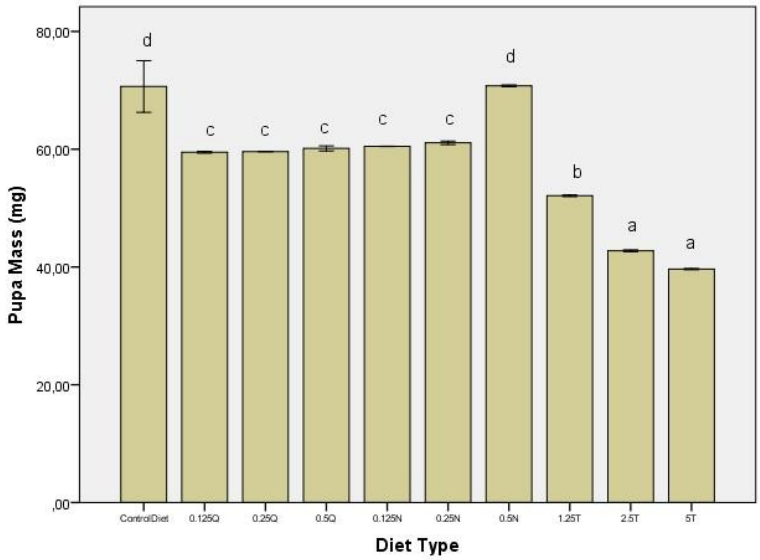

Figure 4. Pupal masses of males fed on different diets (mg). Diets with the same letter are not significantly different.

It was determined that male individuals' pupal lipid amount was less than those of female individuals (Figure 5, Figure 6). While 0.125% quinine concentration causes a decrease in the lipid amount, increasing the concentration to 0.25% increases the lipid amount. No difference was detected between 0.125% and 0.5% nicotine concentrations. The most effective concentration in tannin-containing diets was 0.25% (Figure 6). There was a negative correlation between pupal mass and pupal lipid amount in diets containing nicotine (r= -0.47, p<0.01). A negative correlation was also found between the amount of lipid and the amount of pupal protein (r=-0.36, p<0.05). A positive correlation was found between pupal mass and pupal lipid amount in tannin diets (r=0.92, p<0.01). Food consumption also positively affected pupal lipid amount (r=0.60, p<0.01). However, there was a negative correlation between pupal protein and lipid amount (r=-0.77, p<0.01). In diets containing nicotine, no factor affecting pupal lipid content was detected (p>0.05). While the highest pupal lipid amount in female individuals was in the control diet, the lowest pupal lipid amount was determined in individuals fed a diet containing 5% tannin (Figure 5). In all diets containing secondary metabolite, a decrease in

pupal lipid amount was detected with increasing concentration (Figure 5). There was a positive correlation between food consumption and pupal lipid content in all diets containing secondary metabolite (quinine; r= 0.98, p<0.01; nicotine; r=0.91, p<0.01; tannin; r=0.98, p<0.01). Similarly, there was a positive correlation between pupal mass and pupal lipid content (quinine; r=0.99, p<0.01; nicotine; r= 0.99, p<0.01; tannin; r=0.92, p<0.01). The relationship between pup protein amount and lipid amount was also positive (quinine; r=0.99, p<0.01; nicotine; r=0.99, p<0.01; tannin; r= 0.79, p<0.01).

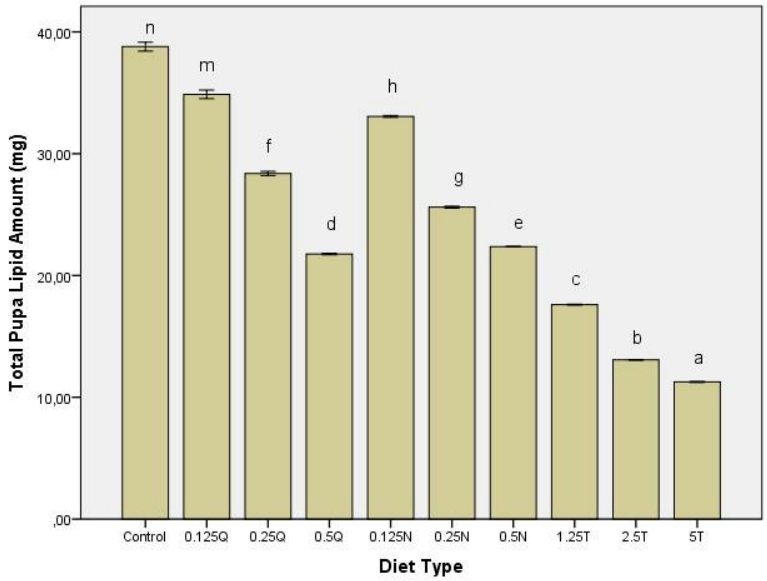

Figure 5. Total pupal lipid amount of female individuals fed on different diets (mg). Diets with the same letter are not significantly different.

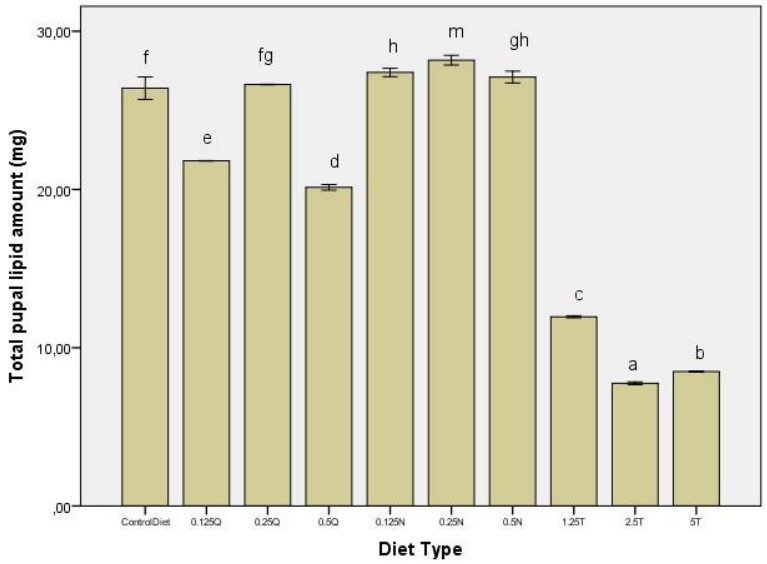

Figure 6. Total pupal lipid amount of male individuals fed on different diets (mg). Diets with the same letter are not significantly different

In females, the crude protein amount of pupae decreased with increasing concentration in diets containing secondary metabolite (Figure 7). Food consumption positively affected pupal protein amount in all diets (quinine; r=0.99, p<0.01; nicotine; r=0.95, p<0.01; tannin; r= 0.66, p<0.01). Again, there was a positive correlation between pupal mass and pupal protein amount in all diets (quinine; r= 0.99, p<0.01; nicotine; r= 0.99, p<0.01; tannin; r= 0.96, p<0.01). In males, pupal protein amounts increased in all diets containing secondary metabolite. (Figure 8).

In males, the increase in nicotine and quinine concentrations caused an increase in pupal protein content (Figure 8). The pupal protein amounts of larvae fed on tannic acid at 1.25% concentration were not different from those of the control group (Figure 8). Increasing the concentration to 2.5% increased the protein amount, while 5% tannin concentration caused a decrease in the pupal protein amount compared to the 2.5% concentration. However, the pupal protein amount in 5% tannin concentration was higher than in the control diet (Figure 8). In males, food consumption in diets containing quinine positively affected pupal protein amount (r= 0.98, p<0.01). However, a negative correlation was found between food consumption and pupal protein in nicotine-containing diets (r=-0.78, p<0.01). In diets containing tannin, food consumption did not affect the amount of protein (p>0.05). A positive correlation existed between pupal mass and protein content in diets containing quinine and nicotine (respectively; r= 0.51, p<0.01; r= 0.99, p<0.01). A negative correlation was found between pupal mass and pupal protein amount in tannin diets (r=-0.46, p<0.05).

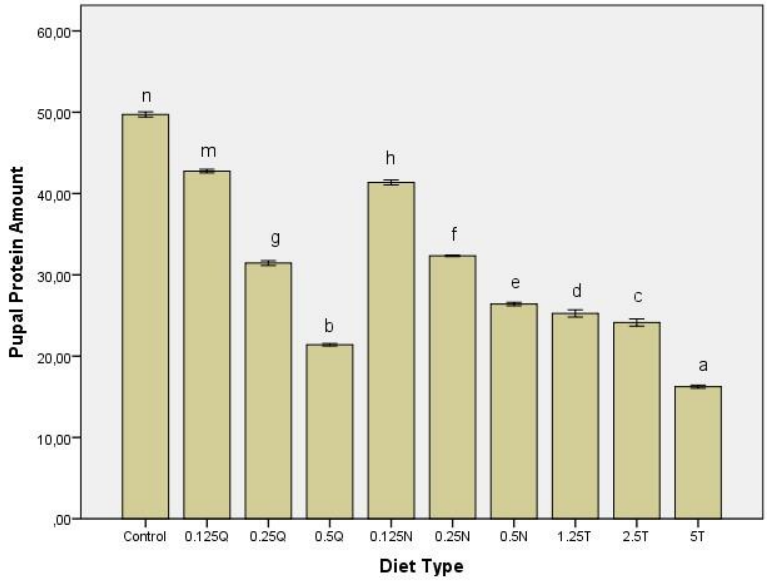

Figure 7. Pupal crude protein amount of female individuals fed on different diets (mg). Diets with the same letter are not significantly different.

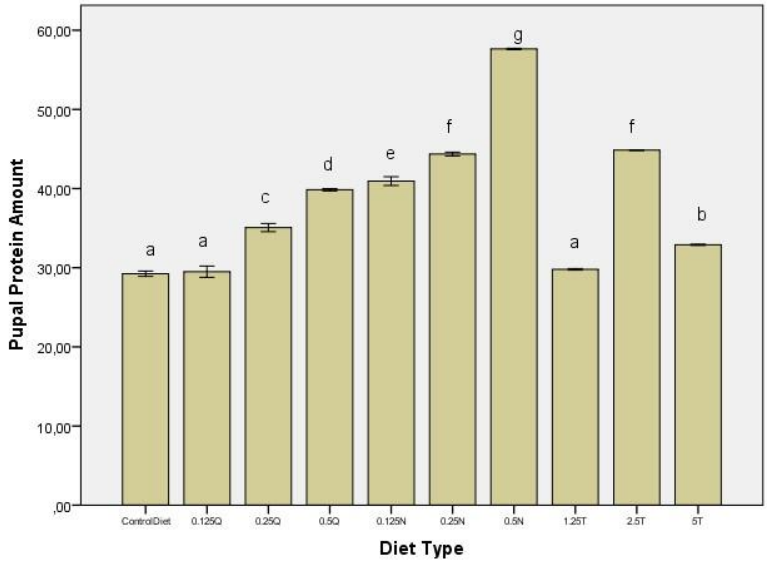

Figure 8. Pupal crude protein amount of male individuals fed on different diets (mg). Diets with the same letter are not significantly different.

4. DISCUSSION and CONCLUSION

Male and female insects may have sex-specific dietary preferences and differences due to differences in nutritional requirements (Carrel & Tanner, 2002). In this study, the food preferences of *M. neustria* female and male individuals were different. Food consumption of female individuals is higher than that of male individuals. Yoder and Grojean (1997) stated that female individuals of the *Gromphadorhina portentosa* (Blattodea:Blaberidae) consume more food than male individuals. Consequently, sexual differences in size tended to increase with increasing body size in these species (Teder & Tammaru, 2005). Therefore, *M. neustria* female individuals may have consumed more nutrients than males. In addition, it was determined that food consumption in *M. neustria* male individuals was less than the control group, except for diets containing nicotine. In female individuals, it was determined that the food consumption in all food types containing secondary metabolites was less than in the control group.

Similarly, Manosalva et al. (2019) stated that alkaloid-containing extracts of *Berberis microphylla* are a nutritional deterrent for *Plutella xylostella* (Lepidoptera: Plutellidae) individuals. The first step in protecting against herbivores of secondary plant metabolites is to be a nutritional deterrent. Insects avoid feeding on alkaloids in the pre-ingestive or post-ingestive stage, primarily because of the bitter taste of the alkaloids. However, it was determined that the food consumption in diets containing 0.5% nicotine in males was higher than in diets containing 0.25% nicotine. Noxious compounds stimulate olfactory (Thompson & Redak, 2007) and gustatory receptor neurons (GRNs) and induce aversive responses in many insects (Munoz *et al*., 2020). Those adapted to alkaloids also cause the gustatory code to change (Munoz *et al*., 2020). The increase in food consumption with an increase in concentration from 0.25% to 0.5% in the quinine-containing diet in male individuals may be related to the change in the gustatory code. Alkaloids are insecticides at low concentrations. They affect the acetylcholine receptor in the nervous system and sodium channels of nerve cell membranes. Since they are not volatile, they are directly toxic to insects (Rattan, 2010). Senthil-Nathan (2013) stated that secondary metabolites affect the nutritional indices of insect populations.

Low food consumption should not be considered alone for food to be classified as a deterrent. Therefore, when pupa weights are examined, in *M. neustria* males, pupal mass and protein content increased, although food consumption at 0.5% concentration was decreased in nicotine-containing diets. Diets containing nicotine are not effective as they cause an increase in pupal mass, although they are a food deterrent. In the literature, it has been stated that secondary metabolites, which are the defense metabolites of plants, cause a decrease in the pupa mass of insects. According to Yuan *et al*. (2022), it was determined that pupal weights of *Spodoptera litura* (Lepidoptera: Noctuidae) individuals fed with different pepper plants decreased with the amount of flavonoids. Similarly, Harvey *et al*. (2007) reported that the pupal mass of *Manduca sexta* (Lepidoptera: Sphingidae) was negatively correlated with nicotine concentration. Pupal mass is indicative of fecundity (Myers *et al*., 2000). Pupal masses of male individuals fed a diet containing 0.5% nicotine did not differ from the control group. Therefore, even if the increase in nicotine concentration reduces food consumption, it is ineffective for males because it may not reduce fecundity. However, other diets containing secondary metabolite may reduce fecundity as they cause a reduction in pupal mass of both female and male individuals.

An increase in pupal protein was observed in male individuals with all secondary metabolites. Dixit *et al*. (2017) stated that the increase in secondary metabolite in plants caused a decrease in the pupal protein in *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *S. litura* individuals. In this respect, the decrease in pupal protein content of male *M. neustria* individuals differs from the literature. Also, in *M. neustria*, male pupal lipid content increased with nicotine concentration. However, as the pupal mass increases, the amount of lipids decreases. Larva stores more protein than lipids in increasing mass. This situation may be due to the use of

protein for development. The increase in storage proteins is significant for holometabolous insects in terms of their use in metamorphosis (Sak *et al*. (2006; 2011). However, insects use lipids for development, flight, reproduction, participation in the structure of cell membranes, and communication through pheromones (Beenakkers *et al*., 1985; Lease & Wolf, 2011).

Interestingly, nicotine poisoning symptoms follow the general sequence of excitation, convulsions, paralysis, death, and mimics as a part of the action of acetylcholine by interacting with the nicotine acetylcholine receptor as the receptor fails to distinguish between nicotine and acetylcholine (Rattan, 2010). However, male larvae differ from the result of Rattan (2010). In diets containing quinine, high food consumption does not affect the pupal mass. This result may be due to the inability of the larvae to use the food. Detoxification of secondary metabolites is a process with an energy cost. Therefore, it caused an increase in energy requirement and decreased food availability in *Spodoptera eridania* (Lepidoptera: Noctuidae) larvae (Cresswell et al., 1992). Secondary metabolites may have reduced the availability of food for *M. neustria* larvae. However, pupal lipid content did not differ from the control group at 0.25% quinine concentration. Bate-Smith (1973) states that secondary metabolites cause enzyme inhibition after a particular concentration or the threshold level required for the sensory deterrent effect is exceeded. Wink (2000) stated that secondary metabolite analogs also interfere with cell signal system components, vital enzymes, and nervous system signals such as neurotransmitter synthesis and receptor activation and block metabolic pathways. Therefore, the nutrients taken after a particular concentration may not be used for development. Although the consumption amount at 0.5% quinine concentration was higher than the control group, the low lipid amount also supports this.

Secondary metabolite caused a decrease in pupal protein amount in female *M. neustria* individuals. However, nicotine has a more significant effect on triggering protein storage than nicotine. In diets containing tannins, more lipids are stored. Proteins are essential for female individuals, especially in egg production (Telang *et al*., 2001). Therefore, the decrease in pupal protein amount with the addition of secondary metabolite may cause a decrease in egg production. Tannins show pharmacologically toxic effects as a nutritional deterrent, binding to digestive enzymes in the intestines of foods and animals, forming lesions in the intestinal epithelium, causing oxidative stress, destroying microbial symbionts, and pharmacologically toxic (Mole & Waterman, 1987; Bernays *et al*., 1989; Schultz & Lechowicz, 1989). They also reduce the availability of proteins. Therefore, it may have triggered lipid storage compared to protein.

Food consumption and pupal parameters decreased in all tannin diets in male and female larvae. Tannins are absorbed in herbivores' midgut and cause high ROS production levels due to oxidative stress (Barbehenn & Kochmanski, 2013). In this case, food availability decreased, causing a decrease in pupal parameters.

As a result, some allelochemicals, as chemical defense compounds, affect organisms molecularly and physiologically (Rattan, 2010). Thus, secondary metabolites are used as insecticides. Many insecticides have been shown to reduce biochemical components (Roya *et al*., 2010; Zhao *et al*., 2016) and lipid parameters (Xu *et al*., 2016) in the animal body. Therefore, since the type of alkaloids affects male and female larvae differently, determining their effects and fighting against them will be beneficial.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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Mahmut Bilgener: Investigation, Resources, Methodology, Analysis, and Writing - original draft. **Nurver Altun**: Methodology, Statistical Analysis, and Writing. Authors may edit this part based on their case.

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Research Article

The Effect of Tapak Liman (*Elephantopus scaber* L.) Extract on *Xa4* Gene Expression in Rice IR64 Infected by Bacterial Leaf Blight (*Xanthomonas oryzae*)

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Abstract: Bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), represents a significant threat to rice (*Oryza sativa*) production. Induce plant resistance has emerged as a promising control strategy. The extract of Tapak Liman (*Elephantopus scaber*) has been considered a promising agent due to its antimicrobial properties, with several of its compounds showing its potential as inducers of plant resistance. This study aimed at elucidating the impact of Tapak Liman extract on the expression of resistance *Xa4* gene in rice that plays a crucial role in the synthesis mechanism leading to cell wall thickening. To explore this effect, we analyzed *Xa4* gene expression using the reverse transcription-polymerase chain reaction (RT-PCR) technique, followed by a semi-quantitative descriptive analysis. Our results demonstrate that the application of Tapak Liman extracts at a concentration of 10 mg/ml significantly upregulates *Xa4* gene expression in the IR64 compared with other concentrations, 1 mg/ml or 5 mg/ml. Furthermore, the observed higher expression of the *Xa4* gene persists until 5 days after pathogen inoculation, which is also implicated with a less developed lesion on rice leaves by 76% compared with the control.

1. INTRODUCTION

Bacterial Leaf Blight (BLB) is caused by the pathogen *Xanthomonas oryz*ae pv. *oryzae* (*Xoo*), which attacks rice (*Oryza sativa* L) around the world, including in Indonesia (Jiang *et al*. 2020). *Xoo* enters plants through wounds on the leaves and then develops in plant tissues to cause symptoms such as spots/lesions parallel to the edges of the leaves and will continue to spread to the entire leaf resulting in the damage to the growing point and the death of the rice plant (Ke *et al*., 2017). Based on the impact caused by this disease, it is necessary to carry out appropriate control to suppress the development of the *Xoo* pathogen.

One of the strategies for controlling the BLB is through the induction of rice plant resistance (Liu & Wang, 2016; Fiyaz *et al*., 2022). A wide variety of biotic and abiotic agents (stimuli) can induce disease resistance in plants (Walter *et al*., 2013). Exogenous application of natural

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or synthetic chemicals to plants can activate their resistance and boost the innate defenses to disease (Nadhira *et al*., 2021; Liu & Wang, 2016; Krzyzaniak *et al*., 2018; Honig *et al*., 2023). Plant extracts are one type of plant-resistance-inducing substance that can be employed (Abo-Elyousr *et al*., 2020).

The Tapak Liman (*Elephantopus scaber*) is an ethnobotanical species in the Asteraceae family containing bioactive compounds that are thought to induce resistance in rice plants to BLB because it can inhibit the growth of Xoo (Rejeki *et al*., 2017). *E. scaber* has been reported to contain biochemical flavonoids, terpenoids, saponins, tannins, carbohydrates, and proteins (Kabiru & Por, 2013). In addition, phenylpropanoids and flavonoids play an important role in plant development by developing resistance to various biotic and abiotic stress (Ramaroson *et al*., 2020).

Induction of plant resistance is also considered to be able to activate genes related to plant resistance responses directly and indirectly, as well as priming cells that cause stronger elicitation of resistance (Walters *et al*., 2005). Rice was identified as having 46 types of natural resistance genes against Xoo (*Xa* or *xa* genes) (Arunakumari *et al*., 2016). Each of these genes has a specific resistance rule to the Xoo pathotype (Jiang *et al*. 2020). The *Xa* genes which have an important function in controlling the Xoo, especially in Asia, are the *Xa4*, *Xa3/Xa26* genes. The *Xa4* gene itself has the function of activating the expression of the *CesAs* gene which can thicken cell walls so that plants are more resistant to Xoo bacteria (Mazerai *et al*., 2018). However, the level of gene expression in each plant varies depending on the variety or cultivar of the plant in expressing the resistance gene. As seen in previous studies, IR64 variety rice is very susceptible to HDB disease strains III, IV, and VIII and has a high incidence rate of 61%, where IR64 expresses the *Xa4* gene before Xoo inoculation, but expresses the *Xa10* gene and *xa13* gene after Xoo inoculation (Nadhira *et al*., 2022).

Therefore, based on the case study above, the expression of the *Xa4* resistance gene needs to be quantified to identify that the *E. scaber* extract can induce the resistance gene of the IR64 variety Rice plant infected with the BLB.

2. MATERIAL and METHODS

2.1. Planting and Seedling Material

Rice IR64 seeds were disinfected using 10% sodium hypochlorite and rinsed with distilled water and soaked for 24 hours. Seeding was carried out on containers filled with paddy soil for 14 days. Rice seeds were then transferred to the planting medium of paddy soil in polybags 15 × 15 × 7 cm to the age of 21 days after planting where each polybag contains 10 plants.

2.2. Preparation of *E. scaber* Extract

A total of 50 grams of simplicial powder of *E. scaber* leaves was mixed with 150 mL of methanol solvent and shaken for 48 hours. The suspension of the *E. scaber* in methanol was then passed through a Whatman paper to obtain a debris-free solution. The solution was evaporated using an evaporator rotary device (Hahnvapor HN 2005 V-N, Korea). The remaining solvent in the extract was evaporated using an oven with a temperature of 45°C until a paste extract was obtained.

2.3. Application of *E. scaber* Extract

The application of *E. scaber* extract was carried out on rice plants aged 14 days after sowing. The doses of *E. scaber* extract given were 1 mg/ml, 5 mg/ml, and 10 mg/ml with an application volume of 10 mL per polybag. The application of salicylic acid (SA) 10 mM was used as a positive control and distilled water as a negative control.

2.4. Preparation of the Inoculum and Inoculation of Pathogens

Xoo strain H01 was obtained from the collection of the Plant-Microbe Interaction Laboratory,

CDAST, University of Jember. Xoo isolates were grown on liquid media yeast extract dextrose at a temperature of 28 °C for 24 hours (Rejeki *et al*., 2021). The inoculum obtained is then diluted with distilled water until it reaches an optical density (OD600) equal a 10^8 CFU per milliliter. Inoculation of the pathogen was carried out on rice aged 7 days after planting (DAP) using the leaf clipping method by dipping sterile scissors in the inoculum suspension which was then used to cut 2-3 cm of the tip of the rice leaf (Nadhira *et al*., 2022).

2.5. RNA Total Isolation

Rice leaves were harvested on day 0 (before Xoo inoculation), day 3rd, and day 5th (after Xoo inoculation). A total of 50-100 mg of rice leaf samples was ground to a fine powder with liquid nitrogen. Total RNA was isolated using Total RNA Mini-kit (Plant) (Geneaid, Taiwan) and pure RNA samples were visualized by electrophoresis on a 1% agarose gel.

2.6. The cDNA (Complementary DNA) Synthesis

The pure total RNAs were reverse-transcribed into complementary DNA (cDNA) using the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The reverse transcription process was carried out by the one-step protocol including gDNA removal according to the manual guideline. Before cDNA synthesis, the 4× DN Master Mix (containing 50:1 gDNA remover) was mixed with a 2 µL RNA template (0.5 µg) and adjusted with the nuclease-free water to a total of 8 µL. About 5 minutes after incubation at 37 °C, about 8 µl of pure RNA was added with 2 µl 5× RT Master mix. Furthermore, incubation was carried out with a program at a temperature of 37 °C for 30 minutes, and then inactivated (reverse transcriptase inactivation) at a temperature of 98°C for 5 minutes.

2.7. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The cDNA sample was subsequently confirmed using polymerase chain reaction (PCR). About 2 µl of cDNA were added with 25 µl of MyTaq HS Red Mix master mix (Bioline, UK) and 2 µl of Forward and Reverse primers of 2 µl each, distilled water added by 19 µl with a total volume of 50 µl. Primers used were Xa4 Forward (5'-ATCGATCGATCGATCTCACGAGG-3') and Reverse (5'-TGCTATAAAAGGCATTCGG-3') responsible for amplicon with the size of 150 bp (Rasmiyana *et al*., 2019). *β-actin* is used as an internal control gene or housekeeping gene to normalize the gene expression with Forward primer 5'-TGTATGCCAGTGGTCGTACCA-3' and Reverse primer of 5'-CCAGCAAGGTCGAGACGAA-3' responsible for amplicon with the size of 121 bp (Nadhira et al., 2022). DNA amplification was carried out in 25 cycles with pre-denaturation conditions of 95 °C for 3 minutes, denaturation of 95°C for 30 seconds, annealing of 53 °C (for *Xa4*) or 56 °C (for *β-actin*) for 30 seconds, temperature elongation of 72 °C for 1 minute, and final elongation of 72 °C for 5 minutes. RT-PCR results are subsequently electrophoresis on agarose gel 1%. The results of the RT-PCR band visualization were observed in 1% of agarose gel and quantified using ImageJ software (Hazman, 2022) by comparing the ratio of the target genes to the housekeeping gene (β-actin gene).

2.8. Evaluation of the Severity of the Disease

The BLB severity of rice plant disease was observed at 14 days after inoculation of the pathogen using a lesion length scale, namely resistant ≤ 3.0 cm; medium resistance 3.0 – 6.0 cm; moderately vulnerable 6.0 – 9.0 cm; and vulnerable to >9.0 cm (Nadhira *et al*., 2022).

2.9. Data Analysis

The data obtained were then carried out analysis using semi-quantitative descriptive analysis. Disease severity data was further statistically analysis using Duncan Multiple Range Test (DMRT) for the significance (p<0.05) using Microsoft Excel 2019.

3. RESULTS

3.1. The *Xa4* Gene Expression

The RT-PCR results showed that the *Xa4* gene indicated different levels of expression according to the *E. scaber* dose treatment and the harvest period both before Xoo inoculation and after Xoo inoculation. It is characterized by the difference in the brightness level of the *Xa4* gene DNA band when compared to *β-actin* as a housekeeping gene (Figure 1A). Gene expression analysis using ImageJ software showed that *Xa4* values tend to fluctuate. The application of *E. scaber* extract at a 10 mg/ml both after and before inoculation of the Xoo pathogen showed the highest *Xa4* gene expression value compared to other treatments, especially when compared to salicylic acid (SA) and dH_2O treatment (Figure 1B). In contrast, the treatment of 1 mg/ml showed the lowest expression of the *Xa4* gene, especially on the fifth day after inoculation (Figure 1B). Gene expression values tended to decrease on the fifth day after pathogen inoculation at the 10 mM salicylic acid (SA) treatment, dH_2O, and 1mg/ml of *E. scaber* dose but increased at the 5mg/ml and 10 mg/ml *E. scaber* dose treatments. In addition, *Xa4* gene expression at the SA of 10 mM treatment also showed the lowest *Xa4* gene expression value at the overall harvest time in all treatments, especially in harvesting before Xoo inoculation and the third day after Xoo inoculation.

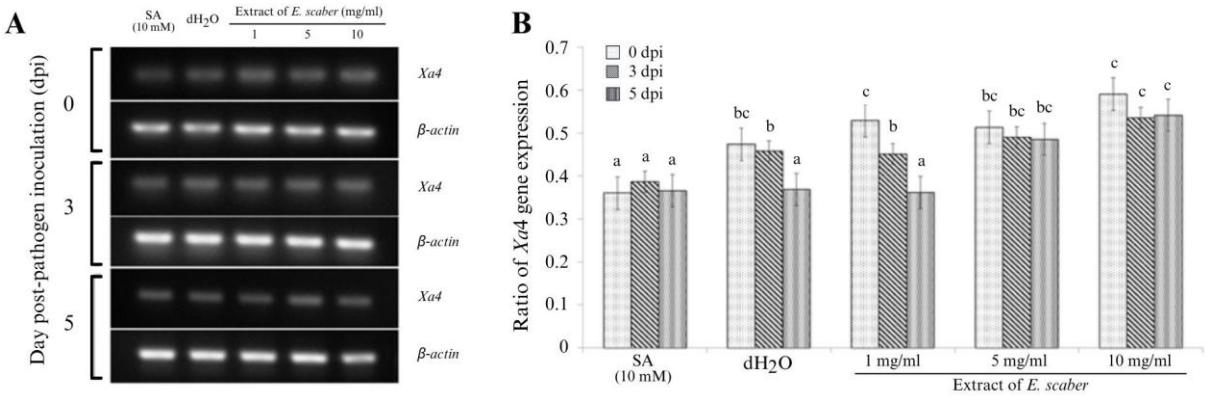

Figure 1. The *Xa4* expression ratio value (A) RT-PCR visualization results of *Xa4* expression compared to *β-actin* gene (B) *Xa4* gene value using ImageJ software; SA is salicylic acid. Different notations above the bars varied significantly by Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

3.2. BLB Disease Severity

Analysis of disease severity in all treatments was shown by the presence of lesions on the tips of the leaves as a symptom of BLB disease (Figure 2A). Data on the severity of the disease showed a significant difference between the treatment with dH_2O with the treatment with *E. scaber* at doses of 5 mg/ml and 10 mg/ml. Leave pre-treated with dH_2O showed the longest lesions with an average of 0.33 cm compare with the *E. scaber* extracts of 5 mg/ml and 10 mg/ml which exhibited the shorter lesions, 0.12 cm and 0.08 cm, respectively (Figure 2B). In addition, the leave pre-treated with 10 mM salicylic acid showed insignificant differences when compared with the leave pre-treated with *E. scaber* extract at the dose of 5 mg/ml and 10 mg/ml (Figure 2B).

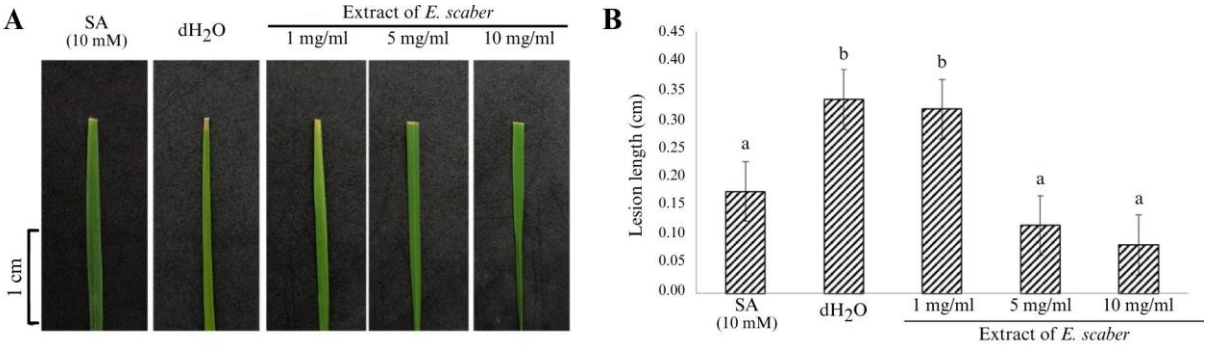

Figure 2. Symptoms of BLB characterized by the appearance of lesions (a) The size of the lesions seen on the tips of the leaves of plants affected by Xoo (b) The average value of the length of the lesions in each treatment; ES is an *Elephantophus scaber*. Different notations above the bars varied significantly by Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

4. DISCUSSION and CONCLUSION

Rice IR64 that are inoculated with the Xoo have symptoms of bacterial leaf blight (BLB) characterized by spots on the shoots or leaf edges (lesions) which then propagate to form pale yellow blight (Ke *et al*., 2017). The lesions are the result of mechanisms of cell death programmed to suppress the pathogen's growth and development in plant cells (Coll *et al*., 2011). In addition, the lesions on the leaves caused by the attack of pathogens are the result of a mechanism of oxidative damage. Oxidative damage will stimulate the occurrence of apoptosis (Demidchik, 2015). The development of lesions as an indication of disease severity shows that there is a difference in the high and low value of disease severity depending on the treatment. The lower severity of the disease under the treatment of *E. scaber* extract indicates that an *E. scaber* extract with a certain concentration can provide a more optimal resistance mechanism in rice against Xoo. This is related to previous studies by Abo-Elyousry *et al*. (2020) and Ashfaq *et al*. (2014) that pre-treating plant extracts such as *Nerium oleander*, *Eucalyptus chamadulonsis* and *Citrullus colocynthis* with certain doses and concentrations reduce the severity of disease in tomato (*Solanum lycopersicum* L.) and chili (*Capsicum annum* L.). In contrast, the higher severity of the disease is caused by the lack of resistance mechanisms of rice plants in resisting the Xoo pathogen, where plants are unable to provide early resistance signaling to pathogen attacks (Herrera-Vásquez *et al*., 2015). The mechanism of resistance to Xoo can be influenced by the expression of resistance genes found in Rice (Zafar *et al*., 2020). The Xa4 gene is one of the BLB resistance genes detected in IR64 rice, whereas in previous studies this gene had a lower gene expression value after inoculation from the Xoo pathogen, which caused plants to become more susceptible to BLB disease (Nadhira *et al*., 2022). Although all disease severity on the treated leaves is in the same resistant category (Figure 1A), however, it shows the differences depending on the treatment representing the resistance, statistically. This indicated that lower severity, a higher resistance response to the pathogen.

The induction of resistance genes through the extraction of the *E. scaber* is intended to increase the expression of the *Xa4* gene, with the aim that the plant is more resistant to Xoo. This can be proven by the pattern of increasing Xa4 gene expression in the treatment of tread dose administration, even before the inoculation of the Xoo pathogen (Figure 1B). The increase in expression of the *Xa4* gene occurs because the *E. scaber* extract contains carotenoid compounds, alkaloids, and flavonoids that have anti-microbial properties (Rejeki *et al*. 2017). This antimicrobial compound is thought to induce the expression of the *Xa4* gene, a type of Wall-Associated Kinase (WAK) gene that works in the thickening of cell walls. The *Xa4* gene can also increase the *Cellulose Synthase* (*CesAs*) gene and inhibit the α-expansin (EXPA) gene resulting in increased mechanical strength and the cell wall of rice plants (Mazerai *et al*., 2018). However, in addition to the effect of increasing *Xa4* expression, there is also the phenomenon

of gene expression value decreasing and then increasing (fluctuating) allegedly due to the growth and development activity of the pathogen so that it affects the activity of the *Xa4* gene. According to Ishihara *et al*. (2019), the decrease in resistance gene expression occurred in the treatment of hot water extract from the *Hypsizygus marmoreus* fungus substrates which influenced the expression of *PR1b* and *PBZ1* genes in rice plants infected with *Pyricularia oryzae*. The *PR1b* and *PBZ1* genes at the beginning of inoculation experienced an increase in gene expression but showed a decrease in the expression value of the *PR1b* and *PBZ1* genes at 2 days after inoculation indicating that a pathogen spread in plants.

The application of *E. scaber* extract can also stimulate the expression of resistance genes through signaling complexes such as reactive oxygen species (ROS) signals and systemic acquired resistant (SAR) transduction signals mediated by SA (salicylic acid) (Chan, 2013; Khataee *et al*., 2019). In addition, the *Xa4* gene stimulates the formation of phytoalexins, molilactone A, and sakruranetin so that plants are more resistant to pathogen infection (Ji *et al*., 2018). On the other hand, the expression value of the *Xa4* gene in SA treatment is smaller than that of dH_2O. It is suspected that the low expression of the *Xa4* gene is due to oxidative stress. The previous studies by Horvath *et al*. (2007) reported that rice plants have a higher basal (endogenous) SA compared to other plants. Accordingly, the application of an exogenous SA on the rice plants experiences faster oxidative stress. Moreover, salicylic acid also stimulates the formation of *NPR1* (*Nonexpressor of Pathogenesis-Related Genes 1*) to transcribe resistance genes as well as the elicitation of resistance due to pathogen infection (Chan, 2013).

In conclusion, this study demonstrated that the *E. scaber* extract at a concentration of 10 mg/ml elicits the most pronounced induction of resistance by increasing the *Xa4* gene expression and consequently slower the BLB symptom development in rice.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Hardian Susilo Addy: Design, analysis, interpretation, and supervision. **Ali Wafa**: Supervision and interpretation. **Nur Habibullah**: Performing the experiment and data analysis. **Hardian Susilo Addy** and **Wulan Arum Hardiyani**: writing the manuscript. All author agrees with the manuscript.

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Research Article

Secondary metabolite estimation and antioxidant potential assessment of purple bell *Thunbergia erecta* (Benth.) T. Anderson

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Abstract: To quantify the aqueous and methanolic extracts for primary and secondary metabolites, and the antioxidant potential of leaf extracts of the *Thunbergia erecta* plant and to adopt them in Ayurvedic medications for various illnesses. Primary metabolites like carbohydrates, proteins, and secondary metabolites such as flavonoids, alkaloids, total phenols, and tannins were estimated using standard procedures. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), hydrogen peroxide, and phosphomolybdenum free radical scavenging activities were employed to evaluate the antioxidative potential. The phytochemical examination of *T. erecta* exhibited the presence of carbohydrates, proteins, amino acids, alkaloids, total polyphenolics, flavonoids, and tannins in significant quantity. The *in vitro* antioxidant potential of the species, clearly verifies that methanolic extract shows prominent antioxidant properties followed by the hydroalcoholic extract. From this study, it can be concluded that this species is effective in scavenging free radicals and may be a powerful antioxidant. The *T. erecta* leaf extract showed the existence of bioactive components which are known to exhibit medicinal activities. The findings of this study suggest that these plant leaves could be a potential source of natural antioxidants that could have great importance as therapeutic agents in preventing various diseases.

1. INTRODUCTION

Oxygen, the most crucial component for the survival of living beings, is extremely reactive and can harm the body's healthy cells as a free radical. Free radicals are produced by oxidation, which can start chain reactions that cause cell damage and even cell death (Moonmun *et al.*, 2017). An antioxidant is a molecule (or an ion, or a relatively stable radical) that is capable of slowing or even preventing the oxidation of other molecules (Pinchuk *et al.*, 2012). The characteristic feature of an antioxidant is the ability to scavenge free radicals due to their redox hydrogen donators and singlet oxygen quencher (Senguttuvan *et al.*, 2014). Both free and non-free radical species are produced by reactive oxygen species (ROS), resulting in hazardous and

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lethal diseases such as diabetes, atherosclerosis, ischemic heart disease, inflammation, immune suppression, neurodegenerative disorders, cancer, and so on. Free radicals such as superoxide and hydroxyl radicals are formed as by-products of the cell's energy production. When the concentration of ROS in the cell rises, it causes oxidative stress. Antioxidants, which are found in nature or as food supplements, counteract these effects in live cells. Several research supports the fact that flavonoids and phenolic compounds are potent antioxidants. Antioxidants scavenge free radicals and mitigate the effects of reactive oxygen species (Ganguly *et al.*, 2021). The continued expansion of the antioxidant market reflects the expectation of curing a wide range of ailments thought to be caused or exacerbated by oxidative stress. Medicinal plants provide a variety of alternative medicines with exciting possibilities. Several sources also say that dietary phytometabolites such as flavonoids, phenols, and tannins have been employed in the prevention and treatment of diseases for millennia (Ganguly *et al.*, 2021).

Thunbergia erecta belongs to the family Acanthaceae, native to the tropical part of Madagascar, Australia, Africa, India, and South Asia. The whole plant is said to be medicinally important by having anxiolytic, sedative (Begum *et al.*, 2019), anticholinesterase, antiaging activity (Refaey *et al.*, 2021), antidiabetic activity, antimicrobial screening (Kusimo *et al.*, 2019), antiurolithiatic (Chandel *et al.*, 2020) and neuroprotective activity (El-Din *et al.*, 2023). Investigations of the *T. erecta* plant uncovered various phytochemical characteristics, the chemical composition of the ethyl acetate fraction of *T. erecta* leaf alcohol extract had significant activity on DOX and Cyclo-induced cognitive impairment in rats. (El-Din *et al.*, 2023). Our review has already covered the various bioactivities *T. erecta* plant (Kochar *et al.*, 2023).

However, much scientific validation has been needed for this species for its medicinal uses. To address this lacuna, the present study was carried out for qualitative and quantitative phytochemical analysis and *in vitro* antioxidant activities of the leaves of *T. erecta* using petroleum ether, methanol, and hydroalcoholic extracts.

2. MATERIAL and METHODS

2.1. Chemicals and Reagents

The chemicals utilized included acetone, ammonia, aluminium chloride, ascorbic acid, ammonium molybdate, anhydrous sodium carbonate, *β*-D-glucose, bovine serum albumin, butylated hydroxytoluene (BHT), chloroform, 1,1- diphenyl-2-picrylhydrazyl radical (DPPH), diethyl ether, ethanol, ethyl acetate, ferric chloride, Folin-Ciocalteu reagent, gallic acid, glacial acetic acid, hydrochloric acid, hydrogen peroxide, methanol, *n*-butanol, *n*-propanol, petroleum ether, phenol, phosphate buffer, potassium acetate, potassium ferricyanide, quercetin, rutin, sodium chloride, sodium nitrate, sodium hydroxide, sodium phosphate, and sodium nitroprusside were of analytical grade and purchased from Merck.

2.2. Collection and Authentication of Plant Material

Fresh leaves of the *Thunbergia erecta* plant were collected from the local area of Wardha, Maharashtra, India. The authentication of plant material was carried out by the Botanical Department of RTMNU Nagpur University, Nagpur. The voucher specimen number is 10421. The collected plant material was washed in tap water to get rid of any contaminant, then dried under shade at room temperature for seven days, and then ground to obtain a coarse powder.

2.3. Organoleptic Study

Organoleptic properties like color, odor, shape, size, taste, and texture were studied (Table 1).

2.4. Physicochemical Investigation

The physicochemical studies were performed on *T. erecta* leaf powder, such as different ash values, extractive values, extractive matter, fiber content, foaming index, and moisture content.

2.4.1. *Determination of ash value*

The plant powdered was analyzed for total ash, sulphated ash, acid-insoluble ash, and water-soluble ash as per standard techniques of WHO quality control methods for herbal materials (Maduka *et al.*, 2020; Singh *et al.*, 2019).

2.4.2. Determination of extractable matter

The extractive value was done on different solvents such as petroleum ether, ethyl acetate, chloroform, acetone, ethanol, methanol, hydroethanolic extract in the ratio (80:20, 60:40, 50:50). A glass stoppered conical flask was filled with approximately 5 g of coarsely powdered air-dried material. 100 mL of the above-mentioned solvents were added. The conical flask was corked and left for 24 hours with continuous shaking. The solvents were then filtered using dry filter paper to ensure that no solvent was lost, and 25 mL of the filtrate was transferred to a tarred flat bottom dish and evaporated to dryness on a water bath. The solvent was dried for 6 hours at 105°C and chilled for 30 minutes in a desiccator before being weighed. The extractable matter quantity in mg/g of air-dried material was calculated (Singh *et al.*, 2019).

2.4.3. Determination of foaming index

Approximately 1 g of coarse powder was precisely weighed and put in a 500 mL conical flask holding 100 mL boiling water. The mixture was kept at a moderate boil for 30 minutes, then cooled and filtered into a 100 mL volumetric flask, with enough water added through the filter to dilute the volume. In ten test tubes with stoppers (diameter: 16 mm; height: 16 cm), the decoction was added in portions of 1 mL, 2 mL, 3 mL, etc., up to 10 mL, and the liquid volume in each tube was adjusted to 10 mL using water. The tubes were stopped and shaken lengthwise for 15 seconds at two shakes per second. The foam was Allowed 15 minutes to rise before measuring its height (Mishra *et al.*, 2016).

The foaming index was calculated using the following formula:

Foaming index = 1000/a

Where a = the volume in mL of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

2.4.4. Determination of moisture content

Moisture content was determined using the standard method described by Maduka (2020), a 1 g sample was precisely weighed and placed in a dry crucible. The samples were dried in an oven at 100-110° C for 2 hours, removed, cooled in a desiccator, and weighed. This process continued until consistent weights were achieved (Maduka *et al.*, 2020). The moisture content of the samples was calculated using the following formula:

$$\% \text{ moisture} = (ab-ac) * 100/ab$$

Where ab = weight of dish + weight of sample before drying (g)

ac = weight of dish + weight of sample after drying (g)

2.4.5. Estimation of crude fiber

A crude sample (2 g) was extracted with petroleum ether to remove fat. After filtration, the material was dried and heated with 200 mL of 0.3M H_2SO_4 for 30 minutes. When the washings were acid-free, it was filtered and washed again with hot water. After heating in 200 mL NaOH for 30 minutes, the residue was filtered and washed in 25 mL boiling H_2SO_4. The residue was placed on a pre-weighed ashtray ("W1", g). After cooling in a desiccator and drying at 130° C for 2 hours, the residue was weighed ("W2", g).

It was burned at 600°C for 30 minutes, cooled in a desiccator, and reweighed ("W3", g). (Maduka *et al.*, 2020; Mishra *et al.*, 2016)

Calculation: Loss in weight = (W2 - W1) - (W3 - W1)

2.5. Extract Preparation

The coarse powder of the leaves of the *T. erecta* plant was defatted with petroleum ether. After that, the defatted plant extract was macerated for 7 days in MeOH and thereafter hydroethanolic solvent (80:20). It was then filtered and concentrated to obtain a dried powdered form of MeOH extract of *T. erecta* (TEME) and hydroalcoholic extract of *T. erecta* (TEHE).

2.6. Preliminary Phytochemical Analysis

The bioactivity of herbal constituents was determined by the phytoconstituent present in it. The petroleum ether, methanolic, and hydroalcoholic extract of *T. erecta* leaves were screened to ascertain the presence of phytoconstituents by using different chemical tests as per standard procedures (Mandal *et al.*, 2013).

2.6.1. The qualitative and quantitative examination of phytoconstituents

T. erecta plant extracts were screened for primary and secondary metabolites such as carbohydrates, proteins, total polyphenols, flavonoids, and alkaloids.

2.6.1.1. Total carbohydrate content. Total carbohydrate content was estimated using the phenol sulfuric acid method by using *β*-D-glucose as standard. 0.1 g of plant sample was hydrolyzed with 5 mL of 2.5 N HCl in a water bath. After cooling to room temperature, the solution was neutralized by adding Na_2CO_3 till the effervescence ceased. The solution was filtered, and the volume was made up to 100 mL with distilled water. Various concentrations of working standard glucose were taken (0.2, 0.4, 0.6, 0.8, and 1 mL). Blank was set with all reagents except the sample. One milliliter of a 5 % phenol solution was added to each test tube. 5 mL of 96 % H_2SO_4 was added and thoroughly mixed, and the combination was maintained at 25-30 ^{0}C for 20 min. At 490 nm, the color change was measured (Jain *et al.*, 2017).

2.6.1.2. Quantification of total protein content. Bovin Serum Albumin working standard solution was prepared by adding BSA in distilled water. The dilutions (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg) were taken in triplicates. 5 mL of reagent mixture (50 mL of 2% $NaCO_3$ in 0.1 N NaOH + 1mL of 1% sodium potassium tartrate + 1mL of 0.5% copper sulphate) was pipetted to all test tubes and incubated for 10 min. 0.5 mL of Folin-Ciocalteu phenol reagent (5 mL of 2 N Folin-Ciocalteu + 6 mL of distilled water) was pipette to each test tube and mixed well immediately. The absorbance of the reacting mixture was taken at 660 nm and the amount of protein was estimated as BSAE/g of fresh weight (Daniel G. & Krishnakumari S., 2014).

2.6.1.3. Determination of total polyphenols. The estimation of total polyphenols of *T. erecta* leaf extract was carried out by using the Folin-Ciocalteu method. Gallic acid was used as standard in a concentration range (50-500 µg/mL). 500 µL of Folin-Ciocalteu reagent (10 %) was mixed with 100 µL of plant extract (1 mg/mL) and incubated in the dark for 20 min. After adding 2 mL of 20 % w/v sodium carbonate and 60 min incubation, the absorbance was taken at 765 nm by UV spectrophotometer. All the determinations were performed in triplicate. The TPC was expressed in mg gallic acid equivalent (GAE)/g dry extract with reference to gallic acid as a standard (Das *et al.*, 2018; Hayat *et al.*, 2020; Kaur *et al.*, 2015).

2.6.1.4. Determination of total flavonoid content. The estimation of total Flavonoid content was done as per the method described by (Hayat *et al.*, 2020). The aluminium chloride method was performed for the estimation of flavonoids. Plant extract (500 µL) and varied concentrations (10-100 µg/mL) of standard (Rutin) were mixed with 1500 µL of methanol, 100 µL of aluminium chloride solution, 100 µL of potassium acetate, and 2800 µL of distilled water. It was held at room temperature for 30 minutes. At 510 nm, the absorbance was measured against methanol as a blank. Using rutin as a standard, the total content of flavonoid compounds was estimated using the equation below (Hayat *et al.*, 2020).

$$C= (c \times V) / m$$

Where C: Total flavonoid content of compounds

c: concentration of rutin

V: volume of extract in mL

m: weight of crude plant extract

2.6.1.5. Determination of alkaloids. Different extracts of the *T. erecta* plant were dissolved in 2 N HCl and filtered. 1 mL of filtrate which was treated thrice with 10 mL of chloroform in a separating funnel. The neutral pH was adjusted by using 0.1 N NaOH solution. 5 mL of BCG (0.1 M) solution and 5 mL of phosphate buffer (pH 4.7) were added. The mixture was shaken thoroughly and was extracted with 1, 2, 3, and 4 mL of chloroform in a 10 mL volumetric flask, and volume was adjusted with chloroform. The absorbance of the formed complex was taken at 470 nm by a UV spectrophotometer. Total alkaloid content was measured from the atropine calibration curve concentration range of 10 to 100 µg/mL and expressed as µg of atropine equivalent/g of dry weight. All determinations were performed in triplicates (Darwish *et al.,* 2021; Das *et al*., 2018; Sulekha Rani & Priti, 2019).

2.7. Antioxidant Assays

2.7.1. *DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay*

The free radical scavenging activity of different extracts of the *T. erecta* plant was determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay described by Jeremiah Oshiomame Unuofin *et al.*, Jelita Rahma Hidayati with slight modification (Hidayati *et al.,* 2020; Unuofin *et al.,* 2017).

The different concentrations of ascorbic acid standard solution (10-500 µg/mL) and test were prepared by diluting the stock solution of DPPH 0.04 mg/mL in methanol. 1 mL of DPPH standard solution was mixed with 3 mL of extracts and kept at room temperature for 30 min. Absorbance was measured at 517 nm. The percentage scavenging of DPPH was measured at different concentrations of extract and standard using the following equation.

$$\text{Inhibition percentage} = (\text{Absorbance of DPPH} - \text{Absorbance of DPPH + Extract}) / \text{Absorbance of DPPH} \times 100\ \%$$

The radical scavenging/inhibition activity of *T. erecta* plant extracts was expressed as an inhibition percentage of DPPH. The inhibition percentage data were plotted to construct the linear regression equation and determine the IC_{50} value. All measurements were performed in triplicate (Khorasani Esmaeili *et al.,* 2015).

2.7.2. *Hydrogen peroxide radical scavenging activity*

The approach described by Atere *et al.*, (2018) was used to evaluate hydrogen peroxide radical scavenging activity. A 1 mL of plant extract at different concentrations (0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL) was combined with 2 mL of hydrogen peroxide solution (20 mM) prepared in 0.1 M phosphate buffer (pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against a blank that contained extracts in sodium phosphate buffer without hydrogen peroxide. Ascorbic acid was used as a working standard (Atere *et al.,* 2018; Saeed *et al.,* 2012). The following formula was used to determine hydrogen peroxide's capacity to scavenge free radicals:

$$\%\ \text{Scavenging activity} = [1- (\text{Absorbance of test} / \text{Absorbance of control})] \times 100$$

2.7.3. *Ferric Reducing antioxidant power assay (assay of reductive potential)*

The potassium ferricyanide ferric chloride technique was used to calculate the ferric reducing antioxidant capacity. 1 mL of plant extract solution in various strengths was mixed with 2.5 mL

of phosphate buffer (0.2 M, pH 6.6) 2.5 and mL of 1% potassium ferricyanide solution was added to the aforesaid mixture, and the solution was incubated at 50 °C for 20 minutes. After completion of incubation 2.5 mL of 10 % (w/v) of trichloroacetic acid was added and the solution was centrifuged at 1000 rpm for 10 minutes. To the 2.5 mL of supernatant solution, 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride solution were added. It was mixed thoroughly, and the bluish color complex formed was read at 700 nm against blank with reference to standard (ascorbic acid) on a UV spectrophotometer. The reducing power of the samples was compared with the reference standard (Chan & Lim, 2006; Li *et al.*, 2020; Rahim *et al.*, 2017).

2.7.4. *Assessment of total antioxidant potential by Phosphomolybdenum method*

The TACs of various extracts of the *T. erecta* plant were calculated using the phosphomolybdenum method as per the method described by Maswada (2013). 1 mL of phosphomolybdenum reagent (4 mM ammonium molybdate, 0.6 M sulfuric acid, and 28 mM Sodium phosphate) was added to 1 mL of plant extract. The reaction solution was incubated in a hot water bath for 90 minutes. The absorbances were recorded at 695 nm by UV spectrophotometer using methanol as a blank. TAC was expressed as equivalents (GAE)/g dry extract with reference to ascorbic acid as a standard. The standard calibration curve was generated by mixing ascorbic acid (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, and 400 µg/mL) with methanol (Doraiswamy & Saminathan, 2020; Maswada, 2013; Moonmun *et al.*, 2017).

2.8. Statistical Analysis

All the experiments were carried out in threes. The data were analyzed in Microsoft Excel and are presented as mean ± standard deviation (n = 3). GraphPad Prism 7 and Sigma Plot 15 for Windows 10 were used to calculate the IC_{50} values.

3. RESULTS and DISCUSSION

3.1. Pharmacognostic Study

Shade-dried leaves of the *T. erecta* plant were evaluated for organoleptic, microscopic, and physical characteristics. The organoleptic characteristics of the *T. erecta* plant are given in Table 1.

Table 1. Organoleptic characters of *T. erecta* leaves.

Organoleptic Characters	Descriptions
Color	Dark green
Odor	No Fragrance
Texture	Glossy
Taste	Bitter
Shape	ovate leaves with entire
Size	Less than 5 cm

3.2. Physicochemical Studies

The ash values, extractive values, moisture content, crude fiber content, and foaming index of dried powdered leaves were investigated. The information congregated from the previous studies is presented in Table 2. The physicochemical analysis performed in this study will help to identify plant adulteration with other species.

Table 2. Physicochemical parameters.

Sr. No.	Parameter	% Values (w/w)
A.	Ash Value	
1	Total ash value	13.7
2	Acid insoluble ash value	3.96
3	Water soluble ash value	7.92
4	Sulphated ash value	18.0
B.	Extractive value	
5	Petroleum ether	2.70
6	Chloroform	4.60
7	Acetone	3.40
8	Methanol	7.30
9	Ethanol	4.20
10	Hydroalcoholic extract (50:50)	28.2
11	Hydroalcoholic extract (70:30)	29.3
12	Hydroalcoholic extract (80:20)	31.5
C.	Moisture content	5.64
D.	Crude fibre content	27.3
E.	Foaming index	166.67

(% w/w = Percent weight by weight)

3.3. Extraction Yield

The extraction yields of extracts of the *T. erecta* plant were determined for petroleum ether, methanolic, and hydroalcoholic extract. The extraction yield was calculated by applying the equation of the weight of the extract to the dry plant's weight. The hydroalcoholic extracts of leaves exhibited a higher yield (22.18 %w/w) followed by methanolic extract (21.65 % w/w). Petroleum ether extract shows the lowest yield (8.82 % w/w).

3.4. Phytochemical Analysis of Leaf Extracts of *T. erecta*

3.4.1 Qualitative analysis (phytochemical screening)

The phytochemical analysis of the extracts was assessed based on a well-established method reported by the literature. (Khandelwal, 2008) The results of the phytochemical study are presented in Table 3. Phytocompounds are highly present in the methanol extract rather than in the hydroalcoholic and petroleum extracts. Among all the phytocompounds alkaloids, flavonoids, polyphenols, saponin, steroids, and tannin show higher concentrations in the methanol extract. The hydroalcoholic extract shows the presence of amino acids, carbohydrates, glycosides, proteins, polyphenols, and tannins.

Table 3. Phytochemical screening of *T.erecta.*

Sr. No.	Phytoconstituents	Petroleum ether extract	Methanolic extract	Hydroalcoholic extract
1	Carbohydrates	-	-	+
2	Amino acids	-	-	+
3	Proteins	-	-	+
4	Tannins	-	-	+
5	Flavonoids	-	+	-
6	Terpenoids	-	+	-
7	Triterpenoids	-	+	-
8	Alkaloids	-	+	-
9	Saponin	-	+	-
10	Polyphenols	-	+	+
11	Glycosides	-	-	+
12	Fatty components	+	-	-
13	sterols	-	+	-

(-) = Negative test; (+) = Positive test

3.4.2. Quantitative analysis of phytochemicals

The carbohydrate and protein content (mg/g of extract) was found to be high in the hydroalcoholic extract followed by the methanolic extract at 560.50 ± 0.0151 and 155.167 ± 0.001, respectively. The plant had significant levels of both carbohydrates and proteins (Figure 1). Phenolic compounds are secondary metabolites that have been extensively investigated in the medicinal plant leaf extract. Phenolic compounds are associated with color, sensory qualities, and nutritional and antioxidant properties. The total phenolic contents of different extracts of *T. erecta* leaves were evaluated using the Folin-Ciocalteu method. The content of phenolic compounds in petroleum ether, methanolic, and hydroalcoholic extracts ranged from 5.174 ± 0.0246, 70.857 ± 0.0469, and 164.492 ± 0.122 GAE/g of extracts, respectively. The hydroalcoholic extract was shown to have a high total polyphenol concentration (Figure 2).

The total flavonoid content was estimated using spectrophotometry and findings were expressed in mg of Ru/g. The highest flavonoid concentration was noted in methanolic extract (Table 4). The number of total alkaloids in plant extracts was calculated and expressed as mg of AE/g of extract based on atropine equivalents. The highest alkaloid concentration was measured in methanol extract at 141.416 ± 0.001 mg/g (Table 4) (Figure 3).

Table 4. Phytoconstituent estimation (mg/g GAE) of various extracts of leaves of *T. erecta.*

Phytoconstituents	Petroleum ether extract	Methanolic extract	Hydroalcoholic extract
Carbohydrates	121.167 ± 0.0287	269.670 ± 0.0035	560.500 ± 0.0151
Proteins	21.667 ± 0.0014	98.583 ± 0.0021	155.167 ± 0.0010
Total polyphenols	5.174 ± 0.0246	70.857 ± 0.0469	164.492 ± 0.1220
Alkaloids	35.291 ± 0.0025	141.416 ± 0.0010	82.625 ± 0.0009
Flavonoids	215.833 ± 0.0233	577.500 ± 0.0272	498.166 ± 0.1070

The values represent the means of three measurements ± standard deviation.

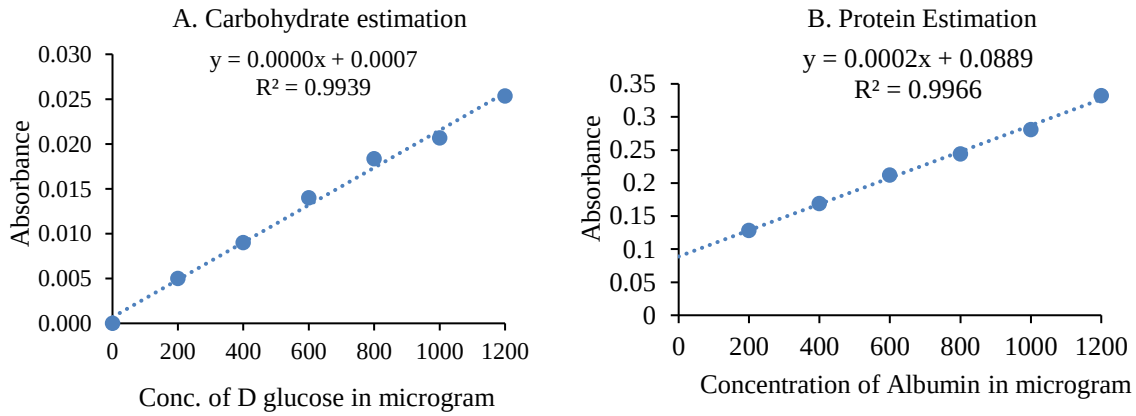

Figure 1. Calibration curve of D-glucose and albumin for primary metabolite A. carbohydrate and B. protein estimation.

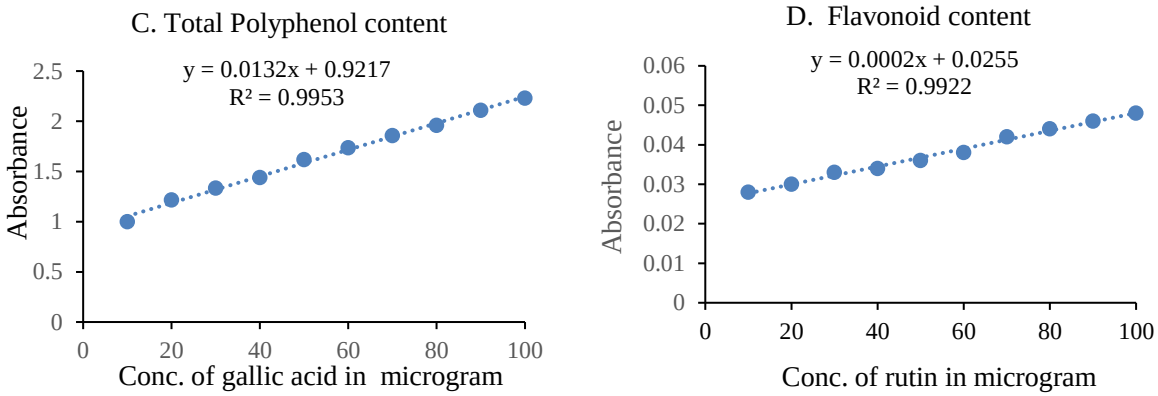

Figure 2. Calibration curve of gallic acid and rutin for C. total polyphenol and D. flavonoid estimation.

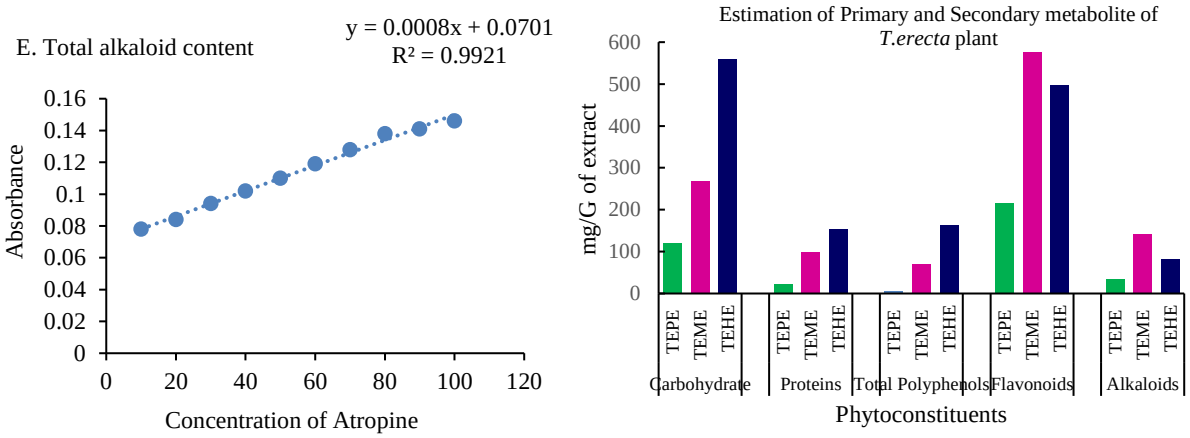

Figure 3. Calibration curve of Atropine for E. Total alkaloid estimation, and Primary and Secondary metabolite estimation of different extracts of *T. erecta*. Results are expressed as the mean ± standard deviation.

3.5. Antioxidant Activity

3.5.1. DPPH free scavenging assay

Figure 4 depicts the effects of *T. erecta* extract on the DPPH free radical in the following order: methanolic extract > hydroalcoholic extract > petroleum ether extract (Table 5). Although the study revealed that the antioxidant potential of the extract was lower than that of ascorbic acid ($p < 0.05$), it also revealed that methanol and hydroalcoholic extracts had a significant amount of antioxidant activity. A substantial amount of polyphenols and flavonoids found in the methanolic extract of plants could be attributed to the observed high antiradical properties of these fractions.

3.5.2. H_2O_2 scavenging activity

The various *T. erecta* plant extracts were evaluated and shown in Figure 4. The ability to scavenge free radicals increases with concentration in both hydroalcoholic and methanolic extracts. Both extracts showed the highest levels of radical scavenging activity, as measured by the IC_{50} value. All data were compared with the IC_{50} value of standard ascorbic acid as shown in Table 5.

3.5.3. Phosphomolybdate assay

Standard ascorbic acid equivalents were used to evaluate antioxidant activity using the phosphomolybdate technique. The methanol extract of *T. erecta* was discovered to have higher antioxidant potential (Table 5). The results demonstrated dose-dependent antioxidant activity

at doses of 10 to 100 g/mL. The methanol extract has a higher IC_{50} value for antioxidant capability than hydroalcoholic and petroleum ether extracts. Strong antioxidants are present in this fraction, which may be due to the presence of phenolic and flavonoid components, as evidenced by the methanol extract's strong antioxidant activity being statistically similar to that of ascorbic acid (Figure 5).

3.5.4. Ferric Reducing antioxidant power assay

In this study, the reducing capacity of the extracts and fractions was performed using Fe^{3+} to Fe^{2+} reduction assay. The flavonoids and phenolics compounds were present in considerable amounts in the extract of plant *T. erecta*. The reducing capacity is shown by all extracts in a concentration-dependent manner (Figure 6).

Table 5. Correlation coefficients ($R^{2)}$ for antioxidant activity relationship of different extracts of *T. erecta*.

Extract/Standard	*DPPH free radical scavenging activity IC_{50} (µg/mL)*		*Phosphomolybdate method*	*H_2O_2 scavenging activity IC_{50} (µg/mL)*	
	IC_{50}	R^2	mg/g	IC_{50}	R^2
Pet. ether	232.98	0.9628	25.12 ± 0.017	131.0	0.9977
Methanolic	44.134	0.9913	111.10 ± 0.047	89.75	0.9954
Hydroalcoholic	81.6885	0.9774	96.158 ± 0.017	99.40	0.9991
Ascorbic acid	33.7115	0.9968	--	62.17	0.9993

Results of triplicate tests, each value represents mean ± SD (n=3).

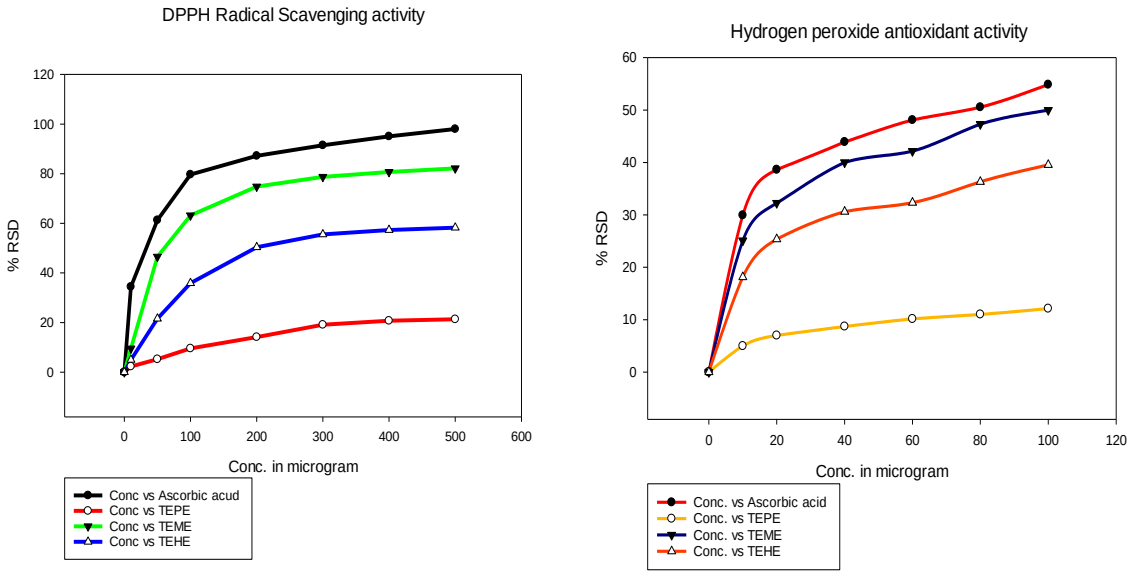

Figure 4. DPPH and H_2O_2 radical scavenging activity of ascorbic acid and leaves extract of *T. erecta* plant. Results were triplicate, each value representing mean ± SD (n=3).

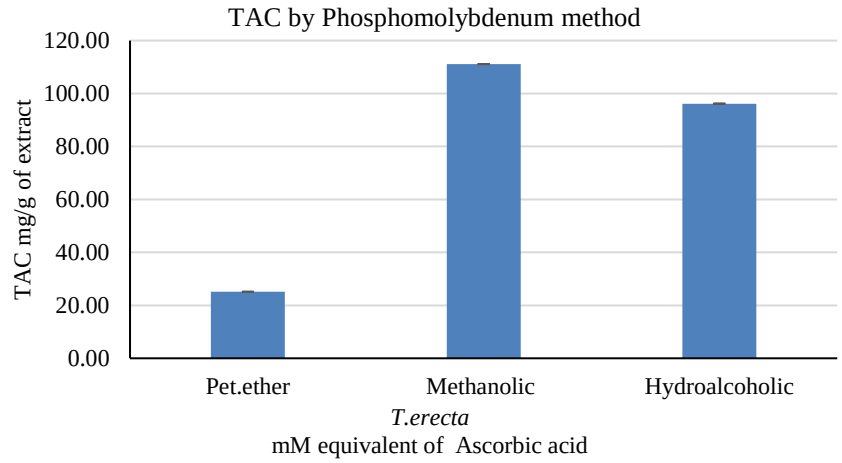

Figure 5. Phosphomolybdate assay of leaves extract of *T. erecta* plant. Results were triplicate, each value represent mean ± SD (n=3).

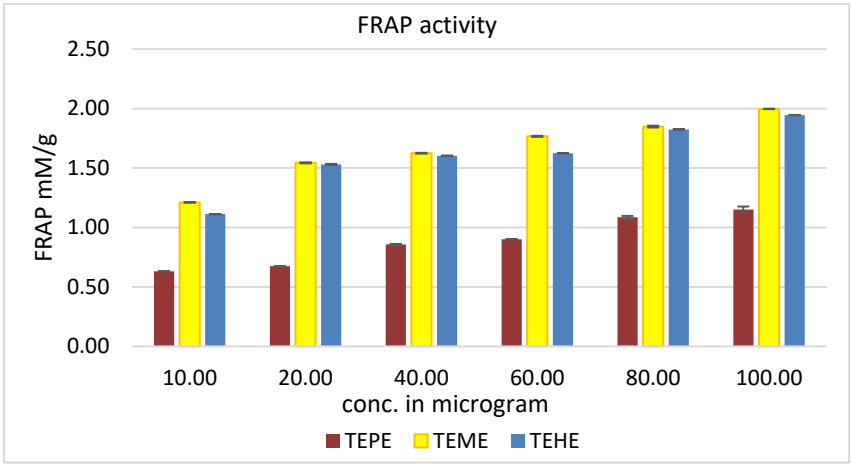

Figure 6. Ferric Reducing antioxidant power assay of leaf extract of *T. erecta* plant. Results were triplicate, each value represent mean ± SD (n=3). Values are mean ± S.D., n = 3, values indicate no significant difference at a level of 5% ($p < 0.05$).

4. CONCLUSION

This study aimed to screen various bioactive compounds for the presence of primary and secondary metabolites and to assess their antioxidant activity. *T. erecta* species is rich in carbohydrates proteins, alkaloids, flavonoids, and polyphenols. The present investigation showed that methanol extract shows a high percentage of flavonoids, polyphenols, and alkaloids whereas the hydroalcoholic extract shows the presence of carbohydrates, proteins, flavonoids, and total polyphenols. The radical scavenging activity showed that the extracts possess potential antioxidant activity when evaluated through various methods. The *in vitro* assays demonstrate that the plant extracts could be a valuable source of an intrinsic antioxidant, which may help in preventing the development of diverse oxidative stress-related diseases. These results support the notion that a diet rich in herbs and plants can aid in the possible reduction of free radicals and could act as a defense against associated disorders. The results of these studies provide scientific development of natural bioactives that have the potential to act as antioxidants for various diseases of the human body.

Further research to isolate individual compounds and their *in vivo* antioxidant activities with different mechanisms is needed. Further studies investigating the isolation and identification of the causative antioxidant components and their mechanisms of action are needed to better understand their ability to combat diseases that significantly influence quality of life.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Nitin Kochar: Methodology, Supervision, Validation, statistical analysis, and manuscript review. **Jayshree C. Vyas:** Formal Analysis, Writing - original draft, experimental studies, manuscript review. **Khushbu Vyas:** Data Analysis, Writing - original draft, experimental studies, manuscript review. **Anil Chandewar:** Definition of intellectual content, data analysis, manuscript review. **Dharmendra Mundhada:** Definition of intellectual content, data analysis, manuscript review.

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Research Article

Phytochemical study and biological activities of *Teucrium mideltense* (Batt.) Humbert.

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Abstract: *Teucrium mideltense* (Batt.) Humbert (*T. mideltense*) is an endemic Moroccan species that grows exclusively in the Oriental High Atlas Mountains of Morocco. In this work, we aim at determining the chemical profile and biological properties of the traditionally used aqueous extract of this plant. HPLC analysis, estimation of the amounts of total phenolic compounds including flavonoids, and in vitro antioxidant activity was evaluated according to the literature procedures (DPPH, ABTS, and FRAP). Additionally, safety assessment was carried out according to the organization for economic cooperation and development guidelines and the anti-hyperlipidemic activity was evaluated in triton-induced hyperlipidemic rat model. Our findings revealed that the aqueous extract of this plant contains significant amounts of phenolic compounds (91.94 mg GAE/gE) including flavonoids (27.41 mg RE/gE). HPLC analysis revealed the presence of vanillic acid, hesperidin, and rutin. Moreover, a considerable in vitro antioxidant effect was evaluated (DPPH IC_{50} = 36.10± 0.02 µg/mL; ABTS IC_{50} = 34.98± 1.31 µg/mL; FRAP EC_{50} = 129.74±2.18 µg/mL). Furthermore, *T. mideltense* extract exerted significant lipid-lowering effects by reducing the levels of total cholesterol (-88.78%), triglycerides (-62.12%), and non-HDL cholesterol (-68.37%). We conclude that the supplementation with the aqueous extract of *T. mideltense* would be effective in lowering lipids under hyperlipidemic conditions.

1. INTRODUCTION

Oxidative stress is a state developed by an imbalance between the formation of free radicals such as reactive oxygen species (ROS) and antioxidant status in the body. This phenomenon has been associated with various health conditions including neurodegenerative and cardiovascular disorders, diabetes mellitus, and numerous other pathological conditions (Sies,

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2020). These consequences highlight the importance of achieving a harmony between the levels of ROS and antioxidant defense system. Cells have intricate biochemical and genetic processes in place to sustain this equilibrium, and it is evident that any disruption to it can result in significant pathophysiological effects (Hayes *et al*., 2020).

Hyperlipidemia is a significant risk factor for cardiovascular diseases. It is characterized by increased levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C), accompanied by reduced levels of high-density lipoprotein cholesterol (HDL-C) in the blood (Hill and Bordoni, 2021). It would be crucial to lower lipid levels in hyperlipidemic people in order to prevent cardiovascular disease and its complications. In the treatment of hyperlipidemia, statins and fibrates are couple of lipid-lowering drugs (Ali *et al*., 2021). Nevertheless, a number of studies have shown that using herbal remedies as an alternative treatment for hyperlipidemia can be effective (El-Tantawy & Temraz, 2019; Gong *et al*., 2020). Indeed, supplementation with the extracts of several medicinal plants have shown promising lipid-lowering effects (Elbouny *et al*., 2022a; Ramchoun *et al*., 2020).

One of the promising genera used in phytomedicine is *Teucrium* (Sadeghi *et al*., 2022). In fact, *Teucrium* L. is a diverse and polymorphic genus of the Lamiaceae family with approximately 434 recognized taxa (Navarro, 2020). The Mediterranean region is the primary distribution area for *Teucrium* species, accounting for over 90% of the total species found worldwide (Blanca *et al*., 2017), in which the main represented areas are Morocco and the lberian Peninsula (Navarro, 2020). In Morocco, there are 57 recognized taxa, comprising 53 species categorized into 8 sections (Fennane *et al.*, 2007). In the traditional medicine of this country, *Teucrium* species are widely used to treat several disorders like diabetes, cardiovascular disease, liver, kidney problems, etc. (El Atki *et al*., 2019; El-Gharbaoui *et al*., 2017). Moreover, recent studies have reported that the species of this genus exert several biological effects including anti-inflammatory (Elbouny *et al*., 2023a), antidiabetic (Asghari *et al*., 2020), antibacterial properties etc. (El Atki *et al*., 2020).

Teucrium mideltense (Batt.) Humbert (*T. mideltense*) is an endemic Moroccan species. This plant occupies a significant part of the Oriental High Atlas, from Midelt-Ayachi (northern limit of its distribution) to the gorges of Oued Dades and Todgha (southern limit of its distribution). It grows between 1500 and 2500 m (El Oualldi et al., 1996; Fennane *et al*., 2007) According to El Oualidi *et al.*, (1996) this species belongs to the *Polium* section subsection *Polium*. Its chemical composition presents an exception related to this section which is the absence of poliumoside the main chemical marker of this section. As far as we know, there are no published reports on the biological activities of this plant. Thus, we aim to reveal the chemical profile and the biological properties of *T. mideltense* aqueous extract (TMAE).

2. MATERIAL and METHODS

2.1. Animals

In this study, we used male *Albino Wistar* rats (250–300 g). Animals were maintained in controlled conditions. Experiments were carried out according to the guidelines of the pharmacological research committee, FSTE, Moulay Ismail University (AREC-FSTE-12/2020).

2.2. Plant and Extraction

T. mideltense aerial parts were harvested in the central High Atlas Mountains, southern Morocco. The botanical identification was carried out in the National Institute of Agronomic Research of Errachidia. A voucher specimen was deposited in the herbarium of FSTE (TM HerbFST # 68) . The aerial parts of the plant were dried for 14 days. Then, 10 g of powdered material was boiled in 1000 mL for 10 min. Next, the solution was filtered and water was eliminated in a ventilated oven (40 °C). The dry extract was collected and put at 4 °C until use.

For *in vitro* and *in vivo* assays, the extract was prepared in distilled water and the appropriate concentrations were prepared based on our preliminary testing.

2.3. High Performance Liquid Chromatography (HPLC) Analysis

T. mideltense extract was analyzed for its chemical profile using the HPLC technique. The analysis was performed using KNAUER apparatus with a column (Eurospher II 100-5 C18. 250 × 4.6 mm) protected by Agilent technologies RP-18 (10 mm × 4.6 mm) pre-column. Columns were placed in an oven set at 25 °C. Formic acid 0.1% (Eluent A) and methanol (Eluent B) were used with a constant flow rate of 1 mL/min. Ten microliters of extract dissolved in methanol were injected. The phenolic compounds were characterized according to their UV–Vis diode array detector at 280 nm spectrum and they were identified by comparing their retention time (RT) values with those of standards. The used standards were gallic acid, vanillic acid, pyrogallic acid, caffeic acid, ferulic acid, cinnamic acid, sinapic acid, chlorogenic acid, hesperidin, rutin, naringin, catechin, epicathechin, quercetin, and kaempferol.

2.4. Determination of Total Phenolic and Flavonoid Contents

2.4.1. *Total phenolic content (TPC)*

Total phenolic content of *T. mideltense* water extract was determined by Folin-Ciocalteu method described by (Singleton & Rossi, 1965). The concentration of total polyphenols, expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g extract), was determined from the gallic acid calibration curve equation.

2.4.2. *Total flavonoids content*

Total Flavonoids of *T. mideltense* water extract was quantified according to Aluminum chloride complex-forming method as described in our previous study (Elbouny *et al*., 2023b). The total amount of flavonoids was expressed as milligram equivalent of quercetin per gram of each extract (mg QE/ g extract) based on the calibration curve of quercetin.

2.5. Antioxidant Activity

2.5.1. *DPPH free radical scavenging activity*

To investigate the radical scavenging potential of *T. mideltense* extract, 2,2-diphenyl-1-picrylhydrazil (DPPH) scavenging activity was tested as described in our previous study (Elbouny *et al*., 2022b). Quercetin (0.38 to 6.09 mg/mL) was used as the standard antioxidant. The scavenging ability of the extracts was calculated as:

$$\%\ \text{Inhibition of DPPH} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.5.2. *ABTS free radical scavenging activity*

The ABTS radical scavenging activity was performed adhering to the method of Pukalskas *et al*. (Pukalskas *et al*., 2002). The absorbance was read at 734 nm and the percentage inhibition was calculated as described earlier for the DPPH test.

2.5.3. *Ferric-reducing antioxidant power test (FRAP)*

The reducing activity of the *T. mideltense* water extract was measured spectrophotometrically with the Oyaizu method (Oyaizu, 1986). Catechin (0.65 - 21.39 µg/mL) was used as a positive control.

2.6. Acute Toxicity

The Organization for Economic Cooperation and Development's OECD N° 423 criteria were used to evaluate the acute oral toxicity of the aqueous extract of *T. mideltense* (OECD, 2002). Three non-pregnant and nulliparous female rats (230 - 250 g) were fasted for 4 hours with free access to water. Then, 2000 mg/kg of TMAE was administered orally. The normal control

group (n=3) was given water orally. Rats were monitored for 30 minutes and two weeks following dosing. Clinical symptoms as well as variations in body weight and mortality were noted throughout this period.

2.7. Anti-Hyperlipidemic Activity

Hyperlipidemia was developed in rats using triton model (Elbouny *et al*., 2023c). This agent was dissolved in saline (200 mg/mL) and administered intraperitoneally to animals (200 mg/kg). Treatments were given orally by gavage after 30 minutes.

Animals were randomly divided into five groups (n=5). The first group (Normal) received an intraperitoneal injection of saline and gavaged with distilled water, the second group (Triton) was injected with triton and gavaged with distilled water, the third group was injected with triton and received 10 mg/kg of simvastatin (Simv 10) which is used as a reference drug, the third (T200) and fourth (T400) groups were injected with triton and received 200 or 400 mg/kg of *T. mideltense* aqueous extract.

After 24 hours, animals had received triton injection, and then were anesthetized; blood was collected from the retro-orbital sinus using heparinized capillaries and plasma samples were obtained by centrifuging the blood at 5000 rpm for 5 minutes. The levels of TGs, TC, and HDL-C in the plasma were determined using enzymatic kits. Non-HDL-C value was determined using the following formula: Non-HDL-C = TC – HDL-C.

2.8. Statistical Analysis

Results were expressed as means ± standard deviation. Data were analyzed using one-way ANOVA test followed by post hoc analysis (Tukey's test). The difference at p value less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Acute Toxicity

The aqueous extract was administered at a dose of 2 g/kg bw, and the findings revealed no clinical harm. Each and every one of the animals involved in the experiment lived after 14 days of observation. Additionally, they continued to act normally, suggesting that the fatal dose (LD50) is greater than 2 g/kg bw. The body weights of the animals did not vary significantly ($p< 0.05$) over the course of the 14-day toxicity assessment (Figure 1). Therefore, based on these findings and the OECD 423 recommendations, we may state that the single dose (2 g/kg bw) of the aqueous extract of *T. mideltense* can be regarded as non-toxic.

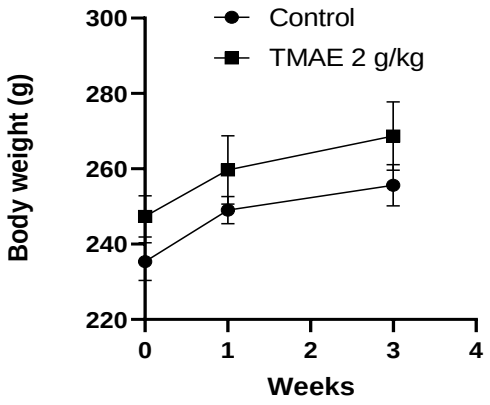

Figure 1. Comparison of weight change between the control group and TMAE treated group.

3.2. Chemical Composition

The result of HPLC analysis is shown in Figure 2. The obtained chromatogram shows that nine compounds were detected in which 3 of them namely vanillic acid, hesperidin, and rutin were identified.

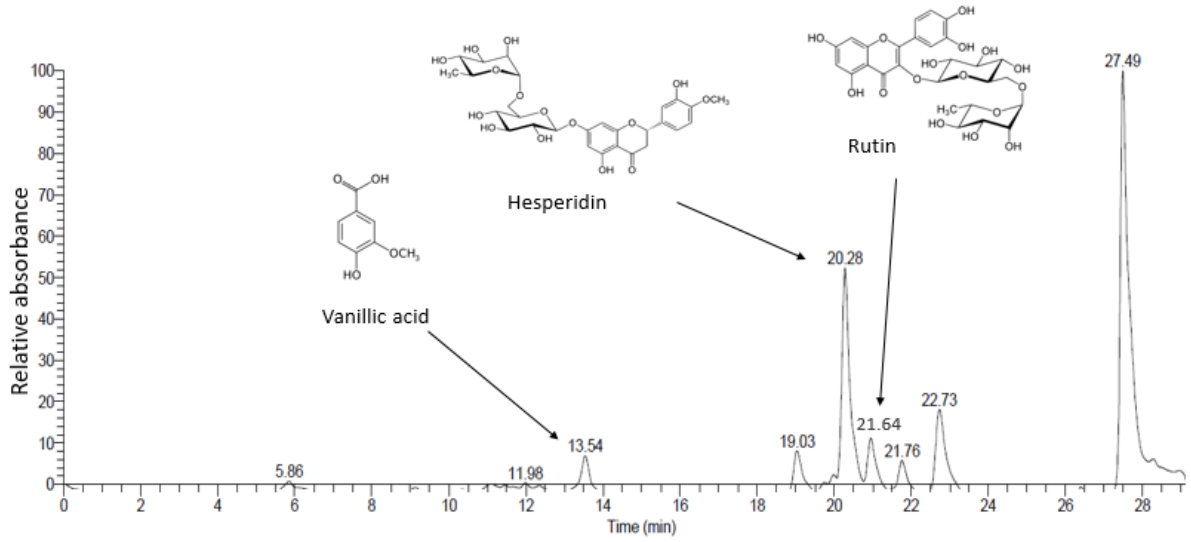

Figure 2. HPLC chromatogram and identified compounds.

3.3. Total Phenolic Content and Antioxidant Activity

The results of total phenolic content and antioxidant activity of TMAE are shown in Table 1. The extract was found to be rich in total phenolic compounds (91.94 mg GAE/gE) including flavonoids (27.41 mg RE/gE). Moreover, the extract revealed considerable antioxidant activity by scavenging DPPH and ABTS radicals and reducing ferric iron. The highest antioxidant capacity is demonstrated against DPPH and ABTS with IC_{50} values of 36.10± 0.02 µg/mL and 34.98± 1.31 µg/mL respectively. However, the antioxidant potential of standards was much higher than that of extract.

Table 1. Total phenolic content and antioxidant activity.

Extract and standards	TPC (mg GAE/gE)	TFC (mg QE/gE)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP EC_{50} (µg/mL)
TMAE	91.94±2.46	27.41±0.37	36.10±0.02	34.98±1.31	129.74±2.18
Ascorbic Acid	-	-	-	2.52±0.02	-
Quercetin	-	-	5.49±0.02	-	-
Catechin	-	-	-	-	13.90±0.03

Values are represented as mean of 3 replicates (± SD). (-): not applicable

3.4. Anti-Hyperlipidemic Activity

The results of anti-hyperlipidemic activity are represented in Figure 3. Triton injection remarkably increased blood lipids when compared to normal group (****: $p<0.0001$). However, TMAE at both doses exerted significant lipid-lowering effects. Indeed, at 400 mg/kg, TMAE lowered the levels of TC (-88.78%), TGs (-62.12%), and non-HDL-C (-68.37%) when compared to the hyperlipidemic group ($^{++++}$: $p<0.0001$). Interestingly, the lipid-lowering effect of *T. mideltense* extract was comparable to that of simvastatin.

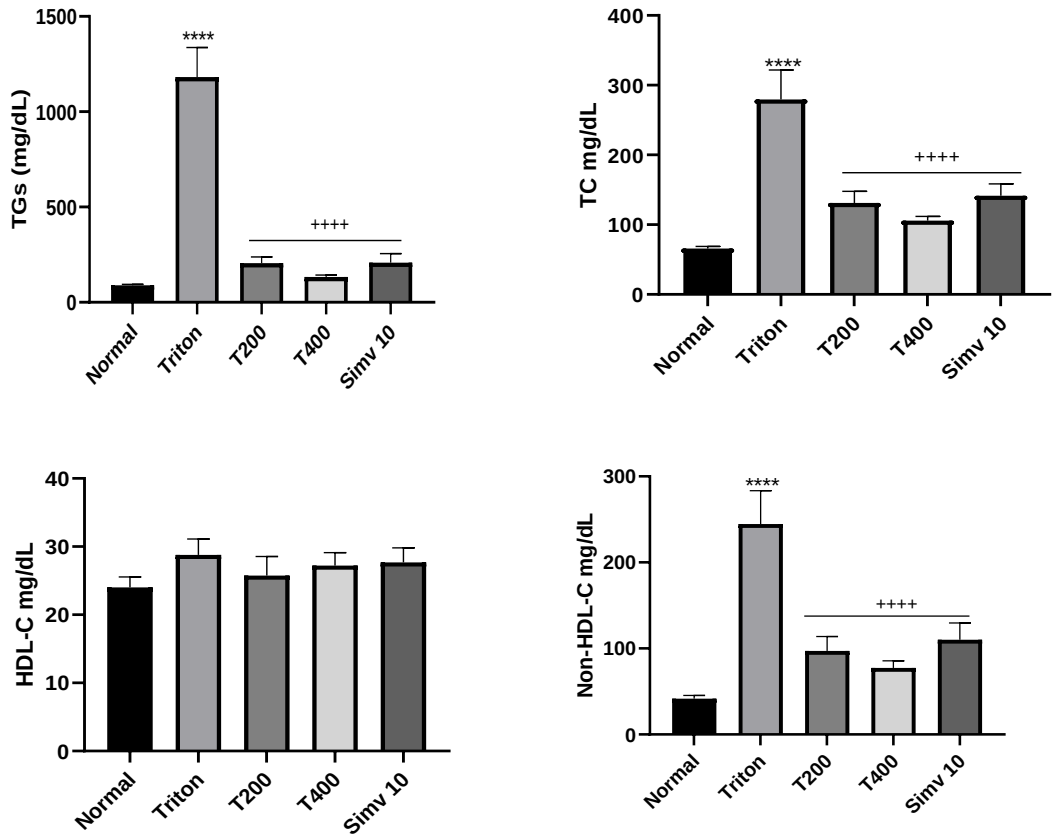

Figure 3. Lipid profile parameters of animals in different groups. Values are expressed as means ± SD. ****: $p<0.0001$ compared to the normal group. + + + +: $p<0.0001$ compared to the hyperlipidemic group according to the Tukey test ($p<0.05$). TC: total cholesterol; TGs: triglycerides; HDL-C: high density lipoprotein cholesterol.

4. DISCUSSION and CONCLUSION

Medicinal plants have been employed for centuries in traditional medicine systems and continue to be utilized in modern healthcare (Michel *et al*., 2020). These plants possess numerous bioactive phytochemicals that exhibit therapeutic properties and can help alleviate symptoms or even cure certain disorders (Ferreira *et al.*, 2014; Shayganni *et al*., 2016). Our findings revealed that TMAE is rich in phenolic compounds including vanillic acid, hesperidin, and rutin in its chemical composition. These compounds were previously reported to exist in the extracts of several *Teucrium* species. For example, vanillic acid ($501.1 \pm 0.6 \times 10^{-4}$ µg/mL extract) was the major compound found in the extract of *T. scordium* from Serbia (Djordjevic *et al.*, 2018). Hesperidin was the most abundant phenolic compound in the ethanolic extract of *T. parviflorum* from Türkiye (5687 ± 159.24 µg/g). Additionally, rutin was the compound found in the largest amount (14.311 ppm) in the methanolic extract of *T. semrae* from Türkiye (Albayrak & Aksoy, 2023).

According to the findings of this study, *T. mideltense* extract has the ability to reduce ferric levels and scavenge free radicals. Interestingly, the radical scavenging effects were more important than the ferric reducing potential, which suggests that TMAE is a stronger proton donor than electron donor. Considering the radical scavenging activity, when compared to other Macedonian *Teucrium* plants namely *T. polium* and *T. chamaedrys*, and *T. montanum* (IC_{50} = 10 - 70 mg/mL), TMAE exibited a high DPPH radical scavenging potential (Tatijana *et al*., 2005). Comparable effect was noted with the aqueous extract of Moroccan *T. polium* (IC_{50} = 0.61 mg/mL) (El Atki *et al*., 2019). On the other hand, other *Teucrium* species such as *T.*

hyrcanicum from Iran (IC_{50} = 0.074 mg/mL) (Golfakhrabadi *et al*., 2015) and *T. chamaedrys* from Romania (IC_{50} = 0.038 ± µg/mL) (Zlatić *et al*., 2017) exerted higher antioxidant potential. Thus, the obtained data revealed that the aqueous extract of *T. mideltense* is rich in phenolic compounds and exerts a considerable antioxidant effect.

We found that the administration of the aqueous extract of *T. mideltense* was able to attenuate triton-induced hyperlipidemia. This effect can be attributed to the presence of the previously mentioned bioactive phenolic compounds in the composition of this plant extract. Indeed, several studies have reported that these phenolic components exert remarkable lipid-lowering effects. For example, high-fat diet (HFD)-fed Sprague-Dawley rats were treated with vanillic acid (30 mg/kg) for 4 months (Chang *et al*., 2015). The results of this study indicate that this phenolic acid alleviated HFD-induced hyperlipidemia through the regulation of lipid metabolism-related proteins and inflammation pathways. Moreover, hesperidin was tested on HFD-induced hyperlipidemic C57BL/6J male mice for 16 months (Li *et al*., 2022). The results revealed that the uptake of hesperidin (0.2%) reduced body and liver weights, improved serum lipid profiles, and attenuated liver dysfunction. The effects obtained were modulated by the regulation of hepatic metabolism and gut bacteria. The hepatoprotective effect of hesperidin against lipids was reported in another scientific investigation (Chen *et al*., 2022). This study revealed that hesperidin significantly decreased in liver damage and blood lipid levels, while providing protection against steatosis in HFD fed-mice *in vivo*. Hesperidin also prevented the *in vitro* accumulation of fats brought on by oleic acid in HepG2 cells. These effects were modulated through the up-regulation of the activity of phosphorylated adenosine monophosphate activated kinase and the reduction of the down-regulation of the expression of sterol regulatory element-binding protein 1c, acetyl coenzyme-A carboxylase, and fatty acid synthase. Moreover, rutin was also reported in several studies to exert significant lipid-lowering effects in animals fed a HFD through the modulation of inflammation pathways (Gao *et al*., 2013), gut microbiota (Peng et al., 2020; Yan *et al*., 2022), and other cellular and molecular targets (Panchal *et al*., 2011; Seo *et al*., 2015). Furthermore, other *Teucrium* plants were reported to exert significant lipid-lowering effects including *T. polium* L. from Iran (Safaeian *et al*., 2018), *T. takoumitense* from Morocco (El-Guourrami *et al*., 2023), and *T. leucocladumon* from Palestine (Bassalat *et al*., 2020). According to our findings and the literature data, the proper administration of *T. mideltense* aqueous extract would be effective in lowering lipids under hyperlipidemic state.

In summary, *T. mideltense* extract was found to be rich in phenolic compounds including flavonoids and exerted considerable antioxidant potential. Moreover, significant lipid-lowering activity was noted for this plant in triton-induced hyperlipidemic rats. The chemical composition of this species contains vanillic acid, hesperidin, and rutin, which could be responsible for these biological effects. However, further studies should be conducted to examine the anti-hyperlipidemic activity of the extract of this plant using chronic experimental models. Additionally, it would be important to identify the mechanisms of action of its lipid-lowering effect.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Hamza Elbouny: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Brahim Ouahzizi** and **Kaoutar Benrahou**: Investigation, Resources. **Abdelmonaim Homrani Bakali**: Investigation, Resources, Visualization, Methodology. **Mohamed Bammou** and **Khalid Sellam**: Visualization, Editing the original draft. **Chakib Alem**: Methodology, Supervision, and Validation.

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Research Article

Metabolite fingerprinting and profiling of two locally cultivated edible plants by using nuclear magnetic resonance

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Abstract: Nuclear magnetic resonance (NMR) spectroscopy is a sensitive technique used to analyse the structure, dynamics, reaction state, and chemical environment of molecules. *Abelmoschus esculentus* and *Lagenaria siceraria* are edible plants used traditionally to treat jaundice, diabetes, weight loss, ulcer, hypertension, heart failure, skin diseases and reduced cholesterol. Therefore, based on the medicinal uses the study was designed to analyze fingerprinting of metabolites of the seeds of the selected plants. The dry seeds were powdered and the metabolites were extracted by socking method with a mixture of methanol/chloroform. The extracted metabolites from seeds were subjected to proton NMR using the noesygpprld pulse sequence. A total 18 peaks were obtained from each spectrum. Among the peaks, three peaks with the highest intensities were analyzed by utilizing NMR. The peak metabolites were determined with the correlation with the correct peak using in built Biological Magnetic Resonance Data Bank (BMRB). The results showed that the obtained data varied from known plant metabolites due to the contamination and interaction between the metabolites. In addition, variants in the metabolites from sample to sample may have been the result of errors or limitations in the study. The data generated from this experiment will be used to help to conduct the advanced research in the near future on the selected edible plant species which will be valuable for many different areas of the scientific community. Plant metabolomics has the potential benefit in the medical field, agricultural industry, and many other areas of our economy.

1. INTRODUCTION

Metabolism is an active set of lifesaving chemicals or chemical reactions in body organisms. The focus of metabolism is the energy conversion from food, to run the circulation systems, to block various secondary metabolic such as proteins, fats, several acids, and polyphenolic ingredients, and eliminate body wastes. All these enzymatic reactions permit an organism to develop and preserve structures, to reproduce and retort against the environment. Metabolism is defined as the total chemical reactions happen in an active organism such as digestion and transportation of substances. Nuclear magnetic resonance (NMR) spectroscopy is a sensitive

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analytical tool that is used for determining the structure of organic materials, physical, chemical and biological properties of matter. Nowadays, several analytical techniques are widely used for drug development, drug discovery, as well as to screen metabolites in various organisms such as bacteria, animals, plants, algae, and cancer cell lines.

Among the analytical techniques, NMR (^{1}H- NMR and ^{13}C-NMR) is one of the sensitive analytical techniques to understand the composition of organisms, drugs-metabolic interaction, drugs-drugs interaction etc. (Sarkar, 2008). Therefore, researchers are using the most advanced NMR analytical techniques to solve the complex problems related to plant and animal metabolism. Recently, the researchers are using overlap NMR data technique with built-in library data to identify various aspects such as 3D structure of the molecule, movement of molecules, and interaction with its environment (Berg *et al*., 2002).

One of the best ways to identify and/or quantify metabolites present in organisms is by using NMR spectroscopy. Metabolites are considered as an intermediate product of metabolic reactions catalyzed by various enzymes that naturally occur within cells (Renneber & Loroch, 2016; Dhanjai *et al*., 2018). Generally, there are two types of metabolites: primary and secondary. Primary metabolites are synthesized by the cell because they are vital including amino acids, alcohols, vitamins, and more. Secondary metabolites are made by the organism, but they are not necessary for primary metabolic activities, although they play important roles in several areas including cell survival, signaling, and defense against predators or the environment. In addition, secondary metabolites also serve as a better tool to distinguish between cellular responses of different species which vary from organism to organism (Roessner & Bowne, 2009). The presence or the concentration of some metabolites may be increased or decreased based on stressors in the environment. The collective study of metabolites, primary and secondary is known as metabolomics. Specifically, metabolomics is defined as the comprehensive analysis of metabolites in a biological specimen (Clish, 2015). This approach has helped us understand what drives significant biological activities in the organism being observed.

Both selected plants are edible. *A. esculentus* belongs to Malvaceae family while *L. siceraria* belongs to the Cucurbitaceae family. They are locally known as okra and bottle gourd and their English names are lady`s finger and calabash long squash. Both plants grow throughout the Asian countries and in some of the EU countries, Australia and the USA. The literature review showed that both selected plants are originated in the South Asian, Ethiopian and West African region. However, they spread throughout the world including Greenland. Both fruits are rich sources of active constituents, nutritional values, minerals, fibers, etc. Based on the above nutritive and bioactive constituents these plants are commercially cultivated dible fruit/seed pods throughout the world. Okra is an annual plant that typically grows 3-5' tall (Figure 1). Bottle gourd is also an annual plant that grows up to 12-16 feet long with large, hairy, broad-ovate, entire to shallowly-lobed, dark green leaves (Figure 1). The okra strains from each country have developed characteristics determined by the climates of the location. The fruits of the plant are rich in vitamins, calcium, potassium and other mineral matters. The mature okra seed is a good source of oil and protein (Kumar, *et al*., 2013). Specifically, it contains unsaturated fatty acids such as linoleic acid (András *et al*., 2005). This fatty acid, which comprises almost fifty percent of okra oils, is vital for human nutrition. The fiber that comes from the mature fruit and stem can also be utilized in the paper industry (Kochhar, 1986). The chemical composition of okra has been thoroughly researched. A multicellular fiber found in okra known as okra bast is the target of many studies. The composition of this fiber is identified as 67.5% a-cellulose, 15.4% hemicelluloses, 7.1% lignin, 3.4% pectic matter, 3.9% fatty and waxy matter and 2.7% aqueous extract (Kumar *et al*., 2013).

Figure 1. Typical picture of Okra and Bottle guard.

Other nutritional values present in okra are carbohydrates, sugars, dietary fibers, fat, protein, water, vitamin A, thiamine (B1), riboflavin (B2), niacin (B3), vitamin, vitamin E, vitamin K, calcium, iron, magnesium, potassium, and zinc. The main constituents of the fiber are a-cellulose, hemicelluloses, and lignin. However, detailed metabolite profiling has not been conducted. Okra seeds are known to contain high amounts of oil (20-40%), which makes it an excellent candidate for essential oilseed (Benchasri, 2012). There are important amino acids in the seeds such as lysine and tryptophan (Kumar *et al*., 2016). Lysine is an essential amino acid whereas tryptophan is important for normal functions of proteins, muscles, enzymes, and also for neurotransmitters production and maintenance (Jenkins *et al*., 2016). The okra seeds also contain oligomeric catechins and flavonol derivatives (Persson *et al*., 2001). Other constituents include hydroxycinnamic and quercetin derivatives, phenolic compounds with important biological properties similar to quartering derivatives, and catechin oligomers, glycol-proteins, procyanidin B2 and B1 (both phenolic compounds), pectin, and rutin (Arapitsas, 2008). Green vegetables are known to contain valuable chlorophyll. Chlorophyllin, a phenolic compound and important component of chlorophyll was studied for its health benefits. These include being an antioxidant, anti-inflammatory and anti-microbial (Mendoza-Núñez *et al*., 2019). Each of these constituents are not only nutritious to humans, but they also play a vital role in the plant's life. There are many properties of okra that are the targets for medicinal uses. These uses include gastro-intestinal aid, endocrine activity, immunoprotective properties, psychological alleviators, and even properties regarding liver detox (Gemede *et al*., 2015). The most notable medicinal properties that have been targeted by researchers are its cardiovascular and endocrine effects. In regard to heart health, consuming okra has shown to assist with reducing cholesterol due to its fiber and the metabolite pectin content (Ngoc *et al*., 2008).

Similarly, phytoconstituents of *Lagenaria siceraria* include carbohydrates such as glucose and fructose, starch, curd fiber, hemi cellulose, cellulose and lagenin. Usually, bitter fruits yield solid foam containing cucurbitacins B, D, G and H, mainly cucurbitacin B. This component is

seen in members of the pumpkin and gourd families. These bitter principles are present in the fruit as aglycones. *Lagenaria siceraria* shows presence of flavone-C glycoside, a well-known antioxidant and anti-tumor agent (Rahman, 2003). *Lagenaria siceraria* seeds contain a wide array of vitamins, minerals, lipids, amino acids, and many other components. These include calcium, magnesium, phosphorous, potassium, vitamin C, niacin and other B vitamins, folate, vitamin A, fatty acids, glutamic acid, aspartic acid, arginine, leucine as well as other amino acids (Roopan *et al*., 2016). Overall, this vegetable provides many important nutrients for human health.

Due to the plethora of nutrients available in bottle gourd, it is used all over the world in traditional and natural medicines. Medicinal properties that have been the targets of research include enzymatic activities of lipase, amylase, and pectinase in the digestive system. Another area is the antimicrobial activity. Antidiabetic activity was also observed. Patients who consumed the juice of bottle gourds showed a marked reduction in cholesterol and blood glucose (Katare *et al*., 2013). Cardioprotective (Adedapo *et al*., 2013), antioxidant (Satvir *et al*., 2012), and anti-inflammatory activities have been observed by monitoring various heart measurements while using bottle gourd powder (Shirsat & Kadam, 2015). There are many more medicinal and pharmaceutical properties of bottle gourd such as anticancer (Thakkar, 2013), analgesic and genetic properties (Roopan *et al*., 2015). Both plants we chose have excellent nutritional values as well as helpful medicinal properties. Understanding their active constituents and important metabolites of the chosen plants will give us new insights into their unique composition. The literature search showed that there is not a single report available on the metabolic identification and profiling of fruits of the selected plants by NMR. Therefore, the objectives of this present study are the isolate the metabolics from the seeds of the selected plants and identify them by using NMR spectroscopy.

2. MATERIAL and METHODS

2.1. Chemicals and Glassware

The solvents were used in this experiment such as methanol, chloroform, and deionized water obtained from E. Merck, Germany. Sodium phosphate buffer and deuterated water were collected from BDH, UK. Other chemicals and solvents were analytical grade. The purities of the chemicals and solvents were above 95%. In addition, all glassware used in this present experiment was purchased from Boroshil, India.

2.2. Instruments

In this experiment, the prepared samples were analyzed by using NMR spectroscopy (Brucker) and centrifuge machine (Model, brand, Country) was used to separate the metabolic from the leaves and seeds of the selected plant species. NMR spectra were noted on a Bruker (400 MHz) instrument in deuterated chloroform with tetramethylsilane as an internal standard (chemical shifts δ, ppm).

2.3. Sample Collection

The seeds of *A. esculentus* and *L. siceraria* were collected locally on (date) during the day time. Both the samples were collected at three different time and places. The collected samples were stored in a plastic bag at room temperature to protect the metabolic change. After collection of the samples the morphological identification was completed based on wikipedia websites (https://en.wikipedia.org/wiki/Okra and https://en.wikipedia.org/wiki/Calabash) and confirmed by local people. Both collected samples were washed with distilled or deionized water. Then, the samples were ground into paste by mortar and pestle. The paste samples were kept in the aluminum foil at ambient temperature for extraction.

2.4. Extraction of Metabolites

Each sample (0.5 g) was taken in a 100 ml conical flask and added a mixture of aqueous solvent (methanol-water: 4:1.25). The samples mixture was vigorously shaken by using a shaker for few minutes. Then chloroform (4 ml) and water (2 ml) were added to the conical flask and shaken by vortexed for 1 minute. The whole mixture was kept in ice bath for 10 minutes. After incubation, the sample was centrifuged at 5000 rpm for 5 minutes. The sample had two layers and the upper layer was transferred to another tube and dried the mixture solvent completely by using a vacuum centrifuge. Finally, the dried extract sample was dissolved with 250 µL 0.1 M sodium phosphate buffer (pH 7.0) in 90% H_2O and 10% D_2O (Wu *et al*., 2008) (Figure 2).

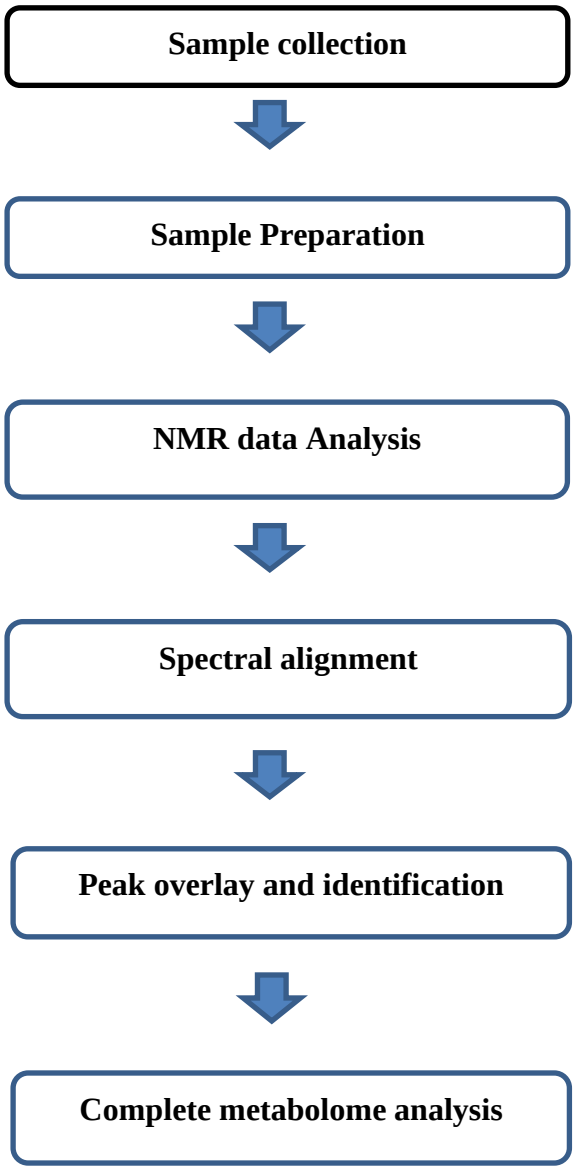


Figure 2. Essential steps involved in NMR based metabolomics studies.

2.5. Analysis of The samples by NMR

The extracted metabolites from okra and bottle guard were analyzed by using NMR with the noesygppr1d pulse sequence. The signals are elucidated by pulses of radiofrequency irradiation and can be related to the contents within the sample (Bligny & Douce, 2001). Utilizing the NMR pulse sequence ensures that all non-exchangeable protons can be detected in a deuterated

buffer solution (Stryeck *et al*., 2018). Deuterated buffers allow for the solvent not to interfere with the sample because the resonance frequency of a deuteron (^{2}H) is vastly different from that of proton (^{1}H). This difference in frequency means there would be no peaks from the solvent in the NMR spectrum. Locking and shimming are also terms used to describe the importance of buffers. Locking refers to keeping the magnetic field stable eliminating the effect of drifting and shimming is to ensure that there is a homogeneous magnetic field over the whole of the sample volume located within the probe's detection coil. If the shimming fails, there will be a distorted line shape leading to poor resolution and decreased sensitivity. Shimming utilizes the coils (shims) around the sample. Applying electrical currents to the coils can make up for any discrepancies in the field homogeneity (Minocha, 2015).

The raw NMR data underwent spectral processing. This process refers to programs in the spectrometer than can be pre-programmed to manipulate certain variables depending on what information we wanted to gather. Along with spectral processing, data processing is also taking place. It is also a part of the pre-processing step. It involves the use of parameters and variables to ensure the data collected is what is desired in the research experiment (Emwas *et al*., 2018).

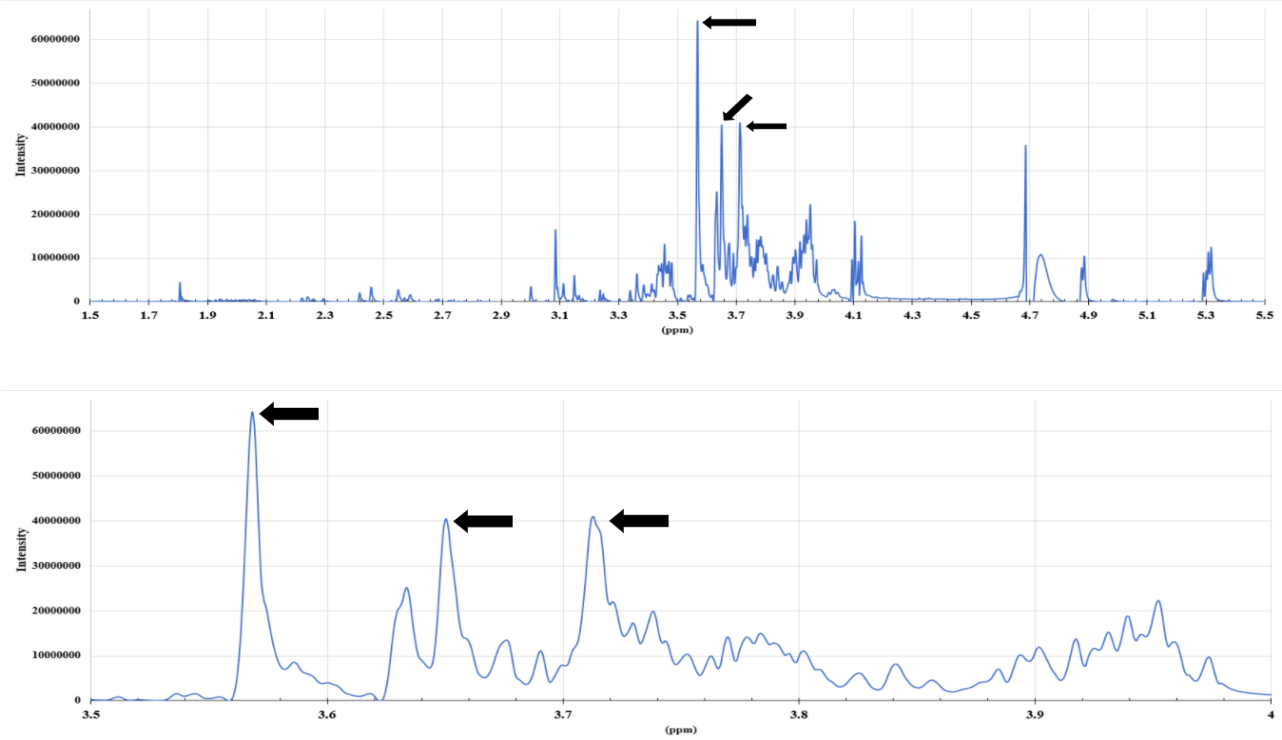
Following the collection of this data from the NMR spectrometer, we then began to analyze the data. The NMR spectroscopy data analysis of the selected plant seeds can be viewed in two different formats that are presented in the result section. The first three sets of samples derived from the *A. esculentus* (okra) and the following three sets of samples are from the *L. siceraria* (bottle gourd). For each of the six samples there are graphs containing the NMR spectroscopy results for the specific sample. The peaks in the spectrums are analyzing and interpreting results of NMR in detail by using the Biological Magnetic Resonance Data Bank (BMRB) and presented in results and discussion section. Based on the BMRM databank as well as previously reported data, it can conclude that which metabolite as a peak is represented in the sample. In addition, based on the BMRB databank and plant metabolomic research, we were able to identify the peaks and discuss their contributions to the plants` inner workings and in near future other areas of the scientific community.

3. RESULTS and DISCUSSION

The people use the fruits of okra and bottle gourd as vegetables as they contain rich sources of bioactive constituents, nutritional values, minerals, fibers etc that can fulfill our body requirements for survival. The previous reports showed that both fruits of the selected plants have significant biological and pharmacological activities due to their active metabolites. All these bioactive compounds play a significant role to protect and treat different ailments. Based on the medicinal benefits, the present work was undertaken to isolate and identify metabolites from the seeds of the selected by using NMR spectroscopy.

3.1. ^{1}H-NMR Spectra of Okra Sample 1

From the spectrum, it showed that several peaks appeared within the range of δ 1.5 to 5.5 ppm. Among the peaks, three peaks at δ 3.5684, 3.6504 and 3.7127 were the major peaks with the highest intensities in the Okra sample 1 as it is presented in Figures 3 and 4.



Figures 3. Proton NMR spectra from okra, *Abelmoschus esculentus* sample one. Figure 4 is an enlarged view of Figure 3. Analyzed peaks are indicated by arrows. X-axis represents the chemical shift (parts per million). Y-axis shows the signal intensity, represented in arbitrary units. The intensity of the signal is proportional to the number of hydrogens that make the signal.

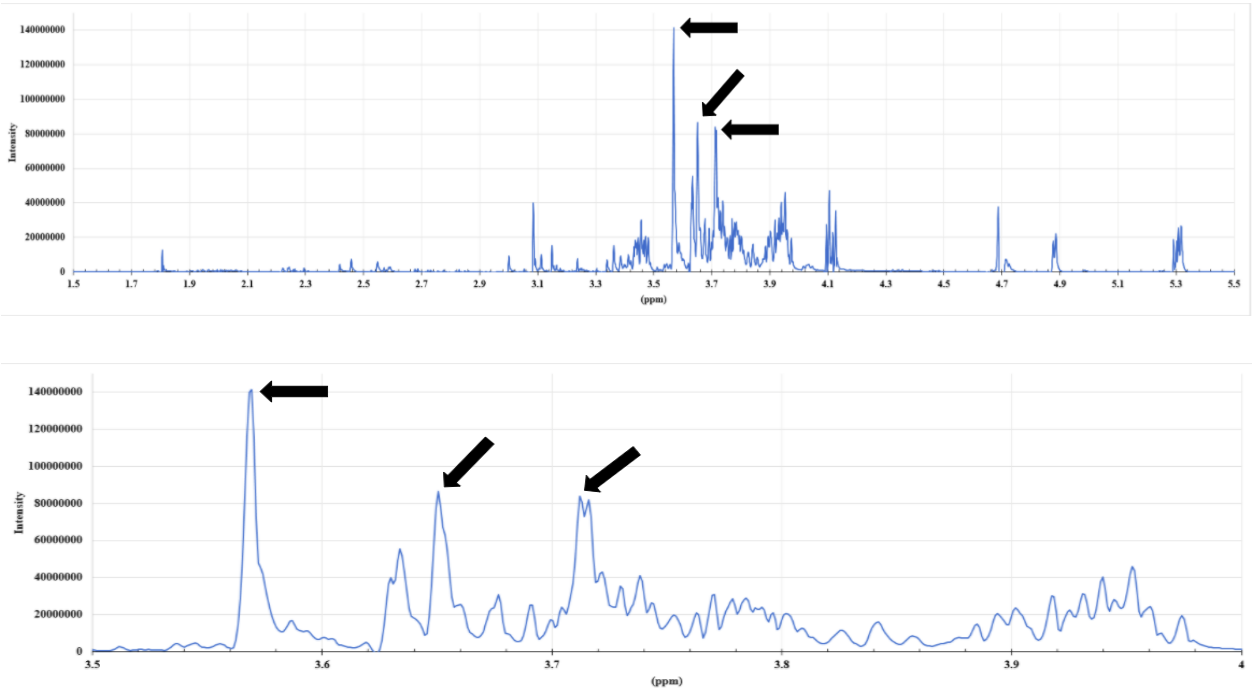


Figure 4. Proton NMR spectra from okra, *Abelmoschus esculentus* sample two. Figure 6 is an enlarged view of Figure 5. Analyzed peaks are indicated by arrows. X-axis represents the chemical shift (parts per million). Y-axis shows the signal intensity, represented in arbitrary units. The intensity of the signal is proportional to the number of hydrogens that make the signal.

The displayed peak at δ 3.5684 ppm had the possibility of being three different substances such as pentoxifylline, aleuritic acid, or H-b-S-OH based on the BMRB databank (Table 1). All of these substances found in Okra sample have been reported earlier by several authors. The bioactive metabolic pentoxifylline is a derivative of theobromine, a methylated xanthine and theobromine is a well-known metabolite that can be found in okra as well as chocolate, tea and cocoa products. Xanthine is a derivative of alkaloids and most closely related to methylated xanthines namely caffeine, theophylline and aminophylline. All of these alkaloid agents are used as a prescription drug to treat relax muscles, particularly bronchial muscles, to stimulate the central nervous system and the kidney to promote diuresis (Zhang, *et al*., 2004). Aleuritic acid is an organic compound derives from the resin. It is a major ingredient of shellac and mainly isolated from plant species. It is used as a starting reagent for manufacturing perfumes. It has also significant health benefits to protect human cell and cure injury (Heredia-Guerrero *et al*., 2017). Lastly, H-b-S-OH according to the BMRB database, falls into the category of b-O-4 dimers and 3-Carbon Sidechains. This compound can be found in the NMR Database of lignin and Cell Wall Model compounds. Usually, it is observed in the plant's cell wall (Biological Magnetic Resonance Data Bank, 2021). Due to the limited resources pertaining to the substance's presence in okra, *A. esculentus*, this peak assigned to compound H-b-S-OH.

Table 1. Summary of peaks and possible matches based on the BMRB databank for sample one of seeds from *A. esculentus*.

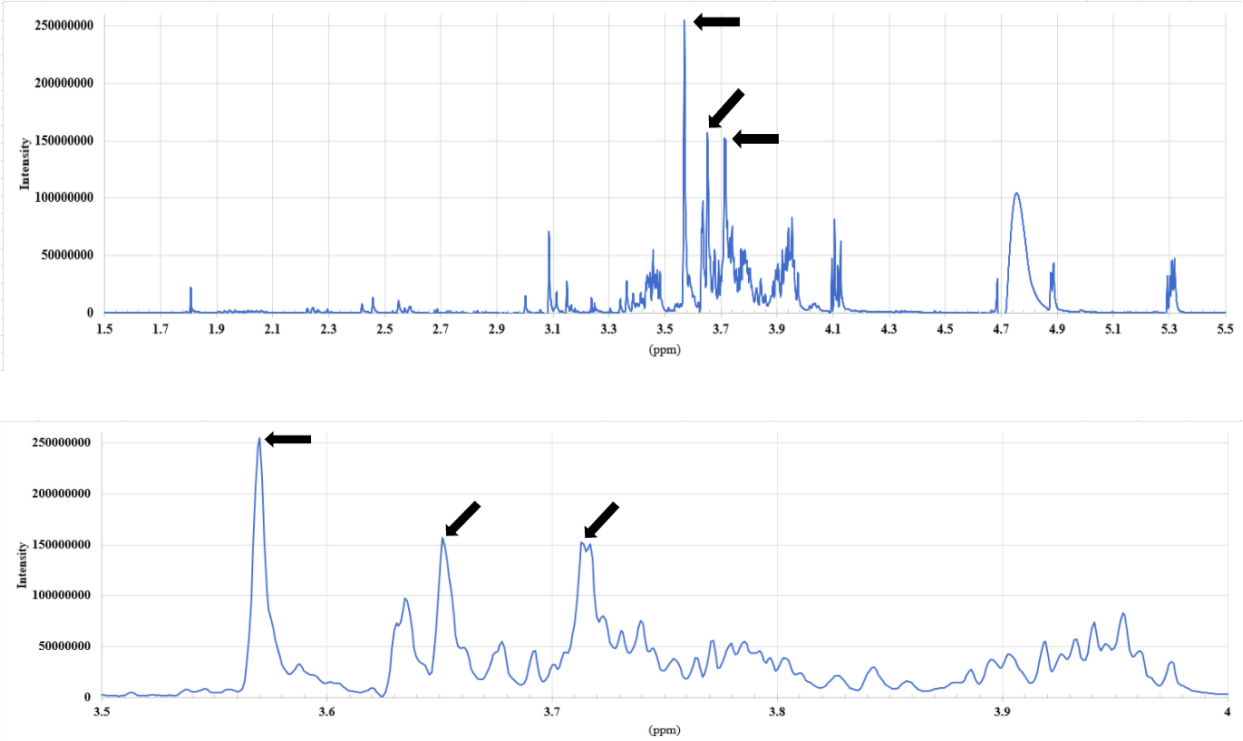
Chemical shift (ppm)	BMRB Databank Results	Predicted results
3.5684	1. Pentoxifylline 2. Aleuritic acid 3. H-b-S-OH	H-b-S-OH
3.6504	1. Lignin_cw_compound_3021 2. Lignin_cw_compound_2028 3. Lignin_cw_compound_2029 4. Lacto-N-fucopentaitolI	Lignin
3.7127	1. Schisandrin 2. L-(-)-Threitol	Lignin

The peak at δ 3.7127 ppm can be attributed to schisandrin, L-(-)-threitol, or lignin_cw_compound_3021 and it is naturally occurring compound found in *Schisandra chinensis*. All those metabolites have significant effects on the human body including hypertension and liver problems (Nasser *et al*., 2020). Recently, L-(-)-threitol has been observed in the edible fungus *Armillaria mellea,* Alaskan beetle, and *Upis ceramboides.* It serves as an antifreeze agent (Huang *et al*., 2015; Wong *et al*., 2019). Lignin_cw_compound_3021 is one of the main components of the cell wall. It acts as a polymer in the cell wall and it is important for many functions including transport, support, and stress resistance. (Liu, *et al*., 2018). Lignin's is a common chemical; therefore, the assigned peak is for lignin (Table 1).

On the other hand, the peak at δ 3.6504 ppm had two candidates, lignin and Lacto-N-fucopentaitolI. The chemical Lacto-N-fucopentaitolI is an oligosaccharide found in human milk. It makes up a very large part of human milk and assist with various functions including immunity (Sotgiu *et al*., 2006). Therefore, once again this peak is also for lignin. Lignin compound is very important for plant structure and cell wall function. Its high prevalence in plants is to be expected.

3.2. ^{1}H-NMR Spectra of Okra Sample 2

The NMR spectrum showed that most of the peaks within the range of δ 1.5 to 5.5 ppm (Figure 5). The peaks at δ 3.5688, 3.6504, and 3.7123 ppm are the major peaks in the okra sample 2 due to the peak intensity. Almost similar results were obtained from the okra sample 2 (Table 2).



Figures 5. Proton NMR spectra from okra, *Abelmoschus esculentus* sample three. Figure 5a is an enlarged view of Figure 5. Analyzed peaks are indicated by arrows. X-axis represents the chemical shift (parts per million). Y-axis shows the signal intensity, represented in arbitrary units. The intensity of the signal is proportional to the number of hydrogens that make the signal

Table 2. Summary of peaks and possible matches based on the BMRB databank for sample one of seeds from *A. esculentus*.

Chemical shift (ppm)	BMRB Databank Results	Predicted results
3.5684	4. Pentoxifylline	H-b-S-OH
	5. Aleuritic acid	
	6. H-b-S-OH	
3.6504	5. Lignin_cw_compound_2028	Lignin
	6. Lignin_cw_compound_2029	
	7. Lacto-N-fucopentaitolI	
3.7127	3. Schisandrin	Lignin
	4. L-(-)-Threitol	
	5. Lignin_cw_compound_3021	

3.3. ^{1}H-NMR Spectra of Okra Sample 3

The NMR spectrum showed most of the peaks within the range of δ 1.5 to 5.5 ppm (Figure 6). The peaks at 3.5699, 3.6516, and 3.7134 ppm are the major peaks in the Okra sample 2 due to the peak intensity. The Okra sample 3 is slightly different from sample 1 and 2 and the obtained results are presented Table 3.

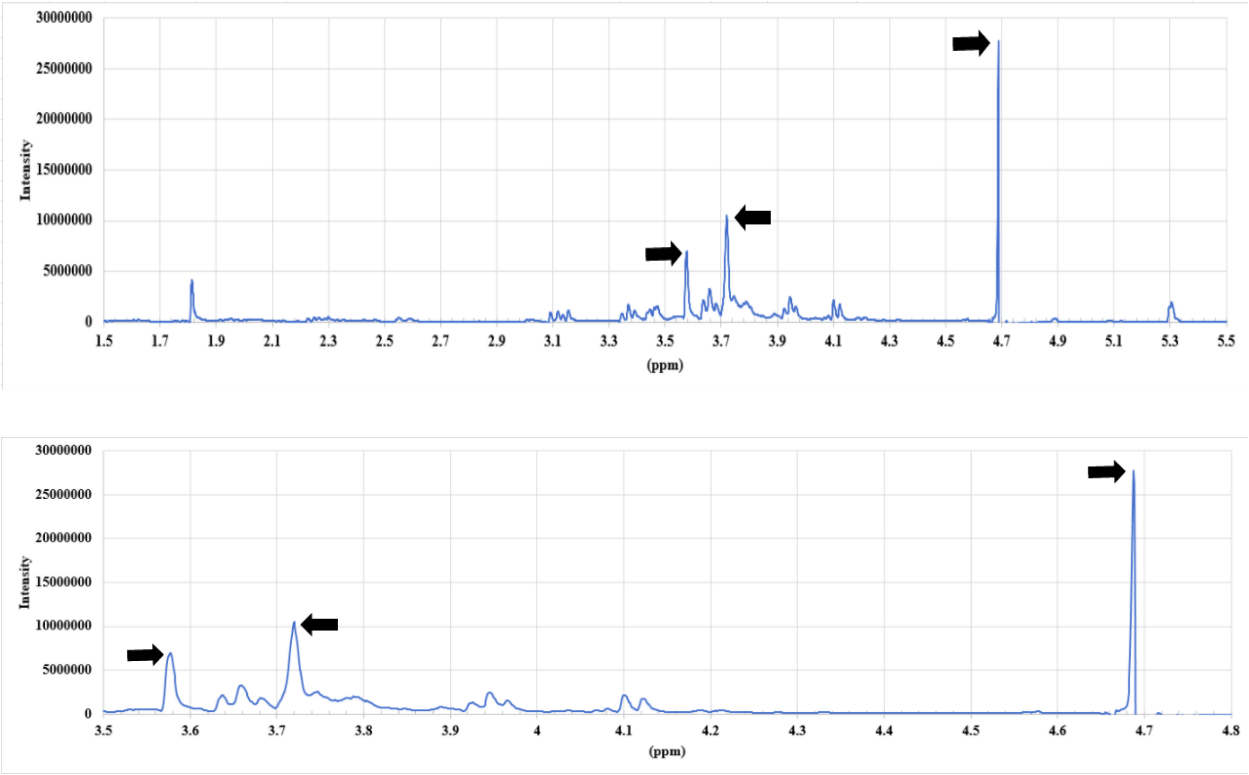


Figure 6. Proton NMR spectra from bottle gourd, *Lagenaria siceraria* sample one. Figure 6a is an enlarged view of Figure 6. Analyzed peaks are indicated by arrows. X-axis represents the chemical shift (parts per million). Y-axis shows signal intensity, represented in arbitrary units. The intensity of the signal is proportional to the number of hydrogens that make the signal.

Table 3. Summary of peaks and possible matches based on the BMRB databank for sample one of seeds from *A. esculentus*.

Chemical shift (ppm)	BMRB Databank Results	Predicted results
3.5684	7. Pentoxifylline 8. Aleuritic acid 9. H-b-S-OH	H-b-S-OH
3.6504	8. L-(-)-Threitol 9. Indole-3-acetic acid 10.lignin_cw_compound_2028	Indole-3-acetic acid
3.7127	6. Bis (2-butoxyethyl) phthalate 7. Rutin trihydrate 8. Schisandrin	Rutin trihydrate

Based on the BMRB databank, the peak at δ 3.6516 ppm indicates the presence of L-(-)-Threitol and lignin _cw_compound_2028 where two possibilities have already been discussed. Another new metabolite Indole-3-acetic acid observed is common naturally occurring plant hormone of the auxin class. Auxins are a class of plant hormones with some morphogen-like characteristics. They are important signaling molecules in the plant. Auxins play an important

role in coordination of growths and behavioral processes in plant life cycles and they are also important for plant body development. (Dhande *et al*., 2012; Woodward & Bartel, 2005). The most likely match for this peak is indole-3-acetic acid. This hormone is vital for plant growth and development.

The third peak in the sample 3 is at δ 3.7134 that indicates the presence of schisandrin that was already discussed. The other two metabolites are biologically important substances. First is bis(2-butoxyethyl) phthalate which is an organic compound derived from 2-butoxyethanol. It is a well-known plasticizer in PVC and PVA and works as an adhesive. It has rarely been found in plants (Cheriti, *et al*., 2006; Thiemann, 2021). The most well-known secondary metabolite is rutin trihydrate. Rutin trihydrate is an organic polyphenolic compound and it has several pharmacological activities, including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities. It is also natural product isolated from several plant species (Ganeshpurkar & Saluja, 2017; Tongjaroenbuangam et. al, 2011). Therefore, the third peak in the NMR spectrum is rutin trihydrate.

3.4. ^{1}H-NMR Spectra of Bottle Gourd Sample 1

In the NMR spectrum of bottle gourd sample 1 showed that many peaks that ranged from δ 1.5 to 5.5 ppm. Among the peaks, three peaks at δ 4.688, 3.7195, and 3.5768 ppm showed the major peaks with the highest intensities in the bottle guard sample 1 that are presented in Figures 9 and 10.

The BMRB databank, displayed that 4.688 ppm peak had the possibility of being three different substances. Betulin, quinidine, or 1-(4-acetoxy-3,5-dimethoxyphenyl)-1,3-diacetoxy-2-[4-(1-acetoxyethyl)phenoxy] propane. Betulin is a naturally occurring triterpene (Boparai, *et al*., 2017). It has a role as a metabolite, an antiviral agent, an analgesic, an anti-inflammatory agent and an antineoplastic agent (Hordyjewska, 2019). The activity of betulin acid has been linked to the induction of the intrinsic pathway of apoptosis and it is being explored for its anti-tumor properties (Fulda, 2008; Zdzisińska *et al*., 2003). The final candidate for this peak is 1-(4-acetoxy-3,5-dimethoxyphenyl)-1,3-diacetoxy-2-[4-(1-acetoxyethyl)phenoxy] propane. The BMRB states that this compound is a lignin. As a lignin, its main functions in the cell are to provide structure and adhesiveness in the wall (Ralph *et al*., 2004). Out of these three options, the best option for our sample is betulin.

Peak two of this sample is 3.7195 ppm. Possible matches for his sample include 2'-fucosyllactose, papaverine hydrochloride, or 6'-sialyllactose sodium salt. 2′-Fucosyllactose (2-FL) is the most abundant fucosylated oligosaccharide in human milk. It has pharmaceutical properties. Human milk oligosaccharides are important components of human milk that prevent infant health (Yu *et al*., 2018). Papaverine is an natural opiate alkaloid isolated from the plant and it is a vasodilator that relaxes smooth muscles in blood vessels to help them dilate. This effect lowers blood pressure and allows blood to flow more easily through your veins and arteries (National Center for Biotechnology Information, 2021; Prabhakara, *et al*., 2010). Lastly, 6'-sialyllactose sodium salt, also known as Neu5Ac-a-2-6-Gal-b1-4-Glc;6'-SL;6'-N-Acetylneuraminyl-D-lactose, is one of the most abundant sialylated (acidic) oligosaccharides found in milk observed in humans and other mammals. It has many immunoprotective properties and aims to protect newborns against pathogens. Based on the data collected, this peak is papaverine hydrochloride.

Another candidate for this peak is dihydroisorescinnamine. It is a naturally occurring Reserpine type alkaloid. A Reserpine is a compound of the alkaloid class used for treating hypertension. The reserpine type alkaloids are common secondary metabolites in gourd species. It is believed that alkaloid toxicity is what helps protect the diseases. (Kumar *et al*., 2012). The final possibility for this peak is lignin_cw_compound_3027. As stated in the above samples,

lignin is a prevalent metabolite thoughout the plant kingdom. However, based on the information the peak is for dihydroisorescinnamine.

3.5. ^{1}H-NMR Spectra of Bottle Gourd Sample 2 & 3

Proton NMR spectra of bottle gourd sample 2 & 3 are shown in Figures 11, and 12. Many peaks that ranged from chemical shift values 1.5 to 5.5 ppm were observed and focused on peaks of 4.695, 3.7213, and 3.5789 ppm because they have the highest intensities in sample two (Table 4). Almost similar metabolites were obtained from the samples 2 & 3.

Table 4. Summary of peaks and possible matches based on the BMRB databank for sample one of seeds from *L. siceraria*.

Chemical shift (ppm)	BMRB Databank Results	Predicted results
4.688	10. Betulin 11. Quinidine 12. 1-(4-acetoxy-3,5-dimethoxyphenyl)-1,3-diacetoxy-2-[4-(1-acetoxyethyl)phenoxy] propane	Betulin
3.7195	11. 2'-Fucosyllactose 12. Papaverine hydrochloride 13. 6'-Sialyllactose sodium salt	Papaverine hydrochloride
3.5768	1. Dihydroisorescinnamine 2. Lignin_cw_compound_3027 3. 3'-Sialyllactose Sodium Salt	Dihydroisorescinnamine

4. CONCLUSION

The benefits of understanding plant metabolomics and the inner workings of plants are vital for many industries. The metabolites that are identified often contribute to other areas of science including agriculture and nutrition, health and medicine, as well as production of biofuels and utilizing alternative energy sources. NMR spectroscopy may be used to assist the development of these areas by discovering new ways to identify and analyze plant metabolites. Our experimental results showed exciting results. In addition, the results are also identified areas of strengths and weaknesses in our study and plan on eliminating as many errors and limitations as possible. The future of plant metabolomics is the ability to identify and quantify the major metabolites of plants effectively and efficiently. The use of NMR spectroscopy is vital in this quest to further explore the world of plant metabolomics.

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Declaration of Conflicting Interests and Ethics

The authors declare that in this review there is no conflict of interest. This present study complies with publishing ethics. Each author is responsible for scientific and legal responsibility for manuscripts published in IJSM.

Authorship Contribution Statement

Megan Huerta: Data curation; Data analysis; **Jyoti Tamang**: Edit manuscript. **Mohammad Amzad Hossain**: Literature survey; Reviewing and Editing. **Gen Kaneko**: Sample collection and data curation **Hashimul Ehsan:** Supervision, Planning, draft writing, interpretation.

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Research Article

GC-MS profiling of anticancer and antimicrobial phytochemicals in the vegetative leaf, root, and stem of *Withania somnifera* (L.) Dunal

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Anticancer,
Withania somnifera.

Abstract: *Withania somnifera* has been used for a long time in traditional medicine. Its crude extract, dried powder, and purified metabolites from mature plants have shown promising therapeutic potential. To further investigate its potential, the detection of phytochemicals with anticancer and antimicrobial properties in the vegetative stage is essential. Hence, this study was done to identify phytochemical constituents using GC-MS analysis for anticancer and antimicrobial activities in the vegetative stage from methanolic extracts of stem, leaf, and root in *W. somnifera*. The air-dried plant parts were extracted with methanol at low pressure to concentrate using a rotary evaporator at 40°C. To identify phytochemicals, Shimadzu GCMSQP2010, Japan, was used with the NIST107.LIB database. The GC-MS identified 35 unique phytochemical peaks at the vegetative stage in *W. somnifera*. In leaves, the antibacterial phytochemicals included cyclotrisiloxane, hexamethyl, with a high abundance, and cyclohexasiloxane, dodecamethyl, with the least abundance. In roots, the phytochemicals 2,2-dimethoxybutane, with high abundance, and cathinone, with least abundance, were found to have antibacterial properties, whereas trans-2,3-epoxyoctane, with high abundance, and 2,2-dimethoxybutane, with least abundance, were found to have anticancer properties. In stem, the antibacterial phytoconstituents octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl, and benzenemethanol, alpha.-(1-aminoethyl), were found to be the most abundant and least abundant, respectively, while arabinitol and pentaacetate had both anticancer and antibacterial activities. At the vegetative stage, GC-MS studies of stem, leaf, and root parts revealed the occurrence of potential phytochemicals for antibacterial and anticancer activities in *W. somnifera*.

1. INTRODUCTION

Withania somnifera (L.) Dunal, a significant Ayurvedic medicinal plant with a variety of therapeutic uses and activities, is a member of the Solanaceae family and is generally referred to as ashwagandha (Dutta *et al.*, 2019). Plant extract and its active constituents from the complete plant, as well as stems, roots, and leaf parts, have been used in the treatment of a broad

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range of ailments in humans (Altemimi *et al.*, 2017). Studies indicate that Ashwagandha has been used for a long time as a tonic, in stress management, in improving cognitive health, in decreasing depression, and in lowering cortisol and blood sugar levels (Salve *et al.*, 2019).

This plant has anti-arthritic, anti-inflammatory, anti-epileptic, anti-coagulant, anti-oxidant, anti-depressant, anti-pyretic, and anti-diabetic effects. It also has palliative properties for growth promotion, analgesia, rejuvenation, and regeneration. The plant is found to positively influence metabolism, act as anti-serotogenic, and have other vital properties (Saidulu *et al.*, 2014). Despite its multidrug constituent and medical use since time immemorial, *W. somnifera* still needs to be extensively studied in terms of antimicrobial and anticancer properties, as well as phytochemical correlation (Musher *et al.*, 2021). Moreover, the herbal products of Ashwagandha sold on the market do not adequately explain the phytochemical constituents of the plant and its medicinal properties, as well as the information about the number of bioactive constituents, the reason for harvesting the selected parts of the plant, and the correct developmental stages of the plant that yield the best products (Salemm *et al.*, 2020).

Hence, examining the phytochemical composition of *W. somnifera* at various phases of growth might shed light on how bioactive components alter in plants as they age. Better therapeutic effectiveness may be ensured by using it to help choose the appropriate developmental stage that produces the maximum concentration of the targeted chemicals. Thus, the investigation of *W. somnifera* bioactive components at various developmental stages using GC-MS is a useful method for understanding the therapeutic qualities of plants, refining cultivation techniques, and guaranteeing product quality. According to prior research, *W. somnifera* should be harvested (Kaur *et al*., 2018; Afewerky *et al*., 2021), depending on its dry weight, harvesting period, and mature plant phytochemical profile (Salemm *et al.*, 2020). However, the phytochemical profile of *W. somnifera* based on plant vegetative growth and developmental phases currently has a gap. Thus, in this study, using GC-MS, the phytochemical constituents at the vegetative stage of the *W. somnifera* stem, leaf, and root parts were identified.

2. MATERIAL and METHODS

2.1. Plant Authentication and Material Collection

W. somnifera seeds were obtained from Zooqa Herbs in Chennai, Tamil Nadu. In the natural soil conditions, the seeds were sown and grown at the Department of Genetics, Osmania University, Hyderabad, Telangana. The plant authentication of *W. somnifera* was done by Dr. A. Vijaya Bhaskar Reddy, Botany Department, Osmania University, Hyderabad. The plant material was placed in the Botany Department herbarium, Osmania University, Hyderabad, with the voucher number: GEN/OU/001-2018-HY.

2.2. Plant Parts Methanolic Extraction

The *W. sominifera* stem, root, and leaf parts were ground coarsely extracted and in a Soxhlet apparatus for 24 hours with methanol (100 ml), followed by air drying. In a rotary evaporator maintained at 40 °C, the extract was concentrated with reduced pressure to produce a semi-solid viscous mass.

2.3. Instrumentation and Sample Analysis in GC-MS

Shimadzu model GCMSQP2010, Japan, was used. The GC-MS system was equipped with the injection port (SPL-1) and injection heat port (INJ-1) to analyse the phytochemicals in the leaf, stem, and root methanolic extracts of *W. somnifera*. An ion source temperature of 230°C and an interface temperature of 250°C were established for a mass-selective detector. A capillary column ZB-5 measuring 30.0 m in length, 0.32 mm in diameter, and 0.25 µm in film thickness was employed in MS analysis running at 70 eV in the electron impact mode. With a 1µl injection volume and a 1.71 mL/min flow rate, the carrier gas 99.9% helium was utilized in a split-less injection (50:1 ratio of split) mode at 250 °C injection temperature. The injection was carried out at a constant linear speed of 47.1 cm/sec, a purge flow of 3 mL/min, and a total flow

of 90.0 mL/min. Initially, 40 °C was maintained in the oven for a duration of one minute. It was elevated to 300 °C gradually at a rate of 10 °C per minute. A total run time of 37 minutes was set for the sample. The mass spectrum range was set at 0–1000 m/z.

2.4. Identification of Phytochemicals

The identification of compounds was done by matching spectra with National Institute of Standards and Technology database library (NIST107.LIB) compounds to determine their names, structures, and molecular weights. Retention time (RT) for GC was used to determine components, and MS fragment interpretation was done by comparison with the NIST107.LIB database.

3. RESULTS

3.1. GC-MS of Vegetative Leaf

In *W. somnifera* vegetative leaf methanol extract, GC-MS identified eighteen unique compounds, as shown in Figure 1. The chromatogram peaks, RT, name of the compound, area of peak, and molecular formulae are presented in Table 1. It indicates presence of compounds ethane, 1,2-dichloro-1-ethoxy- (39.96%); dextroamphetamine (14.79%); 1,3,5-cycloheptatriene (4.29%); propane, 2,2-dimethoxy- (3.87%); alpha.-acetyl-N,N-dinormethadol (3.39%); tartronic acid, 4-(dimethylethylsilyl)phenyl-, dimethyl ester (2.72%); cyclotetrasiloxane, octamethyl- (2.67%); 1,2-Dihydro-2,4-diphenyl-quinazoline (2.4%); 3-ethoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane (1.6%); 1H-indole-2,3-dione, 3-(O-ethyloxi), 1-(tert-butyldimethylsilyl)-5-chloro-, (1.95%); trimethylsilyl ester, 3-methyl-2-trimethylsilyloxy-, benzoic acid (1.71%); cyclotrisiloxane, hexamethyl-(1.69%); norepinephrine, N-(trifluoroacetyl) -N,O,O',O''- tetrakis (trimethylsilyl)- (1.43%); 1,2,3,5,6,7,8,8a - octahydro-4-trimethyl, 1,2-cinnolinedicarboxylic acid (1.36%); silane, [(1,3-diphenyl-1-butenyl)oxy]trimethyl- (1.3%); cycloheptasiloxane, tetradecamethyl- (1.13%); (+)-2-Aminoheptane (0.2%); cyclohexasiloxane, dodecamethyl- (0.17%); and, N-acetyl-2-methylamphetamine (0.12%).

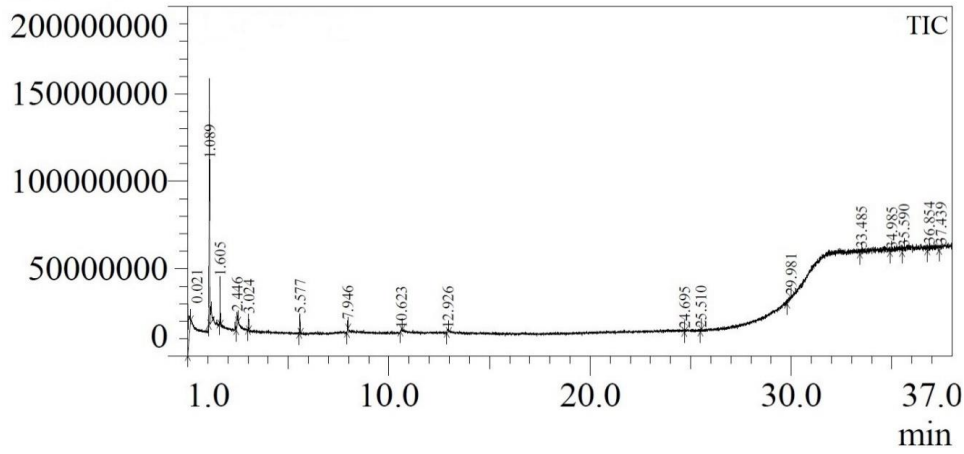

Figure 1. GC-MS chromatogram of methanol-extracted vegetative leaves from *Withania somnifera*, where the x-axis represents run time and y-axis represents abundance.

In *W. somnifera* vegetative leaf methanolic extracts, six compounds with antimicrobial activities were identified through a GC-MS study. The chromatogram peaks, area, compound name, RT, molecular formulae, and therapeutic activity are presented in Table 1. These phytochemicals are 1,3,5-cycloheptatriene (4.29%); cyclotrisiloxane, hexamethyl- (1.69%); cyclotetrasiloxane, octamethyl- (2.67%); N- (trifluoroacetyl)-N,O,O',O' tetrakis (trimethylsilyl) norepinephrine (1.43%); dodecamethyl-cyclohexasiloxane (0.17%); cycloheptasiloxane, tetradecamethyl- (1.13%). There were no proven anticancer compounds identified in the vegetative-stage leaf methanolic extracts of *W. somnifera*.

Table 1. GC-MS identified phytochemicals in *W. somnifera* vegetative leaf methanol extracts.

Peaks	RT	Compound name	Formulae	m/z	Peak area%	Phytochemical group	Therapeutic activity	References
1	0.021	Dextroamphetamine	$C_9H_{13}N$	44.10	14.79	Flavonoid	NA	
2	1.089	Ethane, 1,2-dichloro-1-ethoxy-	$C_4H_8Cl_2O$	34.70	39.96	Organohalogen	NA	
5	1.605	Propane, 2,2-dimethoxy-	$C_5H_{12}O2$	73.10	3.87	Flavonoid	NA	
6	2.446	1,3,5-Cycloheptatriene	C_7H_8	91.15	4.29	Terpenoid	Antibacterial	Yunnikova *et al.*, 2014.
7	3.024	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	207.00	1.69	Terpenoid	Antibacterial	Dahpour *et al.*, 2012.
8	5.577	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	281.15	2.67	Terpenoid	Antibacterial	Keskin *et al.*, 2012.
9	7.946	N,O,O',O' tetrakis (trimethylsilyl) norepinephrine, N-(Trifluoroacetyl) -	$C_{22}H_{42}F_3NO_4Si_4$	73.15	1.43	Alkaloid	Antibacterial	Soliman *et al.*, 2016.
10	10.623	Cyclohexasiloxane, Dodecamethyl-,	$C_{12}H_{36}O_6Si_6$	73.10	0.17	Alkaloid	Antibacterial	Moustafa *et al.*, 2013.
11	12.926	Tetradecamethyl-, cycloheptasiloxane,	$C_{14}H_{42}O_7Si_7$	73.15	1.13	Alkaloid	Antibacterial	Prasathkumara *et al.*, 2021.
12	24.695	(+)-2-Aminoheptane	$C_7H_{17}N$	44.10	0.2	Alkaloid	NA	
13	25.51	N-Acetyl-2-methylamphetamine	$C_{12}H_{17}NO$	44.10	0.12	Alkaloid	NA	
14	29.981	1,2-Dihydro-2,4-diphenyl-quinazoline	$C_{20}H_{16}N_2$	207.10	2.4	Alkaloid	NA	
15	33.485	1-(tert-butyldimethylsilyl)-5-chloro-, 3-(O-ethyloxi, 1H-Indole-2, 3-dione,	$C_{16}H_{23}ClN_2O_2Si$	208.05	1.95	Alkaloids	NA	
16	33.79	Alpha.-Acetyl-N,N-dinormethadol	$C_{21}H_{27}NO_2$	44.10	3.39	Terpenoids	NA	
17	34.985	[(1,3-diphenyl-1-butenyl)oxy]trimethyl-, silane	$C_{19}H_{24}OSi$	281.10	1.3	Terpenoids	NA	
20	35.48	Trimethylsilyl ester, benzoic acid, 3-methyl-2-trimethylsilyloxy-	$C_{14}H_{24}O_3Si_2$	281.10	1.71	Phenolic	NA	
21	35.59	3-Ethoxy-1,1,1,5,5,5 - hexamethyl -, 3 - (trimethylsiloxy) trisiloxane	$C_{11}H_{32}O_4Si_4$	208.05	1.6	Phenolic	NA	

3.2. GC-MS of Vegetative Root

In *W. somnifera* vegetative root methanol extracts, GC-MS identified seventeen compounds, as shown in Figure 2. In Table 2, the peaks RT, compound name, area, and molecular formulae are presented. The predominant compounds found to be were: trans-2,3-epoxyoctane (84.78%); - 3,5,5-tris (trimethylsiloxy) tetrasilo, 3 – isopropoxy - 1,1,1,7,7,7- hexamethyl (2.09%); 2, 2-dimethoxybutane (0.44%); hexadecamethyl-, cyclooctasiloxane, (0.4%); 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-, hexasiloxane (0.26%); ethyl ester, benzeneacetic acid, 3-methoxy-4-[(trimethylsilyl)oxy]- (0.19%); 3,5-dimethyl-, (3,5-dimethylphenyl)methyl ester, benzoic acid (0.18%); benzoxazolinone, 4,5 - dibromo - 6 -chloro - 2 - (0.18%); 1,1,3,3,5,5-hexamethyl-, trisiloxane (0.17%); silane, methyltripropoxy- (0.14%); pentasiloxane, 1,1,3,3,5,5,7,7,9,9-decamethyl- (0.14%); 2-propanone, 1-methoxy-(0.07%); cyclooctasiloxane, hexadecamethyl- (0.4%); allyl(methoxy)dimethylsilane (0.03%); cathinone (0.03%); 5-chloro-6-methyl-, spiro[2.3]hexan-4-one (0.02%); and, 1,3,2-dithiaborinane, 2-ethyl- (0%).

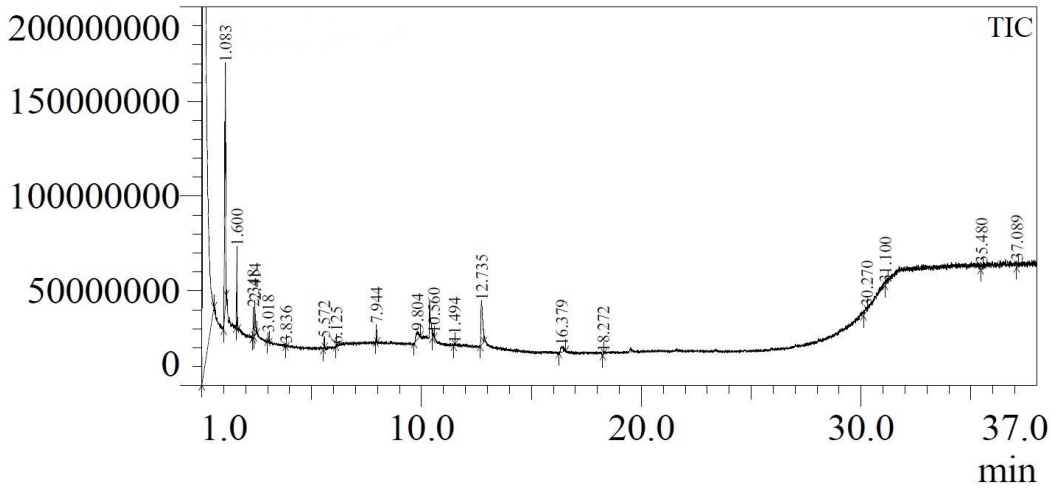

Figure 2. GC-MS chromatogram of methanol-extracted vegetative roots from *Withania somnifera*, where the x-axis represents run time and the y-axis represents abundance.

In *W. somnifera* vegetative root methanolic extracts, twelve compounds with antimicrobial activities were identified through GC-MS. The chromatogram peaks area, molecular formulae RT, name of the compound, and its antimicrobial and anticancer bioactivities are presented in Table 2. The phytochemicals found to be 2,2-dimethoxybutane (0.44%); cyclotrisiloxane, hexamethyl- (0.14%); octamethyl-, cyclotetrasiloxane (0.13%); cyclooctasiloxane, hexadecamethyl- (0.4%); N,O,O',O' – tetrakis (trimethylsilyl) norepinephrine, N-(trifluoroacetyl) - (0.23%); cyclohexasiloxane, dodecamethyl- (0.48%); cathinone (0.03%); 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-, hexasiloxane (0.26%); ethyl ester, 3-methoxy-4-[(trimethylsilyl)oxy]-, benzeneacetic acid (0.19%); 3,5-dimethyl-, benzoic acid (3,5-dimethylphenyl)methyl ester (0.18%); pentasiloxane, 1,1,3,3,5,5,7,7,9,9-decamethyl- (0.14%) and benzoic acid, 3-methyl-2-trimethylsilyloxy-, trimethylsilyl ester (0.19%). The trans-2,3-epoxyoctane (84.78%) was identified as a phytochemical with anticancer properties, and the 2,2-dimethoxybutane (0.44%) was identified as a phytochemical with both anticancer and antibacterial properties.

Table 2. GC-MS identified phytochemicals in *W. somnifera* vegetative root methanolic extracts.

Peaks	RT	Compound name	Formulae	m/z	Peak area%	Phytochemical groups	Therapeutic activity	References
1	0.082	Trans-2,3-Epoxyoctane	$C_8H_{16}O$	53.15	84.78	Fatty acids	Anticancer	Akter *et al.*, 2022.
3	1.138	2-Propanone, 1-methoxy-	$C_4H_8O_2$	73.15	0.07	Flavonoid	NA	
7	2.348	2,2-Dimethoxybutane	$C_6H_{14}O_2$	43.15	0.44	Fatty acids	Antibacterial & Anticancer	Hajjar *et al.*, 2017.
10	3.836	5-chloro-6-methyl-, spiro[2.3]hexan-4-one	C_7H_9ClO	40.05	0.02	Terpenoids	NA	
12	6.125	1,3,2-Dithiaborinane, 2-ethyl-	$C_5H_{11}BS_2$	40.05	0	Organohalogen	NA	
18	11.49	2 - (Dimethylaminomethyl) - 3 - nitrophenol	$C_9H_{12}N_2O_3$	44.10	0.02	Alkaloids	NA	
19	12.74	Hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasilo, 3 - Isopropoxy-1,1,1,7,7,7 -	$C_{18}H_{52}O_7Si_7$	73.10	2.09	Phenolic	NA	
20	16.38	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	73.15	0.4	Alkaloids	Antibacterial	Dahpour *et al.*, 2012.
8	5.572	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	281.20	0.13	Alkaloids	Antibacterial	Keskin *et al.*, 2012.
21	18.27	Cathinone	$C_9H_{11}NO$	44.10	0.03	Alkaloids	Antibacterial	
22	30.27	1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-, hexasiloxane	$C_{12}H_{38}O_5Si_6$	207.10	0.26	Alkaloids	Antibacterial	Majumder *et al.*, 2019.
23	31.1	3-methoxy-4-[(trimethylsilyl)oxy]-, ethyl ester	$C_{14}H_{22}O_4Si$	208.10	0.19	Terpenoids	Antibacterial	Kaviya *et al.*, 2021.
25	31.61	Trisiloxane, 1,1,3,3,5,5-hexamethyl-	$C_6H_{20}O_2Si_3$	207.10	0.17	Alkaloids	NA	
28	35.48	Benzoic acid, dimethylphenyl)methyl ester	$C_{18}H_{20}O_2$	133.10	0.18	Terpenoids	Antibacterial	EL-Zawawy and Mona, 2021.
31	37.09	1,1,3,3,5,5,7,7,9,9-decamethyl-, pentasiloxane,	$C_{10}H_{32}O_4Si_5$	191.05	0.14	Alkaloids	Antibacterial	Amrati *et al.*, 2021.

3.3. GC-MS of Vegetative Stem

In *W. somnifera* vegetative stem methanolic extracts, eighteen unique phytochemicals were identified by GC-MS analysis, as shown in Figure 3. In Table 3, peak area, RT, compound name, and molecular formulae are presented. The phytochemicals identified included benzenemethanol, alpha.-(1-aminoethyl)- (28.07%); 2-butynoic acid (1.47%); 2 hydroxybenzophenone, 3 - tert - butyl - 5 - chloro - (4.96%); 5 - (ethyl-1-amine) bicyclo [2.2.1], heptane (3.25%); 3-amino-1,2-propanediol (2.24%); 2-hydroxy-5-[N,N-dimethylaminomethyl]benzoic acid (0.88%); 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-, heptasiloxane (0.04%); 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-, octasiloxane (0.05%); Arabinitol, pentaacetate (0.05%); cyclononasiloxane, octadecamethyl- (0.15%); 2,4,6,8,10-tetradecapentaenoic acid, 9a-(acetyloxy)-1a,1b,4,4a,5,7a,7b (0.22%); heptasiloxane, hexadecamethyl-(8.88%); northiaden (0.03%); tert-butyld, benzenepropanoic acid, beta.-[(tert-butyldimethylsilyl)oxy]-, (1.45%); benzoic acid, 4-methyl-2-trimethylsilyloxy-, trimethylsilyl ester, (0.96%); silanamine, N-[2,6-dimethyl-4-[(trimethylsilyl)oxy]phenyl]-1,1,1-trimet, (0.65%); 1-propanone, 1,3-diphenyl-3-(trimethylsilyl)- (0.05%); and dihydropyrimidinyl-4 uracil, 5-2-Oxo-6-phenyl-1,2 (0.87%).

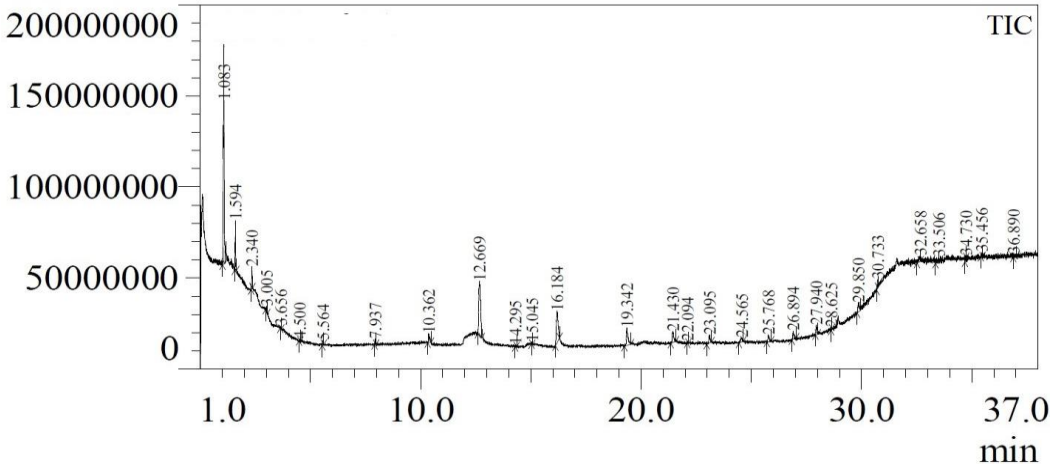

Figure 3. GC-MS chromatogram of methanol-extracted vegetative stems from *Withania somnifera*, where the x-axis represents run time and the y-axis represents abundance.

In *W. sominifera* vegetative stem methanolic extracts, phytochemicals identified with antimicrobial properties are given in Table 3. These included benzeneethanamine, N - (3-methylbutylidene)- (28.07%); 2-pyridinecarboxamide N-oxide (2.3%); 2,2-dimethoxybutane (0.88%); cyclotrisiloxane, hexamethyl- (0.19%); benzenemethanol, .alpha.-(1-aminoethyl)- (0.04%); 2 butynoic acid (0.05%); bicyclo[2.2.1], heptane-5-(ethyl-1-amine) (0.02%); 3-tert-Butyl-5-chloro-2-hydroxybenzophenone (0.05%); cyclotetrasiloxane, octamethyl- (0.15%); cyclohexasiloxane, dodecamethyl- (0.99%); tetrakis (trimethylsilyl) norepinephrine, N-(trifluoroacetyl) - N,O,O',O' – (0.22%); cycloheptasiloxane, tetradecamethyl- (8.88%); 3-amino-1,2-propanediol (0.06%); 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-, cyclooctasiloxane, hexadecamethyl- 5 heptasiloxane (2.8%); 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-, octasiloxane (1.45%); hexadecamethyl, heptasiloxane (0.75%) and, cyclononasiloxane, octadecamethyl- (0.65%). Whereas arabinitol, pentaacetate (0.07%) was identified as a phytochemical with both anticancer and antimicrobial properties.

Table 3. GC-MS identified phytochemicals in *Withania somnifera* vegetative stem methanolic extracts.

Peaks	RT	Compound name	Formulae	m/z	Peak area%	Phytochemical groups	Therapeutic activity	References
2	0.095	Benzenemethanol, .alpha.-(1-aminoethyl)-	$C_9H_{13}NO$	44.05	28.07	Alkaloid	Antibacterial	Diale *et al*., 2021.
3	0.185	2-Butynoic acid	$C_4H_4O_2$	40.05	1.47	Carbohydrate	Antibacterial	Sanabria-Ríos *et al*., 2020.
4	0.195	3-tert-Butyl-5-chloro-2-hydroxybenzophenone	$C_{17}H_{17}ClO_2$	40.05	4.96	Alkaloid	Antibacterial	Fagbemi *et al*., 2021.
5	0.235	Bicyclo [2.2.1], heptane – 5 - (ethyl-1-amine)	$C_9H_{17}N$	44.05	3.25	Amine	Antibacterial	Zielińska-Błajet *et al*., 2020.
6	2.34	2,2-Dimethoxybutane	$C_6H_{14}O_2$	43.05	0.88	Fatty acids	Antibacterial	Hajjar et al., 2017.
10	1.594	3-Amino-1,2-propanediol	$C_3H_9NO_2$	44.05	2.24	Alcohol	Antifungal	Chirumamilla *et al*., 2022.
11	2.34	2-Hydroxy-5-[N,N-dimethylaminomethyl]benzoic acid	$C_{10}H_{13}NO_3$	44.05	0.88	Alkaloid	NA	
13	3.656	1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl- Heptasiloxane	$C_{14}H_{44}O_6Si_7$	73.05	0.04	Organosilicon	Antibacterial	Mukesi *et al*., 2019.
14	4.045	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-octasiloxane	$C_{16}H_{50}O_7Si_8$	73.05	0.05	Alkaloid	Antibacterial	Falowo *et al*., 2017.
15	4.154	Arabinitol, pentaacetate	$C_{15}H_{22}O_{10}$	38.30	0.05	Terpenoids	Antibacterial & Anticancer	Khan *et al*., 2021.
16	3.005	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	73.10	0.19	Organosilicon	Antibacterial	Dahpour *et al.*, 2012.
17	5.564	Cyclononasiloxane, octadecamethyl-	$C_{36}H_{46}O_8$	73.05	0.15	Fatty acids	Antibacterial	Keskin *et al*., 2012.
18	7.937	2,4,6,8,10-Tetradecapentaenoic acid, 9 a - (acetyloxy)-1a,1b,4,4a,5,7a,7b-	$C_{18}H_{54}O_9Si_9$	44.05	0.22	Fatty acids	ND	
20	12.669	Heptasiloxane, hexadecamethyl-	$C_{36}H_{46}O_8$	73.07	8.88	Organosilicon	Antibacterial	Hassan, 2016.
22	15.045	Northiaden	$C_{18}H_{19}NS$	44.10	0.03	Flavonoids	NA	

25	21.43	Beta.-[(tert-butyldimethylsilyl)oxy]-, tert-butyld, benzenepropanoic acid,	$C_{21}H_{38}O_3Si_2$	96.05	1.45	Alkaloid	NA
27	23.095	Trimethylsilyl ester, benzoic acid, 4-methyl-2-trimethylsilyloxy-	$C_{14}H_{24}O_3Si_2$	280.95	0.96	Phenolic	NA
28	24.565	Silanamine, N-[2,6-dimethyl-4-[(trimethylsilyl)oxy]phenyl]-1,1,1-trimet	$C_{14}H_{27}NOSi_2$	73.05	0.65	Alkaloid	NA
29	24.75	1-Propanone, 1,3-diphenyl-3-(trimethylsilyl)-	$C_{18}H_{22}OSi$	281.95	0.05	Fatty acids	NA
30	25.768	5 - (2 – Oxo - 6 – phenyl - 1,2-dihydropyrimidinyl - 4)- Uracil	$C_{14}H_{10}N_4O_3$	281.90	0.87	Alkaloid	NA

4. DISCUSSION and CONCLUSION

W. somnifera-based medicines have been used to treat various human illnesses (Altemimi *et al*., 2017). Most often, the *W. somnifera* roots of mature plants are favoured for a variety of therapeutic uses (Afewerky *et al.*, 2021). The root, stem, and leaf extracts of *W. somnifera* have been shown to be active against several cancers (Yadav *et al*., 2010; Dutta *et al.*, 2019). Also, aqueous and alcoholic extracts of *W. somnifera* leaf, stem, and root have been shown to have antimicrobial properties against a variety of microorganisms (Bisht and Rawat, 2014; Singariya *et al.*, 2011). These phytochemicals with anticancer and antimicrobial properties in *W. somnifera* were found to vary depending on the organ (Lingfa *et al*., 2022). Moreover, their phytochemical status during the vegetative stage is not yet studied in root, stem, and leaf. Hence, it is crucial to understand the bioactive phytochemical distribution in various parts of the plant at various developmental stages, including the vegetative stage of the leaf, stem, and root, for the further development of effective herbal-based formulations.

GC-MS analysis was used in this work to characterize the phytochemicals in *W. somnifera.* The plant parts investigated included the stem, leaf, and root, which were extracted using methanol as the solvent. The choice of methanol as the extraction solvent was based on previous studies that have shown that it yields the highest concentration of alkaloid, flavonoid, phenolic, and terpenoid components in *W. somnifera* (Ruiz-Ruiz *et al*., 2017; Kuppusamy *et al*., 2015; and Chao *et al*., 2014). The extracted samples were then subjected to GC-MS analysis, which involved chromatographic separation, quantification, and identification of the phytochemical compounds. The distribution and existence of antibacterial and anticancer phytochemicals in *W. somnifera* vegetative stage were determined using the GC-MS. The use of GC-MS analysis is advantageous as it is a quick and economical method for evaluating herbal products and provides detailed information on the chemical composition of the plant extracts.

The identified phytochemicals in *W. somnifera* vegetative leaf methanolic extracts, such as 1,3,5-cycloheptatriene (Yunnikova *et al.*, 2014) and cyclotrisiloxane, hexamethyl- have been reported in *Sedum pallidum* (Dahpour *et al*., 2012) to have antibacterial properties. The phytochemical cyclotetrasiloxane, octamethyl-, is also found in *W. somnifera* methanolic stem extracts of reproductive stage (Lingfa *et al*., 2023). In *W. somnifera* vegetative stage leaf methanolic extract: N,O,O',O'- tetrakis (trimethylsilyl), N- (trifluoroacetyl) – norepinephrine phytochemicals were found in red sea cucumber (Soliman *et al*., 2016). cyclohexasiloxane, dodecamethyl in *Argemone ochroleuca* (Moustafa *et al*., 2013). Cycloheptasiloxane, tetradecamethyl- in *Senna auriculaita* (Prasathkumara *et al*., 2021) and reported to have antibacterial properties.

The bioactive phytochemicals benzenemethanol, alpha-(1-aminoethyl)- in *Ribwort plantain* (Haghighi *et al*., 2022), 3-tert-butyl–5–chloro-2-hydroxybenzophenone in the *Tamarindus indica* (Fagbemi *et al*., 2021), cyclotetrasiloxane, octamethyl- in *Olea europaea* (Keskin *et al.*, 2012), N-(Trifluoroacetyl)- N,O,O',O'-tetrakis (trimethylsilyl) norepinephrine in the red sea cucumber *Holothuria atra* (Soliman *et al*., 2016), cyclohexasiloxane, dodecamethyl- in *Argemone ochroleuca* (Moustafa *et al.*, 2013), cycloheptasiloxane, tetradecamethyl- in *Senna auriculata* (Prasathkumara *et al.*, 2021), cyclooctasiloxane, hexadecamethyl- in *Sedum pallidum* (Dahpour *et al*., 2012), heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl- in *Olea europaea* (Mukesi *et al*., 2019), 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-octasiloxane in *Moringa oleifera* (Falowo *et al*., 2017) identified in previous studies to have antibacterial properties are identified in the methanolic extracts of *W. somnifera* vegetative stem. The phytochemicals in methanolic extracts of stem from *W. somnifera*, Cyclononasiloxane, octadecamethyl-, were found to be antibacterial as well as antifungal in *Salvadora persica* (Bratty *et al*., 2020), 3-amino-1,2-propanediol as antifungal in *Solanum khasianum* (Chirumamilla *et al*., 2022). Additionally, the identified phytochemicals in this

study in *W. somnifera,* heptasiloxane, hexadecamethyl- (Bratty *et al.,* 2020), benzeneethanamine, N-(3-methylbutylidene)- (Diale *et al*., 2021), 2-butynoic acid (Sanabria-Ríos *et al*., 2020), and bicyclo [2.2.1] heptane-5-(ethyl-1-amine) (Zielińska-Błajet *et al.*, 2020) have been previously reported to be antibacterial. The antibacterial activity of these compounds' ability may be ascribed to disrupt membranes in bacterial cell or inhibit enzymes that are essentially involved in bacterial growth and replication (Barbieri *et al.,* 2017).

In *W. sominfera,* vegetative root methanolic root extracts, the trans-2,3-epoxyoctane, has been reported to be anticancer (Akter *et al.*, 2022). It is found in the herbal remedy known as Triphala, which is made from the fruits of three different herb species: *Terminalia bellirica*, *T. chebula*, and *Phyllanthus emblica* (Akter *et al.,* 2022). Another phytochemical, 2,2-Dimethoxybutane has been reported to be both anticancer and antibacterial, and it is found in the Saudi Arabian herbal fraction, i.e., in JUN_C2_60% (Hajjar *et al*., 2017). The phytochemical arabinitol pentaacetate identified in vegetative stem methanolic extracts of *W. somnifera* has been shown to have both antibacterial and anticancer properties and was reported in *Abutilon indicum* (Khan *et al*., 2021). The mechanisms by which these anticancer substances work include triggering apoptosis, or programmed cell death, in cancer cells, preventing tumor development and metastasis, and adjusting immune responses against cancer cells (Rahman *et al.,* 2021). To fully understand these phytochemicals' unique modes of action and their uses in antibacterial and anticancer treatments, further studies are required.

The phytochemicals responsible for anticancer and antimicrobial properties in *W. somnifera* at the vegetative stage reveal that phytochemicals found in the leaf, stem, and root parts were trivial in the vegetative stage compared to the reproductive stage (Lingfa *et al*., 2023). In the vegetative stage, the total of phytochemicals with antibacterial and anticancer properties was found to be highest in stem methanolic extracts and least in leaf methanolic extracts. To our knowledge, this is the first GC-MS profiling of stem, leaf, and root methanolic extracts based on the developmental stage, *viz.*, the vegetative stage in *W. somnifera*. This study identified phytochemicals that were not previously reported in the GC-MS analysis of *W. somnifera*.

Most of the phytochemical compounds identified at the vegetative stage from the *W. somnifera* methanolic leaf, root, and stem extracts in this study are commercialised as antibiotics or anticancer medications, such as 1,3,5-cycloheptatriene and cathinone. The identified phytochemical compound 1,3,5-cycloheptatriene from the vegetative leaf methanolic extract of *W. somnifera* is clinically used as an antibacterial compound and is commercially known as triprolidine (Manikandan *et al*., 2019). A study shows that the unsubstituted seven-membered ring structure (triprolidine) has antibacterial activity, and the substitution with a keto group in the 2-position (tropolone) enhanced the bacteriostatic and bactericidal activity (Trust and Bartlett 1975). The identified phytochemical compound from the vegetative root methanolic extract of *W. somnifera* at the vegetative stage, cathinone, is used to develop a commercially available drug known as ciprofloxacin, which is useful to treat a variety of infections, including chancroid, anthrax, respiratory tract infections, and urinary tract infections (Piddock *et al*., 2010). It is a quinolin-4(1H)-one and has substitutions at positions 1, 6, 3, and 7 for piperazin-1-yl, cyclopropyl, carboxylic acid, and fluoro, respectively. Its structural functions are similar to those of a topoisomerase IV inhibitor (ASHP, 2015).

Traditional herbal medicines developed from *W. somnifera* play a significant role in the healthcare system. However, further quality-based product formulation is needed to maximize consumer benefits. Furthermore, since the distribution and amount of bioactive chemicals in plants can change throughout the course of their life cycle, the results emphasize the need of taking the developmental stage into account when analyzing its phytochemical composition. The identification of bioactive phytochemicals with anticancer and antimicrobial properties in the vegetative stage extracts of *W. somnifera* has important implications for the development

of herbal-based products. These phytochemicals could serve as potential leads for the development of new antibiotics and anticancer medications. To separate and purify these substances for usage in therapeutic settings, more investigation is required.

In conclusion, while this study provides valuable insights into the phytochemical composition of *W. somnifera* at the vegetative stage, it is important to note that the study focused on methanolic extracts and specific phytochemicals. Further investigations could explore other extraction methods and analyze a broader range of phytochemicals. Additionally, future studies could investigate the biological activities of the identified phytochemicals and their potential mechanisms of action. Understanding the pharmacological properties of these compounds could pave the way for the development of novel herbal-based therapies.

Plant Authentication

The plant material (*Withania somnifera*) was identified and authenticated by Dr. A. Vijaya Bhaskar Reddy, Assistant Professor, Department of Botany, Osmania University, Hyderabad. The plant was deposited in the herbarium of the Department of Botany, Osmania University, Hyderabad, with the voucher number GEN/OU/001-2018-HY.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Lali Lingfa: Investigation, resources, formal analysis, statistics, and writing the draft. **Aravinda Tirumala**: Resources, visualisation, writing, formal analysis**. Ankanagari Srinivas**: Concept, editing and writing, investigation, resources, supervision, and validation.

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Research Article

In-Vitro Bioactivity Evaluation of Hydrangenol Extracted from *Hydrangea macrophylla* (Thunb.) Ser. Leaves

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Abstract: *Hydrangea macrophylla* plant, native to Japan and Korea, has been attracting scientific attention due to its potential applications in both food science and health-related research. In this investigation, dry *Hydrangea* leaves were utilized as the source material. Subsequent to comminution and thermal treatment at 70 °C for an 18-hour duration, followed by a 30-minute ultrasonic bath extraction and a 5-minute centrifugation at 5000 rpm, hydrangenol was isolated through preparative HPLC. The investigation involved assessing the antioxidant capacity of hydrangenol, its impact on the activity of α-amylase and α-glucosidase enzymes, and its ability to prevent enzymatic browning. Quantification of antioxidant capacity, determined through TEAC (Trolox Equivalent Antioxidant Capacity), showed values from 1.8 to 3.2 mmol TE/mmol. Likewise, the ORAC (Oxygen Radical Absorbance Capacity) values were in the range of 16.5-27.0 mmol TE/mmol. Total phenolics content (Folin-Ciocalteu test) yielded a range of 7.1-11.2 g GAE (Gallic Acid Equivalents) per 100 g. Examining α-amylase inhibition, hydrangenol demonstrated a 52% inhibition (IC50: 3.6 mg/mL), whereas acarbose (positive control) displayed a higher inhibition of 99 % (IC50: 0.51 mg/mL). Regarding α-glucosidase inhibition, hydrangenol exhibited a 51% inhibition (IC50: 0.97 mg/mL), while acarbose displayed a 46% inhibition (IC50: 2.1 mg/mL). Additionally, the activity of PPO was suppressed by 61% at hydrangenol concentrations of 1 mg/mL and 2 mg/mL, and by 46% at a concentration of 4 mg/mL.

1. INTRODUCTION

Recent advances in health and nutrition have sparked a renewed fascination with natural compounds possessing antioxidant properties. A diet abundant in these antioxidants holds the potential to positively impact human health, lowering the risk of ailments such as cardiovascular diseases, cancers, and age-related macular degeneration (Singh & Goyal, 2008). Phenolic compounds, commonly referred to as polyphenols, are remarkable secondary metabolites synthesized by plants. They are characterized by the presence of one or more phenolic rings with attached hydroxyl groups. These structurally diverse compounds originate from plant pathways such as pentose phosphate, shikimate, and phenylpropanoid (Gianmaria *et al.*, 2011). Polyphenols can be categorized into specific groups based on the strength of their phenolic

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rings, which encompass phenolic acids, flavonoids, stilbenes, and lignans (Abbas *et al.*, 2017). *Hydrangea macrophylla* subsp. serrata (Thunb.) is a deciduous shrub native to Japan and Korea, known for its historical use in making Amacha, a ceremonial tea that derives its name from the Japanese words "甘" for sweet and "茶" for tea (Moll *et al.*, 2022). The fermentation process applied to its leaves enhances sweetness by increasing the content of the aglycon phyllodulcin, contributing to the unique flavor of this traditional tea, often referred to as "tea-hortensia" (Matsuno *et al.*, 2008). In recent years, *H. macrophylla* has gained significant attention in the fields of the food industry and pharmacology, primarily due to the presence of dihydroisocoumarins (DHC), hydrangenol (HG), and phyllodulcin (PD) (Yasuda *et al.*, 2004). The remarkable chemical diversity inherent in isocoumarins, stemming from their intricate chemical substitution patterns, underlies their extensive range of biological and pharmacological activities. These versatile compounds have been extensively studied and thoroughly documented for their potent antimicrobial, antifungal, insecticidal, antioxidant, anti-cancer, anti-inflammatory, and anti-diabetic properties, as confirmed by research from Tianpanich *et al.* (2011), Das *et al.* (2021), and Krohn *et al.* (2001). Diabetes, a chronic condition resulting from insufficient insulin levels and reduced sensitivity (Bhandari et al., 2008), is characterized by elevated blood sugar levels after meals, especially in type 2 diabetes mellitus (T2DM) (Ch'ng *et al.*, 2019). Controlling elevated blood sugar levels after meals entails regulating the function of enzymes responsible for breaking down carbohydrates within the digestive tract. Two pivotal enzymes in carbohydrate digestion are α-amylase (1,4-α-D-glucan-glucanohydrolase, EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) (Dona *et al.*, 2010). α-Amylase, a critical enzyme, breaks down complex substances by breaking α-1,4-glucan linkages present in starch, maltodextrins, and similar carbohydrates (Truscheit *et al.*, 2010). α-Glucosidase, which includes maltase, α-dextrinase, and sucrase, is located on the surface of intestinal mucosal cells and cleaves glucose from polysaccharides through hydrolysis of the α-1,4-glycosidic bond. This process aids in the digestion of dietary starch and related carbohydrates, transforming them into absorbable glucose units within the human intestine (Vocadlo *et al.*, 2008).

Moreover, in their 1998 research, Adams *et al.* emphasized the multifaceted nature of DHC, showcasing its broad spectrum of applications in both the food industry and the development of skin-lightening formulations. The primary objective of this study is to conduct a meticulous examination and assessment of the biological activities associated with HG derived from *H. macrophylla* plant leaves. These activities include evaluating its antioxidant capacity, potential anti-diabetic properties, and its ability to inhibit browning processes.

2. MATERIAL and METHODS

2.1. Chemicals

All chemicals were of analytical quality and the solvents for chromatography were of HPLC-grade. HPLC-grade water was produced using a MicroPure instrument (Thermo Electron LED GmbH, Niederelbert, Germany). Moreover, the chemicals were of the highest quality available (95-99%) and were used without purification. Hydrangenol (8-hydroxy-3-(4-hydroxyphenyl)-3,4-dihydroisochromen-1-one, ($C_{15}H_{12}O_4$)) as reference material was from BOC Sciences, Shirley, USA. The PD (8-hydroxy-3-(3-hydroxy-4-methoxyphenyl)-3,4-dihydro-1H-2-benzopyran-1-one, ($C_{16}H_{14}O_5$)) was purchased from abcr GmbH, Karlsruhe, Germany. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH), phosphate buffered saline (PBS; pH 7.4, 75 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), HCl and Folin-Ciocalteu phenol reagent (FCR) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Fluorescein and 3, 4, 5-trihydroxybenzoic acid (gallic acid) were bought from Fluka (Buchs, Switzerland). α-Amylase and acarbose were purchased from Sigma-Aldrich, Germany. α-Glucosidase (2000

U) was purchased from NEOGEN Europe Ltd, Scotland, UK, 4-methyl catechol ($C_7H_8O_2$) from Thermo Fisher Scientific GmbH, Dreieich, Germany, p-nitrophenyl-α-D-glucopyranoside ($C_{12}H_{15}NO_8$) from CalbioChem (Merck), Darmstadt, Germany, and Polyvinylpolypyrrolidone K25 from Sigma, Taufkirchen, Germany, and Triton X-100 ($C_{34}H_{62}O_{11}$) from Riedel-de Haën, Seelze, Germany.

2.2. Description of the Samples

Hydrangea macrophylla leaves were kindly provided by Hansabred GmbH Co. KG (Dresden, Germany). The experimental procedure involved crushing *Hydrangea* leaves in a mortar for 5 minutes. Next, 0.1 g ± 0.01 of the crushed material was weighed in triplicate and transferred to a 15 mL falcon tube. Subsequently, 2 mL of HPLC water was added, and the mixture was vortexed for 1 minute. The samples were then subjected to fermentation at 70 °C for 18 hours using a vacuum oven (Heraeus, Hanau, Germany). After the fermentation process, the samples underwent a 30-minute extraction using 1 mL of MeOH and 1 mL of 70% EtOH in an ultrasonic bath (Sonorex RK 100, Bandelin, Berlin, Germany). Finally, the extraction was followed by centrifugation at 5000 rpm for 5 minutes using a Centrifuge 5702 R (Eppendorf, Hamburg, Germany). The supernatant obtained from the centrifugation was carefully removed and transferred to a 5 mL volumetric flask. The final volume of 5 mL was obtained by adding 70% EtOH. 2 mL of this solution was centrifuged again at 14000 rpm for 5 minutes.

2.3. Identification, Quantification and Isolation

2.3.1. LC-MS/MS analysis

The study followed Ernawita *et al.*'s 2019 separation method, conducted at a constant 30 °C temperature using a Kinetex C18 column (150 x 2.1 mm, 5 µm), Phenomenex Ltd, Aschaffenburg, Germany) preceded by a C18 pre-column. Separation was achieved with a mobile phase of 0.3% formic acid in HPLC water (A) and acetonitrile (B) using a gradient elution profile: starting at 10% A for 2 minutes, increasing to 20% A at 12 minutes, maintaining 35% A from 22 to 25 minutes, and returning to 10% A from 27 to 37 minutes. The flow rate was 0.5 mL/min, and the analysis was conducted at 30 °C. Each sample injection used 20 µL, and detection employed a Shimadzu UV detector (Shimadzu, Duisburg, Germany). Qualitative analysis for substance validation was done with the API 2000 MS/MS system in negative ion mode (APCI) (AB Sciex, Darmstadt, Germany) at an evaporation temperature of 400 °C. Using Multiple Ion Scan with [M-1]-ions at m/z 285.3 and m/z 255.3, dihydrochalcones (DHC), specifically phyllodulcin and hydrangenol, were confidently identified. Peak assignment in the UV chromatogram was qualitatively performed by comparing mass spectra.

2.3.2. *Fractionation*

The study employed a specialized preparative HPLC reversed-phase system, including the Merck-Hitachi L-7100 pump, L-7400 UV detector, L-5025 column thermostat, and Shimadzu Chromatopac C-R6A integrator. Separation and fractionation at a column temperature of 20 ± 1 °C utilized a C18 column (250 x 8 mm, Nucleodur, Macherey-Nagel, Düren, Germany), with a mobile phase (flow rate: 4.0 mL/min) of 0.3% formic acid in HPLC-water (A) and acetonitrile (B). The dynamic gradient elution involved 70% A from 0 to 20 minutes, transitioning to 60% A from 20 to 30 minutes, and finally reaching 5% A from 31 to 35 minutes. The injection volume was 800 µL, and detection occurred at 254 nm using a UV detector. Fractionation consisted of repeated isolation and sample collection. These fractions were subsequently concentrated in a rotary evaporator at 30 °C under reduced pressure (approximately 20 Pa). The resulting residue was re-dissolved in MeOH and stored at -25 °C for further analysis.

2.4. Antioxidants Capacity

2.4.1. Samples preparation

This study focuses on the quantitative evaluation of the antioxidant capacity of HG, which was isolated using preparative HPLC. HG was dissolved in 10 mL ethanol. Dilutions were prepared (factors: 200, 100, 50, 20, 10, and 5) and adjusted to 1 mL with HPLC-grade water.

2.4.2. Determination of total phenolic contents (TP)

In this study, total phenolics (TP) were evaluated in a 96-well microtiter plate using the Folin-Ciocalteu method (Al-Yafeai *et al.*, 2018), a colorimetric oxidation/reduction reaction. Specific components were placed in separate wells: the blank well contained 30 µL of HPLC water, while other wells held gallic acid monohydrate standard solutions (ranging from 8.51 to 170.12 mg/L) and various dilutions of the HG extract. Subsequently, 150 µL of Folin-Ciocalteu's 1:10 diluted reagent and 120 µL of a sodium carbonate solution (75 g/L) were added to each well, ensuring thorough mixing with the samples. The microplate was then shielded from light and incubated at room temperature for 2 hours. After incubation, the absorbance of each sample was measured at 740 nm using a microplate reader set at 30 °C. The total phenolic content was quantified in terms of gallic acid equivalents (GAE) in mg per 100 g of the sample**.**

2.4.3. Hydrophilic trolox equivalent antioxidant capacity (H-TEAC) assay

Within this investigation, the hydrophilic TEAC assay was utilized, relying on $ABTS^{\bullet+}$ cation radicals. This well-established method for assessing antioxidant radical-quenching capabilities (Al-Yafeai *et al.*, 2018) involved preparing the $ABTS^{\bullet+}$ radical by incubating ABTS and potassium peroxodisulfate solutions. The resulting $ABTS^{\bullet+}$ working solution was freshly diluted with phosphate buffer. In a 96-well microtiter plate, various samples were arranged, including HPLC water (as the blank), Trolox standard at different concentrations, and the methanol/water extract of HG. The HG dilutions were mixed with the $ABTS^{\bullet+}$ working solution, and the reduction in $ABTS^{\bullet+}$ radical absorption at 730 nm due to antioxidant reaction was monitored. Antioxidant capacity was quantified in Trolox equivalents (TE) in mmol per mmol, providing valuable insights into the radical-scavenging potential of the antioxidants.

2.4.4. Hydrophilic oxygen radical absorbance capacity (H-ORAC) assay

The H-ORAC assay, as per Al-Yafeai *et al.* (2018), involves a reaction between the peroxyl radical and a fluorescent probe, generating a non-fluorescent product that can be readily quantified using fluorescence. The analysis begins by preparing a fluorescein working solution (1.2 µM) through a 1:100 dilution of a stock solution of fluorescein (0.12 mM) with phosphate buffer (75 mM, pH 7.4). In a 96-well plate, HG dilutions (10 µL), the fluorescein working solution (25 µL), and buffer (100 µL) are combined. After incubation at 37 °C for 10 minutes, the reaction is started by addition of 150 µL of a freshly prepared AAPH solution (129 mM). Over 120 minutes at 37 °C, fluorescence intensity is measured (excitation: 490 nm, emission: 510 nm), with readings taken every 60 seconds. To assess antioxidant capacity, we evaluated the rate of non-fluorescent product formation over time. The protective effects of the antioxidants are quantified by calculating the integrated area under the fluorescence decay curves (AUC). Results are expressed in Trolox equivalents (TE) as mmol TE/mmol, providing insights into the antioxidant potential of the tested samples.

2.5. Anti-Diabetic Potential

The study aimed to assess HG's anti-diabetic potential by examining its impact on α-amylase and α-glucosidase activities, with acarbose as a positive control for experimental consistency.

2.5.1. α-Amylase assay

This assay was done according to Ernawita *et al.* (2016), slightly modified. Various control and sample groups were established. The negative control group contained phosphate buffer, starch,

and α-amylase, while the positive control group contained only starch and phosphate buffer. To ensure accuracy and minimize interference, blanks were prepared for each HG serial dilution, consisting of HG dilutions, starch, phosphate buffer, and α-amylase. Isolated HG was dried with nitrogen, re-dissolved in 10 mL of MeOH, and then prepared as triplicate 1 mL samples, which were dried again and re-dissolved in a 10% DMSO and MeOH mixture, generating serial dilutions with phosphate buffer (pH 6.9). Subsequently, 200 µl of different sample dilutions were combined in 1 mL Eppendorf tubes to prepare test samples and corresponding blank values. Each tube had 400 µl of starch added and was vortexed for 30 seconds. The tubes were incubated in an orbital shaker at 37 °C for 5 minutes. After incubation, 200 µl of α-amylase was added to each Eppendorf tube (except for the blank and positive control samples), resulting in a final volume of 800 µL in each tube. The mixture was vortexed for 30 seconds and further incubated in the orbital shaker at 37 °C for 15 minutes. After the incubation, the solution was transferred into 15 mL plastic tubes containing 800 µL of HCl. The color reaction was initiated by iodine reagent (1000 µL). The samples were then transferred to cuvettes, and absorption readings were measured at 630 nm using a spectrophotometer. These meticulous steps allowed for a comprehensive assessment of HG's inhibitory effects on α-amylase activity, indicating its potential as an anti-diabetic agent. The α-amylase inhibitory activity was calculated using Equation (1).

Equation (1):

$$\text{Inhibition of α-amylase \%} = [1 - (\frac{A630\ blank - A630\ sample}{A630\ starch\ solution - A630\ \text{negative control}})] * 100$$

2.5.2. α-Glucosidase assay

The experimental procedures in this study involved the utilization of various crucial reagents. A 0.1 M phosphate buffer at pH 6.8 (25 °C) was skillfully prepared by combining two solutions: Solution A (0.2 M NaH_2PO_4 H_2O) and solution B (0.2 M $Na_2HPO_4 2H_2O$). A p-nitrophenyl-α-D-glucopyranoside (p-NPG) solution at a 2.5 mM concentration was made by dissolving p-NPG in H_2O. Notably, this solution demonstrated stability over two weeks when stored within the temperature range of 0 °C to 5 °C. Additionally, a new enzyme, α-glucosidase (with an activity unit of 120 U/mg and a concentration of 1000 U/mL), was introduced. To maintain consistency in the experimental procedures, the enzyme's activity was adjusted to 0.2 U/mL by dissolving it in a 20 mL phosphate buffer. The inhibition potential of HG on α-glucosidase was evaluated according to Li *et al.*, (2018), with slight adjustments. In this assay, each sample dilution (0.2 mL) was combined with 250 µL of α-glucosidase (0.25 U/mL) in a pH 6.8 phosphate buffer solution and 500 µL phosphate buffer, and incubated at 37 °C for 5 minutes. Subsequently, 1 mL of 2.5 mmol/L p-NPG in phosphate buffer solution was added, and the reaction was allowed to proceed for additional 15 minutes at 37 °C. The reaction was stopped with 1 mL of 0.1 mol/L Na_2CO_3. After further incubation at 37 °C (15 minutes), the absorbance was measured at 405 nm. The α-glucosidase inhibitory activity was calculated using Equation (2).

Equation (2):

$$\text{Inhibition of α-glucosidase \%} = \left(\frac{A405\ control - A405\ sample}{A405\ control}\right) * 100$$

2.6. Polyphenol Oxidase Activity (PPO)

PPO catalyzes polyphenol oxidation to create colored products. Monitoring 420 nm absorbance changes assesses HG extract effects on PPO activity, offering insights into its significance and inhibition.

2.6.1. Extraction of polyphenol oxidase

In this study, the extraction method as described by Kschonsek *et al.* (2019) was utilized to isolate PPO. The extraction buffer, comprising 0.2 M phosphate buffer (pH 6.5) containing disodium hydrogen phosphate dihydrate (1.272 g), sodium dihydrogen phosphate monohydrate (1.774 g), polyvinylpolypyrrolidon (PvPP) (1 g), and Triton X-100 (250 µL), with a final volume of 100 mL using HPLC water, was employed. To initiate the extraction process, approximately 2 g ± 0.015 g of apple samples were precisely weighed in triplicate and transferred to individual 50 mL falcon tubes, maintaining a constant temperature of 4 °C. Subsequently, 6 mL of the extraction buffer was added to each tube, ensuring thorough mixing for the uniform distribution of the buffer. The homogenization process was repeated three times for one minute each, using an ultra-turrax operating at 7600 rpm, with the apparatus immersed in an ice bath to maintain low temperatures. After homogenization, the mixture underwent centrifugation at 4 °C for 30 minutes at 8000 rpm using a Heraeus Multifuge 1S-R centrifuge. The resulting supernatant was carefully collected and transferred to 15 mL falcon tubes. An additional centrifugation step was then performed for 10 minutes at 4 °C and 8000 rpm to ensure the proper separation of the lower phase. The collected lower phase was used for the determination of PPO activity. This passive extraction method effectively retrieved PPO from the samples, providing reliable enzyme activity data for further analysis.

2.6.2. *Examination of HG's inhibitory properties on polyphenol oxidase*

The inhibitory effect of PPO activity by HG from *H. macrophylla* was investigated using an approach modified from the method described by Bobo *et al*. (2022). HG extracts were prepared at different dilutions (4 mg/mL, 2 mg/mL, and 1 mg/mL) in phosphate buffer by dissolving approximately 40 mg of the extracts in 10% DMSO/MeOH. The experimental setup involved arranging the microplate into specific configurations: (A) A combination of 120 µL of substrate solution (0.033 M 4-methylcatechol) and 40 µL of PPO in phosphate buffer (pH 6.8). (B) A blank for the "A" wells, which consisted of 160 µL of buffer. (C) A mixture of 80 µL of substrate solution (0.033 M 4-methylcatechol), 40 µL of HG at different concentrations containing 10% DMSO, and 40 µL of PPO in phosphate buffer (pH 6.8). (D) A blank for the "C" wells, created by combining 120 µL of buffer and 40 µL of the inhibitor. The measurement of PPO activity was initiated immediately after adding the substrate. The enzymatic reaction, converting colorless 4-methylcatechol to orange 4-methyl-o-benzoquinone, was passively monitored by measuring absorbance changes at 420 nm over time at 25 °C. This process was executed using the BMG LABTECH FLUOstar OPTIMA plate reader and the Optima computer program (BMG LABTECH, Ortenberg, Germany) for over 100 measurement cycles over a duration of one hour, fifty-seven minutes, and fifteen seconds. Each cycle, lasting 24.75 seconds, included a double orbital shaking of the 96-well plate for one second at 120 rpm before each measurement. To ensure accuracy and reproducibility, all determinations were made in triplicate. Data were analyzed by using the Optima Data Analysis program (BMG LABTECH). An increase in absorbance of 0.001 was considered as 1 unit (U) of enzyme activity. The substrate turnover rate (U/(min*g)) was determined by calculating the slope of the absorption curve within the linear phase of the reaction. The linear phase began a few seconds after adding the substrate and lasted for a brief period before transitioning into a non-linear phase. The extent of PPO inhibition was determined by calculating the percentage of inhibition using Equation (3).

Equation (3):

$$\% \text{ PPO inhibition} = [(((A - B) - (C - D)) / (A - B)) \times 100]$$

3. STATISTICAL ANALYSIS

The data analysis in this study was conducted using Prism program for Windows, version 7.0, developed by GraphPad Software, Inc., San Diego, CA, USA. All experimental analyses were performed in triplicate, and the results are presented as mean ± standard deviation (SD). To compare data from different analyses, a one-way ANOVA (analysis of variance) followed by the Student-Newman-Keuls post-hoc test (S-N-K) was employed, enabling the identification of significant differences ($p < 0.05$) between multiple groups. Significant differences ($p < 0.05$) between two groups were identified by Paired T-test. Correlations between variables were assessed using Pearson's correlation coefficient (R), with the precision of the methods evaluated using the coefficient of determination (r^2). The relative inhibitory activity (%) and IC50 values were calculated from dose-response inhibition plots created using the GraphPad software, specifically using the dose-response inhibition model (log (inhibitor) vs. normalized response-variable slope).

4. RESULTS

4.1. Quantification and Identification of DHC

The results obtained from the preliminary analysis of *H. macrophylla* leaf extract using chromatography showed multiple peaks at varying retention times. The substances underwent qualitative validation using the API 2000 MS/MS system in negative ion mode (APCI). HG (22.52 min) and PD (24.55 min) were successfully identified through the Multiple Ion Scan with [M-1]- ions at m/z 255.3 and m/z 285.3, respectively. In this study, a calibration curve precisely quantified HG and PD levels, especially at lower concentrations. Consistency is maintained through triplicate analysis, while standard deviation enhanced result reliability, reinforcing the study's robustness. The research uncovers quantitative variations in DHC (HG and PD) content within *H. macrophylla.* Figure 1 shows the levels of HG (192 ± 3 mg/100 g) and PD (37 ± 3 mg/100 g) in a series of examined samples from *H. macrophylla.* The remarkable difference between HG and PD raises questions regarding the determinants affecting DHC levels in *H. macrophylla.*

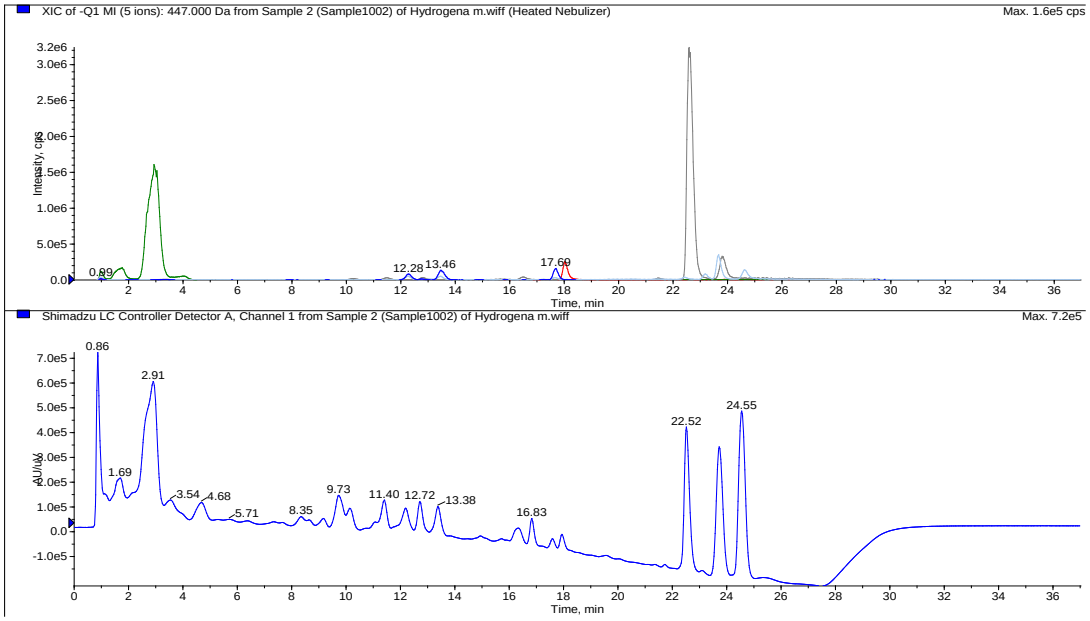

Figure 1. MS and UV chromatograms of *H. macrophylla* leaves extract. The UV analysis was conducted at 214 nm, detecting hydrangenol at 22.52 min and phyllodulcin at 24.55 min.

4.2. Fractionation of DHC

Utilizing preparative HPLC under optimized conditions, HG was successfully extracted from the leaf extract of *H. macrophylla* to ensure optimal purity. Three distinct chromatographic peaks emerged at 15.3, 21.4, and 22.3 minutes, to be further analysed. The identity and purity of HG for the peak at 15.3 minutes was confirmed by LC-MS/MS.

4.3. Biological Activities of HG Isolated from *H. macrophylla* Leaves Extract

4.3.1. Total phenolic contents and hydrophilic antioxidant capacity

In the realm of total phenolics content evaluation, the Folin-Ciocalteu method is employed to harness distinct redox properties. This approach facilitates the quantification of HG's total phenolics content (reducing capacity). The tabulated data within Table 1 illustrate variations in TP levels across diverse dilutions of HG, indicating a statistically significant increase ($p < 0.001$). The highest activity was observed in the factor 100 dilutions, at 11 ± 0.7 g GAE/100 g, while the lowest value was shown in the factor 5 dilutions, measuring 7.0 ± 1.4 g GAE/100 g. In the course of investigating various dilution factors of HG, an intriguing revelation was brought forth: a coefficient of variation of 15% was obtained (Table 1). Notably, the examination of different dilution factors of HG still revealed a significant enhancement in antioxidant capacity, as illustrated in Table 1. Both TEAC and ORAC values continued to exhibit clear increases, with robust statistical analysis confirming the significance of these changes ($p < 0.001$ for TEAC and $p < 0.05$ for ORAC). In a similar vein, the dilution effects were considered to be stronger at a coefficient of variation greater than 10% as presented in Table 1.

Table 1. Total phenolic content and antioxidant capacity of HG in *H. macrophylla* leaf extracts.

Dilution Factor	TP [g GAE/100 g]	H-ORAC [mmol TE/mmol]	H-TEAC [mmol TE/mmol]
200	$9.0 \pm 0.5^{b,c}$	19.0 ± 1.1^{a}	3.2 ± 0.20^{d}
100	11.0 ± 0.7^{e}	25.0 ± 2.1^{c}	2.3 ± 0.07^{c}
50	10.0 ± 0.6^{d}	26.0 ± 2.5^{c}	2.3 ± 0.03^{c}
20	9.0 ± 0.1^{c}	27.0 ± 1.0^{c}	2.1 ± 0.08^{b}
10	8.2 ± 0.2^{b}	$21.0 \pm 1.5^{a,b}$	2.0 ± 0.03^{b}
5	7.0 ± 1.4^{a}	15.0 ± 0.1^{a}	1.7 ± 0.05^{a}
Mean ± SD	9.1 ± 1.4	23.0 ± 4.3	2.3 ± 0.5
CV %	15.2	19.1	23.5

The data are presented as mean ± standard deviation (SD) for three replicates (n = 3); along with the coefficient of variation (CV). Significance of differences between samples ($p < 0.05$) was determined using one-way ANOVA followed by the Student-Newman-Keuls post-hoc test, with different letters (a/b/c/d/e) within a column indicating statistically significant distinctions. TP: Total phenolics content, ORAC: Oxygen radical absorbance capacity, TEAC: Trolox equivalent antioxidant capacity, GAE: gallic acid equivalents, TE: Trolox equivalents.

4.3.2. Anti-diabetic potential

Research is currently being conducted to explore compounds that inhibit the enzymes responsible for carbohydrate digestion, with the objective to delay the release of glucose into the bloodstream as a potential strategy for hyperglycemia control. In Figure 2a, the contrasting inhibitory effects of α-amylase activity by acarbose and HG are brought to attention. Acarbose showed an inhibition of 58% with an IC50 of 0.51 mg/mL. Its inhibitory effect was almost linear increasing up to 2.5 mg/mL, eventually reaching 90% inhibition at 10 mg/mL. Conversely, HG displayed significant inhibition, resulting in a 52% reduction with an IC50 of 3.6 mg/mL. These findings highlight concentration-dependent distinctions in their inhibitory capacities.

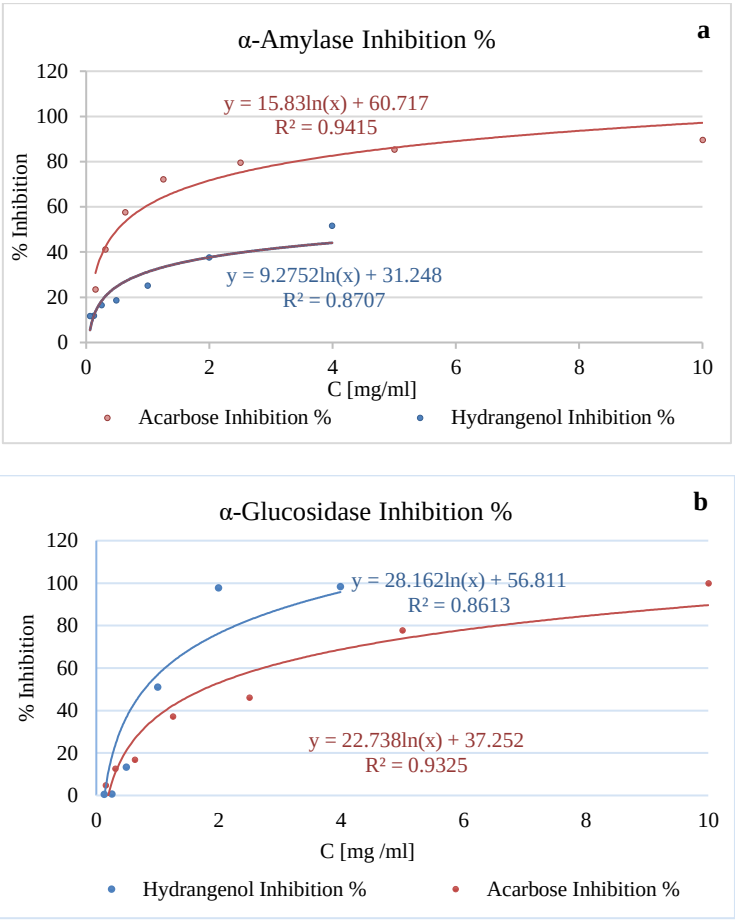

Figure 2. Comparative inhibition effects of HG from *H. macrophylla* leaf extract and acarbose at various concentrations (mg/mL) (a) α-amylase and (b) α-glucosidase.

Furthermore, Figure 2b unveils a strikingly consistent inhibition pattern in α-glucosidase activity. Acarbose displayed notable inhibition at 46% with an IC50 of 2.1 mg/mL, characterized by a distinct non-linear trend that captures attention. On the other hand, HG showcased an exceptional inhibition of 51% with an even lower IC50 of 0.97 mg/mL, underscoring its pronounced efficacy in controlling α-glucosidase activity. These results shed light on the potential of HG as a promising candidate for hyperglycemia management.

4.3.3. Anti-browning potential

One goal of this study was to evaluate the potential of HG to inhibit the PPO enzyme, a critical factor regarding food quality and color preservation. The importance of color in consumer choices becomes particularly evident when selecting food items or skincare products. Our investigation has produced significant and noteworthy findings: At concentrations of 1 mg/mL and 2 mg/mL, HG displayed substantial inhibition of PPO activity, with inhibition rates reaching 61%. Conversely, at a concentration of 4 mg/mL, a slightly reduced inhibition rate of 46% on PPO activity was observed. These results emphasize the considerable inhibitory potential of HG in relation to PPO activity, underscoring its promise as a valuable tool for regulating enzymatic browning processes and maintaining the visual appeal of food products (Table 2).

Table 2. Inhibitory impact of HG sourced from *H. macrophylla* leaf extracts on PPO activity.

	M ± SD	CV %	Inhibition %
PPO	0.6 ± 0.07	12	
HG 1 mg/mL	0.2 ± 0.02	7	61
HG 2 mg/mL	0.2 ± 0.02	10	61
HG 4 mg/mL	0.3 ± 0.03	11	47

The data are displayed as the mean browning intensity ± standard deviation (SD) across different concentrations in the analysis, accompanied by the coefficient of variation (CV) expressed as a percentage.

5. DISCUSSION and CONCLUSION

The significant difference between the contents of HG and PD in Figure 1 raises questions regarding the factors affecting DHC levels in *H. macrophylla*. The observed fluctuations in DHC levels within *H. macrophylla* could be attributed to two key factors. Firstly, seasonal changes and leaf age, as reported in previous studies, may influence these variations (Moll *et al.*, 2021). Secondly, genetic factors in *H. macrophylla* plants appear to play a crucial role in determining DHC content. Specific genotypes and genetic traits within these plants may be responsible for the observed differences. This genetic influence is further supported by the distribution of HG and PD among various species within the *Hydrangea* genus. HG is found widely across species, while PD is predominantly concentrated in specific species, highlighting the genetic underpinnings of DHC variability. It is plausible that distinct gene sets or clusters regulate the biosynthetic pathways of these compounds, resulting in varying expression levels among different species (Moll *et al.*, 2022).

Within the context of assessing antioxidant capacity and total phenolic contents, this study enhances our comprehension of how the antioxidant efficacy of HG is impacted by diverse dilution factors, providing insights into its performance under varying conditions. HG, a naturally occurring dihydroflavonol classified within the flavonoid subgroup, assumes a pivotal role in antioxidant functions. Flavonoids, such as HG, possess the capability to counteract reactive oxygen species, a fundamental mechanism contributing to their antioxidant potential. This is achieved through two primary pathways: the immediate neutralization of free radicals via hydrogen atom donation and participation in single-electron transfer reactions (Procházková *et al.*, 2011). Another notable attribute of flavonoids lies in their capacity to chelate transition metal elements. By forming chelates with metal ions in the human body, flavonoids effectively shield these ions from oxidation. Specific flavonoids can also chelate trace metal ions like Fe^{2+} and Cu^{+}, which play essential roles in oxygen metabolism and free radical formation (Malešev & Kunti 2007). Moreover, the observation that the coefficient of variation was higher than 10%, as demonstrated in Table 1, is noteworthy. This result is in accordance with the findings reported by Hengst *et al.* (2009), adding further support to the idea that a coefficient of variation exceeding 10% indicates a substantial impact of the dilution factor on antioxidant capacity. This observation is consistent with the research of Híc and Balík (2012), reaffirming that the dilution factors of materials have indeed influenced the results.

Acarbose, originating as a fermentation byproduct of actinoplanes species, functions as a competitive inhibitor targeting both α-amylase and α-glucosidase enzymes. Its primary role centers on the management of type 2 diabetes, achieved by effectively impeding the activity of glucosidases present in the upper gastrointestinal tract (Kim *et al.*,1999). This inhibitory effect is dosage-dependent, resulting in delayed absorption of glucose and a reduction in postprandial hyperglycemia. However, the administration of acarbose is often associated with gastrointestinal side effects, predominantly characterized by flatulence, and on occasion, instances of soft stools or abdominal discomfort (Rosak and Mertes, 2012). Research is

exploring compounds that inhibit carbohydrate-digesting enzymes to delay glucose release into the bloodstream, a potential strategy for controlling hyperglycemia.

The inhibitory effects against α-amylase and α-glucosidase, as observed in this study, align with the findings of Li *et al.* (2018), who also reported similar outcomes for flavonoid-rich extracts. Highlighting the distinctions between acarbose and HG enhances our understanding of complex interaction dynamics, enriching our comprehension of enzymatic inhibition mechanisms and their broader implications in the biological context.

Enzyme inhibitors, chemical compounds that hinder or completely suppress enzymatic catalysis, come in different types. Reversible apoenzyme inhibitors, for instance, fall into three categories: competitive, uncompetitive, and noncompetitive (or mixed-type) (Sharma, 2012). Uncompetitive inhibitors bind to a site on the enzyme-substrate complex being different from the substrate's site. Competitive inhibitors, on the other hand, occupy the same site as the substrate on the enzyme. Mixed inhibitors interact with both the enzyme and the enzyme-substrate complex. In the non-competitive or mixed mechanism, the enzyme undergoes a conformational change into an inactive state, leading to an inability to bind to the substrate or release the product. The competitive mechanism in contrast involves the reversible blockage of the active site, preventing substrate molecules from binding (Nelson & Cox, 2005).

The inhibitory effectiveness of phenolic compounds in various modes (mixed, uncompetitive, competitive) is intricately linked to their molecular structures (Kim *et al.*, 2009). Phenolic acids' inhibitory potential is heavily influenced by the presence of hydroxyl and methoxy groups within their aromatic ring (Malunga et al., 2018). In contrast, flavonoids, with additional hydroxyl groups, exhibit superior inhibition of α-glucosidase activity (Di Stefano *et al.*, 2018). The introduction of more aromatic hydroxyl groups through glucoside substitutions further enhances enzyme inhibition (Şöhretoğlu *et al.*, 2018). Molecular docking studies indicate that phenolic compounds establish interactions with both active and allosteric sites, primarily through hydrogen bonding, hydrophobic interactions, and van der Waals forces, facilitating their binding with enzymes (Di Stefano *et al.*, 2018). It's worth noting that p-coumaric acids are notably effective in inhibiting α-glucosidase through mixed noncompetitive inhibition (Li *et al.*, 2009)

Enzymatic browning, common in fruits and vegetables, results from the oxidation of phenolic compounds catalyzed by the PPO enzyme. Tyrosinase, a key enzyme, triggers this process when it interacts with polyphenols in the presence of oxygen, disrupting cell structure. The PPO enzyme is classified into two categories: EC1.14.28.1 (tyrosinase, cresolase, and monophenol monooxygenase) and EC1.10.3.1 (o-diphenol oxygen oxidoreductase, diphenol oxidase, and catechol oxidase) (Moon *et al.*, 2020; Mayer, 2006; Hurrel & Finot, 1984). Simultaneously, roughly 15% of the global population utilizes skin-whitening agents to diminish melanin production, the pigment responsible for skin color and protection against UV radiation. Nevertheless, the excessive accumulation of melanin stemming from factors like UV exposure and certain medications can lead to the formation of pigmented patches, giving rise to aesthetic concerns (Loizzo *et al.*, 2012 and Briganti *et al.*, 2003).

These results illustrate the temporal dynamics of browning intensity. The tabulated data accentuates the prominent inhibitory efficacy of HG in relation to PPO activity, thereby emphasizing its potential utility as a modulator of enzymatic browning processes. Within the domain of the flavonoid subclass, HG manifests as an inherent dihydroflavonol, characterized by the presence of hydroxyl groups, thereby assuming a central regulatory position in anti-browning mechanisms. Notably, these polyphenols boast hydroxyl groups that can engage in electron donation to intermediate quinones, effectively impeding the oxidation process. Furthermore, enzymes stemming from phenolic compounds exhibit the ability to chelate metal ions, particularly Cu^{2+}, at both binding and catalytic sites of the PPO enzyme. Consequently,

the formation of hydrogen bonds between these phenolic derivatives and the active sites of enzymes leads to a reduction in enzyme activity (Sae-leaw *et al.*, 2019).

In summary, this study thoroughly investigated the biological potential of hydrangenol, a key bioactive compound derived from *Hydrangea macrophylla*. Employing rigorous analytical techniques and varying hydrangenol concentrations, the research revealed significant antioxidant properties and notable enzyme inhibition activities, including anti-diabetic and anti-browning effects. These results feature the manyfold applications of hydrangenol across various scientific fields. As a result, this research provides a solid foundation for future investigations in this scientific area.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Ahlam Al-Yafeai: Conception and experimental design, Experiment execution (LC-MS/MS analysis), Fractionation and anti-diabetic potential, Evaluation (antioxidant capacity, PPO), Manuscript preparation and data analysis, Manuscript review and editing. **Barbara Schmitt**: Fractionation and anti-diabetic potential. **Angelika Malarski**: Experiment execution (LC-MS/MS analysis). **Volker Böhm**: Conception and experimental design, Manuscript review and editing.

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Research Article

Effect of solvent polarity of phytochemical analysis of different Moroccan sites of *Opuntia ficus-indica* (L.) Mill. pads

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Abstract: The current study focuses on the study of phenolic compounds in methanol, dichloromethane, and chloroform extracts as solvents with increasing polarity, in order to quantify the secondary metabolites of *Opuntia ficus indica* pads collected from three Moroccan sites (Oulad Boubker, Imzouren, and Skoura). The findings showthat betalains and carotenoids were present, in varying amounts depending on the study area. The methanolic extracts had the concentrations of compounds. When analyzing the chemical composition using Gas Chromatography - Mass Spectrometry, it was found that linoleic acid was the unsaturated fatty acid accounting for 14.279%. Palmitic acid, fatty acid derivatives and alkanes were also detected in all regions. Among the extracts Imzouren had the concentration of fatty acids (24.874%) followed by Oulad Boubker (13.907%) and Skoura (13.319%). However, oleic acid was detected only in the extract of Skoura. Hence, we were able to confirm that the chemical differences among the various *Opuntia* extracts were primarily influenced by the selection of the extracting solvent and the prevailing climatic and geographical factors.

1. INTRODUCTION

Opuntia ficus-indica (OFI) popularly named prickly pear, originally from Mexico, was introduced in North Africa (Morocco, Algeria, Tunisia) around the 16th century (Boutakiout, 2015). The economic importance of this plant lies in the production of the fruit for human consumption and its use for animal feed purposes. This plant holds significance due to its adaptation to prevailing environmental and climatic conditions, as well as its ability to withstand various soil and climate variations (Hernández-Urbiola *et al.*, 2011), its nutritional properties, and the use of its waste to produce biogas (Inglese *et al.*, 2018).

The Green Morocco Plan, a novel agricultural development approach, presents a tangible chance for the advancement of the cactus industry (Bouzoubaâ *et al.*, 2014). The different parts of OFI (pads, fruits, and flowers) present a variability of shape, color, weight, content of acids

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antioxidant compounds (phenols, flavonoids, betaxanthin and betacyanine), etc. These different parameters depend on the cultivar and are strongly influenced by the environment (Bouzoubaâ *et al.*, 2014; Mouas *et al.*, 2021; Piga, 2004). The composition of polyphenols in prickly pear and its derived products have been thoroughly identified, encompassing compounds such as phenolic acids, flavonoids, and lignans (Mena *et al.*, 2018; Serra *et al.*, 2013). In addition, the fruit contains large amounts of betalains (Mena *et al.*, 2018).

The present study aims at quantifying the secondary metabolites and identifying the chemical composition of the pads using increasing polarity solvents and three sites present in the regions of Driouche, Al Houceima and Ouarzazate in Morocco.

2. MATERIAL and METHODS

2.1. Sample Collection

The mature pads of *Opuntia ficus indica* (OFI) were collected, between October and November 2021, from three sites that differ by their earth's topography and the climate. These are Imzouren's site, which is a town located in the province of Al Hoceima in the northern coast of the Mediterranean in the Tangier Tetouan Al Hoceima region, Oulad Boubkar's site, which is a Moroccan rural commune in the subdivision of Driouch, in the Oriental region, and Skoura's site, located 40 km from Ouarzazate in the South of Morocco in the Sousse Massa region.

2.2. Plant Material

Mature and healthy pads were selected, placed in a dark and humid place, and transported to the laboratory where they were cleaned and stripped of their spines, disinfected with 10% alcohol, and then rinsed using distilled water before being cut into tiny cubes. Then, the plant material was dried in the oven at 60°C under ventilation for 18 to 20 hours with the verification of the stability of the weight of the material during the drying process. The dried material was finely powdered and stored away from light and in darkness.

2.3. Preparation Of Extracts

Solid-liquid extraction is performed with methanol (MeOH), chloroform and dichloromethane as solvents of increasing polarity following the approach outlined by the International Organization for Standardization with some modifications (ISO, 2003). The powder of pads from different locations (10g) is macerated in 100ml of solvents for 48h in the cold and dark. The filtrates containing the extracts were retrieved through filtration and then preserved in opaque containers, being stored at 4°C until they were employed.

2.4. Phenolic Compounds Analysis

The method for determining the total polyphenol content of the extracts, as described by Singleton *et al.* (1999), utilizes gallic acid as a standard. The results are expressed as gallic acid equivalents per gram of the dry matter (mg GAE/g DM).The quantification of total flavonoid content was carried out following the procedure outlined by Djeridane *et al.*, while the flavones and flavonols were measured using the method of (Kosalec *et al.*, 2005). The results of these flavonoids are stated as mg quercetin equivalent per gram of pad's dry matter (mg QE/g DM) referring to the calibration curve of quercetin as a standard. The quantification of condensed tannins was determined using the acidified vanillin method adapted by (Boutakiout *et al.*, 2015), and the hydrolysable tannins were measured using the Willis method (Willis *et al.*, 1998), and the results are presented in terms of milligrams of catechin equivalent per gram of dry plant material (mg CE/g DM) with reference to the catechin calibration curve.

2.5. Extraction And Quantification of Betalains

Extraction of betalains was performed according to the protocol described by Castellanos-Santiago and Yahia, which consists of shaking a mixture of 100 mg of pad's powder and 20 ml of citrate-phosphate buffer (pH 6.5) for 10 min in the dark and then centrifuging at 12000xg at

15 °C for 15 min (Castellanos-Santiago & Yahia, 2008). The recovered supernatant is filtered and the absorbance is measured at 538 nm for betacyanins and at 480 nm for betaxanthantins.

2.6. Extraction And Determination of Carotenoids

The protocol for carotenoid extraction used was the one outlined by (Sass-kiss *et al.*, 2005). 4g of pad's powder was homogenized for 15min with a solvent mixture (10 ml) (hexane/acetone/ethanol) (1V:2V:2V), and then centrifuged at 5500rpm at 4 °C for 15 min. The upper hexane layer, which holds the pigment, is removed and its absorbance is measured at 430 nm.

2.7. GC-MS

In the analysis of the pad powder extract using GC-MS, 1 µL of each extract was automatically injected via an autoinjector into a BRUKER 456 GC EVOQ gas chromatograph. This system was equipped with an RXL-5SIL MS column from BURKER, GERMANY, and coupled to a GC mass spectrophotometer (3Q: triple Quadrupole) operating in electron impact mode. The scan range covered m/z values from 10 to 600 atomic mass units. The column temperature was initially set at 35 °C and gradually increased to 300 °C at a rate of 5 °C/min, remaining at that temperature for 10 minutes. Helium (He) was used as the carrier gas at a flow rate of 1.5 mL/min, and the injection chamber was maintained at a temperature of 300 °C (Lahlou *et al.*, 2014).

2.8. Statistical Analysis

The experimental analysis of the data was done using IBM SPSS version 23. The data were presented as the mean and standard deviation (SD) from three separate experiments conducted in triplicate (ANOVA), followed by Tukey test. We considered $p \leq 0.05$ to be statistically significant.

3. RESULTS

In this research, the content of total phenolic compounds measured in OFI pads is significantly close between the three extraction solvents, as well as between the three study sites (Tables 1, 2, and 3). MeOH extracts are richer in total polyphenols compared to dichloromethane and chloroform extracts. However, chloroform provided the lowest concentrations of these compounds for all three study sites.

The content of condensed and hydrolyzable tannins in OFI pads revealed fairly close values for the three extraction solvents as well as for the three study sites (Table 4, 5). The pads contain an amount of betalains ranging from 48.98±0.02 to 49.07±1.32 mg per 100 g dry weight (DW) depending on the study site (Table 6).

We contrasted the chemical makeup of extracts obtained using MeOH, dichloromethane, and chloroform from pads of the OFI cactus. These pads were sourced from three distinct Moroccan geographical areas (as shown in Tables 7, 8, and 9. The initial findings from the GC-MS examination of the MeOH extract indicated the existence of various fatty acids and their derivatives. Their amounts varied depending on the location, climatic conditions and maturity of the plant. The primary components of these pad extracts consisted of linoleic acid, present in quantities varying from 6.281 % to 14.279 %, subsequent to palmitic acid (3.077 to 4.740 %), fatty acid derivatives (28.160 to 65.567 %) including alcohol functions and fatty acid esters, terpenes (2.486 to 15.054 %), phytosterols (1.273 to 15.615 %) and other compounds (6.950 to 17.531 %). Among the samples, Imzouren's extract exhibited the greatest proportion of linoleic acid (14.279 %), succeeded by Oulad Boubker and Skoura with 8.614 % and 6.281 % respectively. Oleic acid was detected only in the extract of Skoura with a value of 1.260 %.

Table 1. Total polyphenol contents in extracts of *O. ficus indica* pads (mg GAE/g DM).

Solvents	Oulad Boubker	Imzouren	Skoura
Dichloromethane	24.02±0.05 ^{d}	22.97±0.08 ^{b}	23.25±0.03 ^{c}
Chloroforme	23.06±0.06 ^{bc}	22.25±0.09 ^{a}	22.82±0.04 ^{bc}
MeOH	24.24±0.22 ^{d}	24.15±0.07 ^{d}	24.32±0.15 ^{d}

Letters a-d signify that there is a statistical difference ($p < 0.05$) among each individually tested parameter.

Table 2. Contents of flavonoids in the extracts of *O. ficus indica* pads (mg QE/g DM)

Solvents	Oulad Boubker	Imzouren	Skoura
Dichloromethane	11.10 ± 0.09 ^{abc}	11.25 ± 0.04 ^{bc}	11.40 ± 0.01 ^{c}
Chloroforme	10.95 ± 0.06 ^{ab}	10.85 ± 0.06 ^{a}	10.90 ± 0.09 ^{a}
MeOH	12.66 ± 0.08 ^{e}	12.61 ± 0.12 ^{e}	12.10 ± 0.08 ^{d}

Letters a-e signify that there is a statistical difference ($p < 0.05$) among each individually tested parameter.

Table 3. Contents of flavones and total flavonols in the extracts of *O. ficus indica* pads (mg QE/g DM).

Solvents	Oulad Boubker	Imzouren	Skoura
Dichloromethane	6.23±0.05 ^{ab}	6.48±0.02 ^{b}	6.51±0.02 ^{b}
Chloroforme	6.08±0.07 ^{a}	5.94±0.05 ^{a}	6.02±0.04 ^{a}
MeOH	7.79±0.17 ^{d}	7.29±0.04 ^{c}	6.98±0.13 ^{c}

Letters a-d signify that there is a statistical difference ($p < 0.05$) among each individually tested parameter.

Table 4. Contents of condensed and hydrolysable tannins in the extracts of *O. ficus indica* pads (mg CE/g DM).

Solvants	Oulad Boubker	Imzouren	Skoura
Chloroforme	0.14±0.03 ^{a}	0.15±0.04 ^{a}	0.15±0.03 ^{a}
Dichloromethane	0.17±0.03 ^{a}	0.14±0.03 ^{a}	0.13±0.03 ^{a}
Methanol	0.18±0.02 ^{a}	0.16±0.04^{a}	0.16±0.04 ^{a}

The letter "a" indicates that there is no statistical difference ($p < 0.05$) between the tested samples.

Table 5. Contents of hydrolysable tannins in the extracts of *O. ficus indica* pads (mg CE/g DM).

Solvants	Oulad Boubker	Imzouren	Skoura
Chloroforme	0.07±0.01 ^{a}	0.08±0.004 ^{a}	0.07±0.01 ^{a}
Dichloromethane	0.09±0.03 ^{a}	0.07±0.01 ^{a}	0.06±0.01 ^{a}
Methanol	0.10±0.02 ^{a}	0.09±0.01 ^{a}	0.09±0.01 ^{a}

The letter "a" indicates that there is no statistical difference ($p < 0.05$) between the tested samples.

Table 6. Betalains and carotenoids contents of *O. ficus indica* pads (mg/100g DW).

Parameters	Oulad Boubker	Imzouren	Skoura
Betalains (betacyanins and betaxanthantins)	49.07±1.32 ^{a}	48.98±0.02 ^{a}	49.01±0.01 ^{a}
Carotenoids	13.71±0.15 ^{b}	13.52±0.08 ^{b}	13.09±0.07 ^{c}

Letters a-c signify that there is a statistical difference ($p < 0.05$) among each individually tested parameter.

Table 7. Chemical composition of methanolic extracts of *O. ficus indica* pads.

Compounds	RT (min)	Formula	Area %		
			Oulad Boubker	Imzouren	Skoura
3-Furaldehyde	8.158	$C_5H_4O_2$	3.879	0.254	14.326
2-Methyltetrahydrothiophene	10.89	$C_5H_{10}S$	-	11.345	-
4-oxo-5-methoxy-2-penten-5-olide	14.403	$C_6H_6O_4$	-	-	0.882
Undecane	22.203	$C_{11}H_{24}$	1.179	1.792	0.585
Oleic Acide	24.71	$C_{18}H_{34}O_2$	-	-	1.260
5-Hydroxymethylfurfural	28.418	$C_6H_6O_3$	62.570	23.124	24.818
Thymol	31.779	$C_{10}H_{14}O$	0.362	-	-
2-methoxy-4-vinyl-phenol	32.049	$C_9H_{10}O_2$	-	1.929%	-
Stearyl alcohol	49.245	$C_{18}H_{38}O$	0.700	-	-
Dibutyladipate	50.09	$C_{14}H_{26}O_4$	1.340	2.204	1.974
Neophytadiene	52.588	$C_{20}H_{38}$	1.980	1.257	12.170
Phytol	53.354	$C_{20}H_{40}O$	1.629	1.228	2.884
Palmitic acid	56.705	$C_{16}H_{32}O_2$	3.077	4.740	4.149
Methyl 9,10-octadecadienoate	60.53	$C_{19}H_{34}O_2$	-	-	0.374
Methyl 8,11,14-heptadecatrienoate	60.689	$C_{18}H_{30}O_2$	-	-	0.513
Linoleic acid	61.758	$C_{18}H_{32}O_2$	8.614	14.279	6.281
Elaidamide	67.942	$C_{18}H_{35}NO$	-	0.918	0.992
11,14-Octadecadienoic acid, methyl ester,	69.573	$C_{19}H_{34}O_2$	-	-	0.743
methyl hydroxylinolenate	69.741	$C_{19}H_{32}O_3$	-	-	0.824
Octocrylene	75.256	$C_{24}H_{27}NO_2$	0.956	1.915	2.180
methyl hydroxylinolenate	75.638	$C_{19}H_{32}O_3$	-	-	0.328
Oleamide	78.337	$C_{18}H_{35}NO$	2.216	5.954	-
Cholesterol	80.232	$C_{27}H_{46}O$	-	-	0.457
Clionasterol	84.512	$C_{29}H_{50}O$	1.273	-	4.643
α,-Tocopheryl acetate	85.416	$C_{31}H_{52}O_3$	-	-	0.479
E Vitamin	85.436	$C_{27}H_{46}O_2$	0.714	2.211	-
β,-Sitosterol	88.749	$C_{29}H_{50}O$	-	10.406	10.972

Table 8. Chemical composition of dichloromethane extracts of *O. ficus indica* pads.

Compounds	RT (min)	Formula	Area %		
			Oulad Boubker	Imzouren	Skoura
Nonadecane	47.756	$C_{19}H_{40}$	5.298	3.128	5.915
Dibutyl adipate	50.031	$C_{14}H_{26}O_4$	7.285	4.727	7.427
Neophytadiene	52.535	$C_{20}H_{38}$	4.148	-	6.693
Octadecane	55.16	$C_{18}H_{38}$	-	2.666	-
Palmitic acid	56.677	$C_{16}H_{32}O_2$	4.197	4.301	7.902
Butylated Hydroxytoluene	57.501	$C_{15}H_{24}O$	-	3.643	8.808
Octocrylene	75.025	$C_{24}H_{27}NO_2$	-	2.897	-
Elaidamide	78.077	$C_{18}H_{35}NO$	-	-	12.176
9-Octadecenamide	78.16	$C_{18}H_{35}NO$	39.387	-	-
1-Dotriacontanol	83.048	$C_{32}H_{66}O$	-	25.484	-
Triacontane	85.196	$C_{30}H_{62}$	5.193	23.316	19.430
Vitamine E	85.383	$C_{27}H_{46}O_2$	4.496	5.331	6.258
β-Sitosterol	88.62	$C_{29}H_{50}O$	9.620	12.849	22.798
Hentriacontane	89.05	$C_{31}H_{64}$	4.252	-	-
Octatriacontyl pentafluoropr	90.796	$C_{41}H_{77}F_5O_2$	-	3.661	-

Table 9. Chemical composition of chloroform extracts of *O. ficus indica* pads.

Compounds	RT (min)	Formula	Area %		
			Oulad Boubker	Imzouren	Skoura
2,4-Di-tert-butylphenol	40.487	$C_{14}H_{22}O$	-	4.031	5.976
Docosane	47.755	$C_{22}H_{46}$	-	3.661	-
Nonadecane	47.756	$C_{19}H_{40}$	-	-	5.456
Dibutyl adipate	50.027	$C_{14}H_{26}O_4$	11.778	4.925	-
Hexanedioic acid, dibutyl ester	50.031	$C_{14}H_{26}O_4$	-	-	6.236
Neophytadiene	52.518	$C_{20}H_{38}$	3.173	-	3.897
Tricosane	54.911	$C_{23}H_{48}$	0.967	3.116	-
Hexacosane	54.914	$C_{26}H_{54}$	1.298	7.714	8.199
Eicosane	62.211	$C_{20}H_{42}$	2.431	-	-
Phytol	71.438	$C_{20}H_{40}O$	1.100	-	-
Hentriacontane	72.897	$C_{31}H64$	2.173	-	-
Heneicosanoic acid, methyl ester	73.717	$C_{22}H_{44}O_2$	1.257	-	-
palmitic acid	75.735	$C_{16}H_{32}O2$	2.759	-	-
7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	76.459	$C_{17}H_{24}O_3$	1.452	-	-
Elaidamide	78.083	$C_{18}H_{35}NO$	8.932	-	12.529
Tetratriacontane	82.935	$C_{34}H_{70}$	0.316	-	-
Linoelaidic acid	83.168	$C_{18}H_{32}O_2$	1.345	-	-
Heptadecane	84.427	$C_{17}H_{36}$	0.743	-	-
9-Octadecenoic acid	84.624	$C_{18}H_{34}O_2$	6.553	-	-
Triacontane	85.121	$C_{30}H_{62}$	-	33.559	36.894
β—Sitosterol	88.62	$C_{29}H_{50}O$	-	10.525	20.814
Octocrylene	102.944	$C_{24}H_{27}NO_2$	3.380	-	-
Heptacosane	105.481	$C_{27}H_{56}$	4.001	-	-
Nonacos-1-ene	108.749	$C_{29}H_{58}$	1.593	-	-
Squalene	108.999	$C_{30}H_{50}$	7.001	-	-

4. DISCUSSION and CONCLUSION

The total polyphenol content of pads from the Oulad Boubker site is the highest, with a concentration of 24.24±0.22 mg GAE/g DM, followed by those from the Skoura and Imzouren sites, which have concentrations of 24.32±0.15 and 24.15±0.07 mg GAE/g DM, respectively. These values are close considerably with those found by (Msaddak, 2018) (24.85 mg EAG/g DM), for the methanolic extracts of pads of the same species. On the other hand, a much higher value (63.54±1.13 mg GAE/g DM) was reported by Moussaoui, (2020). Alternative research carried out by Gallegos-Infante *et al.*, on Algerian pads of the same species showed a total polyphenol content of 63.54±1.13 mg GAE/g DM (Gallegos-Infante *et al.*, 2009). According to De Santiago *et al.* (2018), this value ranged from 1.7 mg/g DM to 180 mg GAE/g DM.

Concerning solvents, the results presented in Table 2 reveal that the total flavonoid contents of MeOH extracts of OFI pads are the highest ranging from 12.10±0.08 mg QE/g DM to 12.66±0.08 mg QE/g DM followed by those of dichloromethane extracts which are between 11.10±0.09 mg EQ/g DM and 11.40±0.01 mg QE/g DM; on the other hand the lowest values are those obtained from chloroform extracts, and the same for flavones and flavonols.

In addition, all three sites have significantly close values for each solvent separately. Comparing the locations, the lowest concentrations of total flavonoids are those obtained by

pads from the Imzouren site with values ranging from 10.85±0.06 mg QE/g DM to 12.61±0.12 mg QE/g DM, followed by those of Skoura's site with values between 10.90±0.09 and 12.10±0.08 mg QE/g DM, while the pads from Oulad Boubker's site are the richest in flavonoids with concentrations ranging from 10.95±0.06 mg QE/g DM to 12.08±0.53 mg QE/g DM. when it comes to flavones and flavonols, Oulad Boubker's site has the highest values from 6.08±0.07 to 7.79±0.17 mg QE/g DM (Table 3).

In other similar studies, a higher value was revealed by Moussaoui (18.07±0.77 mg QE/g DM) for flavonoids and (12.20±0.07 mg QE/g DM) for flavones and flavonols (Moussaoui, 2020), while Guevara-Figueroa *et al.* (2010), who worked on pads of two varieties of OFI, found lower values (9.8 mg and 5.9 mg QE/g DM).

MeOH extracts always contained higher amounts of tannins than those determined by dichloromethane and chloroform extracts with values ranging from 0.16±0.04 to 0.18±0.02 mg CE/g DM, from 0.13±0.03 to 0.17±0.03 mg CE/g DM and 0.14±0.03 to 0.15±0.03 mg CE/g DM for condensed tannins, and 0.9±0.1 to 0.10±0.02 mg CE/g DM, 0.06±0.01 to 0.09±0.03 mg CE/g DM, and 0.07±0.01 to 0.08±0.004 mg CE/g DM for hydrolyzable tannins. The quantity of tannins found in the Oulad Boubker site is between 0.14±0.03 to 0.18±0.02 mg CE/g DM for condensed tannins, 0.07±0.01 to 0.10±0.02 mg CE/g DM for hydrolysable tannins, followed by Imzouren's site with values ranging from 0.14±0.03 to 0.16±0.04 mg CE/g DM, from 0.07±0.01 to 0.09±0.01 mg CE/g DM for condensed and hydrolysable tannins respectively, while the lowest values concern Skoura's site with contents ranging from 0.13±0.03 to 0.16±0.04 mg CE/g DM for condensed tannins and from 0.06±0.01 to 0.09±0.01 mg CE/g DM for hydrolysable tannins.

These findings align with the research conducted by Boutakiout in 2015, on pads from five different regions in Morocco (Khouribga, Beni Mellal, Bejaad, Oued Zam and Kelaa), where he found values that varied between 12.10±0.21 to 18.23±0.36 mg ATE/100ml for condensed tannins and 1.24±0.02 to 1.33±0.15 mg ATE/100ml for hydrolysable tannins. Similarly, Moussaoui revealed values of 0.18±0.02 and 0.1±0.03 mg ATE/g DM (Moussaoui, 2020), while the content of condensed tannins in pad juice, recorded by Boutakiout, was 0.01 mg CE/mL (Boutakiout, 2015).

The content of Betalaines and carotenoids in pads from Imzouren site is the lowest compared to other sites with a value equal to 48.98±0.02 mg/100g DW. These values are very close to those found by Moussaoui (48.13±2.2 mg/100g DW) for Algerian pads of the same species (Moussaoui, 2020). The extraction yield of betalains is proportional not only to the variety, part or tissue selected and its growth stage, but also to the extraction technique used and its parameters. These factors even cause heterogeneous distribution and variation of betalains, individual structures and betacyanin/ betaxanthin ratios in different parts of *Opuntia* (Cai *et al.*, 1998; Sanchez-Gonzalez *et al.*, 2013; De Wit *et al.*, 2019). In 2019 De Wit *et al.*, also identified the presence of betalains in pads of *Opuntia*, South African, as they reported that the levels of these pigment depend on the cultivar.

The value of carotenoids in Moroccan pads of OFI observed in this study ranged from 13.09±0.07 to 13.71±0.15 mg/100g DW depending on geographical locations (Table 6). The highest value was found in Oulad Boubker (13.71±0.15 mg/100g DW), followed by Imzouren (13.52±0.08 mg/100g DW) and Skoura (13.09±0.07 mg/100g DW), noting that these values are significantly close. In a similar analysis carried out by (Jaramillo-Flores *et al.*, in 2003; and Bensadón *et al.*, in 2010, the authors reported the presence of carotenoids in *Opuntia* pads with values ranging from 2 to 23.18 mg/100g DW, and they indicated that these values can be increased depending on the heat treatment used. For the variants related to the development conditions of the plants, the polarity of the carotenoids influences their solubilities in the extraction solvents and the extraction itself (Tsao *et al.*, 2004; Dias *et al.*, 2009). Ethanol

remains the best solvent for extraction of polar carotenoids while hexane is the best choice for extraction of apolar carotenoids (Amorim-Carrilho *et al.*, 2014).

However, a very small amount of fatty acids was present in the dichloromethane extracts with values ranging from 4.197 to 7.902% of palmitic acid, fatty acid derivatives (19.609 to 46.671 %), alkanes (14.744 to 29.109 %), phytosterols (9.620 to 22.798 %), tocopherols (4.496 to 6.258 %) and other compounds (3.661 to 15.501%). The extract of Oulad Boubker has the lowest amount of fatty acids (4.197%), followed by Imzounren (4.301 %) and Skoura (7.902 %). The extracts of Oulad Boubker and Imzouren are richer in fatty acid derivatives with 46.671% to 33.108 %, than the extract of Skoura with 19.609 %.

In addition, a moderate amount of alkanes was found in the chloroform extracts with values ranging from 21.507% to 50.548% and a small amount of fatty acid derivatives (4.925 % to 25.347 %). However, a very low amount of fatty acid was exclusively detected within the extract of Oulad Boubker with nearly 10.657 % and traces of ketone with 1.452 %. The terpenes were presented only in the two extracts of Oulad Boubker and Skoura with values respectively of 13.171 % and 3.897 %. While phytosterols were found in the two extracts of Imzouren and Skoura with values of 10.525 % and 20.814 % respectively.

On a different note, Algerian pads of OFI showed the presence of seventeen alkaloids, including Isoquinoline, imidapyrazole and pyrazolo-benzothiazole derivatives (Guevara-Figueroa *et al.*, 2010). Conversely, another study identified the occurrence of palmitic acid (19.81 to 24.27 %), linoleic acid (66.57 to 72.39 %), stearic acid (2.37 to 9.61 %) and myristic acid (0.97 to 2.91 %) in another species of *Opuntia* (*Opuntia dillenii*) (Loukili *et al.*, 2021), this study is consistent with the one done by Alsaad *et al.* (2019). However, a similar study indicated an alternative fatty acid characteristic of Chinese *Opuntia dillenii* including linolenic acid (66.56 %), palmitic acid (19.78 %), stearic acid (9.01 %) and linoleic acid (2.65 %) (Liu *et al.*, 2009).

The prevalence of linoleic acid as the primary constituent in the *Opuntia ficus* plant implies potential health advantages associated with it. In human tissues, linoleic acid is chiefly transformed into arachidonic acid (ARA), α-linolenic acid (ALA), and docosahexaenoic acid (DHA) (Waleed *et al.*, 2017). Linoleic acid and its related group of fatty acids are collectively known as omega-6 fatty acids.

Multiple experimental and medical investigations have indicated the potential positive impact of including linoleic acid in a diet. Especially, if these benefits encompass the enhancement of cardiovascular well-being, notably the improvement of plasma lipid profiles, and a better glycemic control by increasing insulin resistance. Moreover, a higher dietary intake or elevated tissue levels of linoleic acid have been correlated with a decreased likelihood of cardiovascular disease and the development of metabolic syndrome or type 2 diabetes. Nevertheless, further clinical trials are necessary to comprehensively evaluate the underlying mechanisms that connect the observed health benefits with the intake of this essential fatty acid (Marangoni *et al.*, 2020).

The molecule profile reported in this study showed that pad varieties from distinct sites exhibit diverse types or compositions of molecules.

In our study, experiments on animal models are necessary to investigate and confirm the beneficial effect of the *Opuntia ficus* plant and its role in metabolism.

5. CONCLUSION

In this work, our results showed the presence of a proximal difference in phenolic compounds of OFI pads between the three geographical locations studied. Phenolic compounds were found to be higher in extracts from Oulad Boubker, followed by Skoura and Imzouren. While betalains, carotenoids and alkaloids were found to be very low. Quantification of these

compounds showed that MeOH extracts had the highest concentrations, followed by dichloromethane and chloroform. Chemical composition analysis by GC-MS demonstrated that the extracts of the studied OFI pads from three locations contained a significant proportion of fatty acids derivatives and alkane, as well as a small proportion of fatty acids. Linoleic acid was the predominant fatty acid detected, with a maximum content of 14.279 %, followed by palmitic acid (maximum value of 7.902 %).

Therefore, Cladodes extracts contain compounds that may be useful for functional food and cosmetic applications. Moreover, the research also indicated that the chemical diversity of the various *Opuntia* extracts was mainly influenced by the selection of the extraction solvent and the prevailing climatic and geographic factors.

In the future, it would be prudent to continue biotechnological research, improve our grasp of its genetic variability using molecular biology techniques, and conduct more complete studies into its agronomic worth.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Sara Razzak: Conception, Methodology, Resources, Visualization, Software, Data Collection and Processing, Analysis and Interpretation, and Writing -original draft. **Marouane Aouji**: Visualization, Analysis and Interpretation, and Software. **Chaima Sabri:** Visualization, Correction and Literature Review. **Hiba Benchehida:** Writing Translating and Literature Review. **Mariame Taibi:** Methodology and Visualization. **Youness Taboz:** Supervision, Correction and validation, Critical Review

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Research Article

Inhibition of carbonic anhydrase and cholinesterase enzymes by acetone extract of *Bryoria capillaris* (Ach.) Brodo & D.Hawksw.

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Abstract: In traditional medicine, people commonly consume *Bryoria capillaris* (BC) as flour and tea, particularly in Northeast Anatolia, where it is one of the most prevalent lichen species. This study aimed to obtain an acetone extract of BC and investigate its inhibitory effects on carbonic anhydrase (CA) I, CA II, acetylcholine esterase (AChE), and butyrylcholine esterase (BChE) enzymes. We determined IC50 values of BC for each enzyme to measure the level of inhibition. The IC50 values for CA I and CA II were 8.77 µg/mL and 7.56 µg/mL, respectively. Acetazolamide, a specific CA I and II inhibitor, had IC50 values of 1.65 and 0.016 µg/mL, respectively. The IC50 values of BC for AChE and BChE were 7.96 and 8.58 µg/mL, respectively. Galantamine had IC50 values of 4.68 and 16.07 µg/mL for AChE and BChE, respectively. These results indicate that BC extract has a high potential to provide new drug candidates for all the tested enzymes, particularly for BChE.

1. INTRODUCTION

A lichen is usually described as the iconic example of symbiosis, but it is a minuscule ecosystem. A fungus and one or more photosynthetic partners comprise the prominent symbiosis. Other parties, such as lichenicolous fungi, bacteria, and even invertebrates, can live under or within this symbiosis. Thus, the symbiosis in question can become complicated beyond its disposition.

The secondary metabolites produced by this complex structure have unique natures. Most of these secondary metabolites are not synthesized by other organisms, even by the fungal partner cultured outside the symbiosis. Although about 1000 secondary metabolites have been isolated from lichens so far (Elix, 2014; Furmanek *et al*., 2022b), the number of those tested for their biological activities is relatively small. However, the number of studies on the bioactivities of

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lichen-derived secondary metabolites has increased significantly in recent years (Adenubi *et al*., 2022).

Bryoria capillaris (Ach.) Brodo & D. Hawksw. (BC) is a pendulous, filamentous, and widespread lichen found wrapped around tree trunks and branches in the coniferous and deciduous forests of the world. It is commonly known as "horsehair lichen" (Smith, 2009). Researchers reported antibacterial (Karagoz *et al*., 2018; Tas *et al*., 2019; Yılmaz Sarıözlü *et al*., 2016), antifungal (Furmanek *et al*., 2022a, 2022b, 2022c), anti-cancer (Goncu *et al*., 2020; Ozturk *et al*., 2021; Tripathi *et al*., 2022; Varol, 2018), antioxidant and antigenotoxic (Tas *et al*., 2017; Tufan-Cetin & Cetin, 2021; Turkez *et al*., 2014) effects of BC.

Enzyme inhibitors play a vital role in the management of various medical conditions (Şentürk, 2017). Carbonic anhydrases (CAs, EC 4.2.1.1) are a family of metal-containing enzymes that catalyze the conversion of carbon dioxide to bicarbonate and protons. These enzymes are found in a wide range of living organisms and are encoded by eight different gene families (Akocak & Supuran, 2019; Supuran, 2023). In humans, 15 forms of the alpha-CA gene family have been identified. Among these, the CA I and CA II isoforms are particularly common in all tissue types. The study of how carbonic anhydrase activity can be modulated is of great importance for the development of new treatments for a wide range of clinically significant diseases (Supuran, 2008; Yaseen *et al*., 2016). For example, inhibitors of specific forms of the enzyme, such as CA I and II, have been used to create new drugs for conditions such as epilepsy, edema, and glaucoma. Therefore, the discovery of new inhibitors of carbonic anhydrase isoenzymes holds great promise as a potential therapeutic strategy (Arslan *et al*., 2020; Özil *et al*., 2019; Yaseen *et al*., 2016).

Acetylcholinesterase (AChE) is a particularly important enzyme, as it plays a crucial role in regulating the signaling process within the cholinergic system. AChE breaks down acetylcholine (ACh), a neurotransmitter that is involved in memory formation and the functioning of motor neurons. Inhibiting the activity of AChE can have a significant impact on the treatment of diseases related to the cholinergic system (Comert Onder *et al*., 2022; Hampel *et al*., 2018).

AChE is located in the postsynaptic membrane of neurons and is responsible for breaking down the neurotransmitter ACh to terminate signal transduction. BChE, on the other hand, is primarily produced in the liver and is found in various bodily fluids and tissues, such as blood plasma and in the central and peripheral nervous systems. Clinical studies have demonstrated that inhibitors of AChE can boost ACh levels at cholinergic synapses and enhance cholinergic activity. While ACh is primarily broken down by AChE, BChE is believed to have only a minor role in regulating ACh levels in the brain. However, it has been found to play a crucial role in drug metabolism and the removal of toxins from the body. Targeted inhibitors can be used to treat motor neuron diseases such as dementia, myasthenia gravis, and Alzheimer's disease by decreasing the activity of AChE and BChE (Başaran *et al*., 2022; Comert Onder *et al*., 2022; Hampel *et al*., 2018).

In this study, inhibitory effects of BC acetone extract against cholinesterase enzymes (AChE and BChE) and carbonic anhydrases (CA I and II) were investigated in order to establish a steady biochemical basis of some of its therapeutic actions.

2. MATERIAL and METHODS

2.1. Lichen Material

BC was collected from Uzunoluk forest in Oltu county of Erzurum province, Turkey, in 2011. The lichen was identified according to literature (Smith, 2009). After drying in the shade, and removal of debris and foreign material, 10 g of BC was ground into a coarse powder and macerated with acetone (3 x 100 ml) at room temperature. Extracts were filtered and pooled

together. Acetone was removed in a rotary evaporator under reduced pressure at 40 °C. The residue (crude extract) weighed 200 mg (yield 2%).

2.2. Enzymes and Substrates

The enzymes and substrates used in this study were obtained from Sigma-Aldrich Company (USA, Lot numbers are as follows; AChE C1682, BChE B4186, CA I C4396, CA II C6624, 4-nitrophenyl acetate [NPA] N8130, 5,5'-Dithiobis-(2-Nitrobenzoic Acid) [DTNB] D218200, acetylthiocholine iodide A5771, S-butrylthiocholine chloride B3128).

2.3. Carbonic Anhydrase I And II Inhibition

The activity of these isoenzymes was measured using spectrophotometry by observing the change in absorbance at 348 nm as 4-nitrophenyl acetate (NPA) was converted to 4-nitrophenolate (NP) over 3 minutes at 25ºC. The reaction mixture consisted of 1.4 mL of 50 mM Tris-SO_4 buffer at pH 7.4, 1 mL of 3 mM NPA, 0.5 mL of water, and 0.1 mL of enzyme solution, for a total volume of 3.0 mL (Verpoorte *et al*., 1967).

2.4. Cholinesterase Enzymes Inhibition

The assay system employed comprised a sample of the BC acetone extract, with a volume ranging from 5 to 60 mL, in conjunction with 200 mL of buffer, specifically 1 M Tris-HCl buffer for the assay of AChE and PB for the assay of BChE, both at a pH of 8.0. Additionally, 50 mL of 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) (0.5 mM) and 50 mL of acetylthiocholine iodide/S-butrylthiocholine chloride (10 mM) were incorporated, as well as 10 mL of the enzyme, with a concentration of 0.28 U/mL for the AChE assay and 0.32 U/mL for the BChE assay (Ellman *et al*., 1961). The reaction was initiated by adding the enzyme, and the system was prepared at room temperature within a quartz cuvette. A control was also performed, consisting of all the afore mentioned chemicals, except for the inhibitor.

2.5. General Enzyme Inhibition Studies

Inhibition activities of BC acetone extract on CA I/II, AChE, and BChE were defined using the spectrophotometric methods. In this analysis, acetazolamide (AZA) for CA I/II, galantamine (GAL) for AChE/BChE were used as reference molecules.

In this study, stock solution of the extract under investigation was prepared by dissolving it in dimethyl sulfoxide to achieve a concentration of 1 mg per mL. The resulting stock solution was then meticulously diluted one thousand fold with distilled water. To evaluate the inhibitory activity of these extract on the enzymes under examination, measurements were conducted at seven distinct concentrations. The methodology employed in this study has been previously described in detail in prior studies (Arslan *et al*., 2020; Cavdar *et al*., 2019; Özil *et al*., 2019).

3. RESULTS

IC50 values obtained for BC acetone extract is summarized in Table 1 and Figures 1-4.

Table 1. IC50 values obtained with BC acetone extract and reference drugs for the enzymes tested.

Enzyme	BC Extract	Reference molecules
CA I[a]	8.77 ± 0.12 µg/mL	1.65 ± 0.03 µg/mL
CA II[a]	7.56 ± 0.10 µg/mL	0.016 ± 0.001 µg/mL
AChE[b]	7.96 ± 0.09µg/mL	4.68 ± 0.31 µg/mL
BChE[b]	8.58 ± 0.11 µg/mL	16.07 ± 1.04 µg/mL

[a]Acetazolamide was used as a specific inhibitor and reference drug for CA I and II isoenzymes.
[b]Galantamine was used as a specific inhibitor and reference drug for AChE and BChE enzymes (Faraone *et al*., 2019).

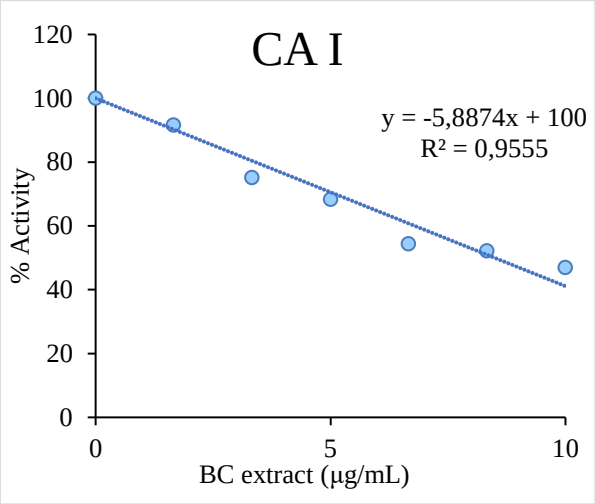

Figure 1. IC50 plot for CA I and BC extract.

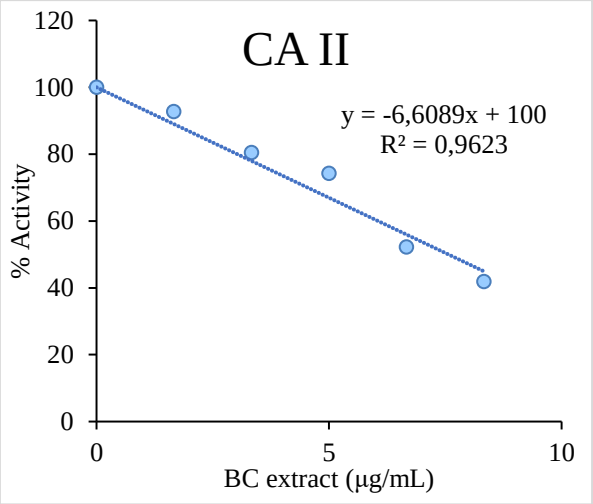

Figure 2. IC50 plot for CA II and BC extract.

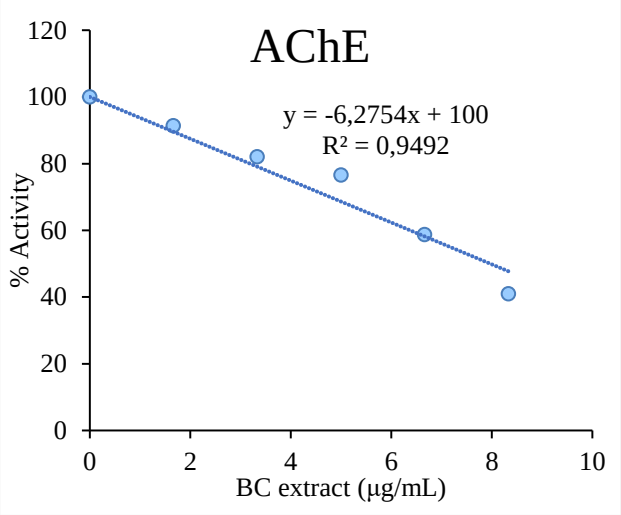

Figure 3. IC50 plot for AChE and BC extract.

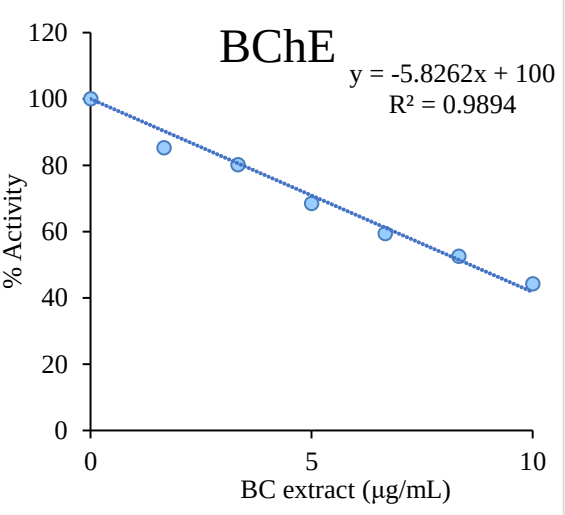

Figure 4. IC50 plot for BChE and BC extract.

4. DISCUSSION and CONCLUSION

B. capillaris contains barbatolic acid and alectorialic acid as major compounds (Culberson, 1969; Culberson, 1970; Culberson *et al*., 1977). Previously, Areche *et al*. (2022) reported that barbatolic acid isolated from *Himantormia lugubris* was an effective inhibitor of AChE and BChE (IC50 values 17.42 ± 0.03 and 23.95 ± 0.02 µg/mL, respectively). In our study BC extract presented a better inhibition of these enzymes, suggesting that synergistic effects may occur due to presence of other chemical compounds.

In another study (Yañez *et al*., 2023) barbatolic acid was estimated to be a moderate inhibitor of Cytochrome P450 (CYP) isoenzymes with calculated binding energies ranging between -6 to -9.2 kcal/mol. Authors concluded that tested lichen substances, including barbatolic acid, could exert antioxidant activity by interacting with the CYP system. As oxidative stress is implied in so many pathological conditions (Liguori *et al*., 2018), antioxidant molecules or mixtures, the BC extract in our case, may provide a means of preventing or treating such conditions.

Methanol extracts of different mushroom, plant and honey samples were obtained by Sahin *et al*. (2012). In the study, IC50 studies on CA I/II were performed with these extracts. The

authors determined that the IC50 values of the extracts for CA I were between 0.52 and 36.66 µg/mL. The IC50 values of these extracts for the CA II enzyme were found to be between 0.49 and 24.02 µg/mL. In our study the IC50 value of BC for the CA I isoenzyme was determined as 8.77 µg/mL. This value suggests a very effective inhibition value when compared to one of the strongest known inhibitors, AZA (IC50: 1.65 µg/mL). For CA II isoenzyme, the IC50 value was determined as 7.56 µg/mL, which is seen to be a moderately effective result when compared to AZA.

CA inhibitors (CAIs) have established roles as diuretics and antiglaucoma drugs. However, recent findings indicate that CAIs promise potential as novel anti-obesity, anticancer and anti-infective drugs (Supuran, 2008). According to our results, *Bryoria capillaris* may provide molecules that serve as effective CAIs, therefore deserves further investigation on this field.

The IC50 of the BC extract tested against the AChE enzyme was found to be 7.96 µg/mL. For the reference molecule galantamine, the AChE IC50 value was determined as 4.68 µg/mL. The IC50 value of BC extract inhibitory activity on BChE enzyme was determined as 8.58 µg/mL, and the IC50 of galantamine was found as 16.07 µg/mL. It was determined that BC extract has an effective inhibition for cholinesterase enzymes.

Butyrylcholinesterase acts as a backup for acetylcholinesterase by hydrolyzing acetylcholine that has diffused out of nerve synapses (Lockridge *et al*., 2011). In management of Alzheimer's disease (AD), first choice for the treatment is the AChE inhibitor. However, AChE inhibitors have some flaws, such as insufficient long-term treatment effect and dose limitations. Recent studies revealed that BChE inhibitors or double inhibitors (molecules that inhibit both AChE and BChE) have better effects on AD, and the side effects are lower than those of specific AChE inhibitors. Dual target cholinesterase inhibitors have become a new hot spot in the research of anti-AD drugs (Zhou & Huang, 2022). In the patient with AD, a potent selective BChE inhibitor may produce significant increases of brain ACh levels without triggering severe peripheral or central cholinergic adverse effects (Giacobini, 2001). In this perspective, BC extract presents high potential as a source of anti-AD molecules as it inhibits AChE comparable to galantamine, and BChE better than galantamine.

In conclusion, the present study demonstrates that BC extract has a significant therapeutic potential due to its inhibition effects on CA I, CA II, AChE, and BChE enzymes. The IC50 values obtained in this study indicate that the extract has a moderate to high level of inhibition on these enzymes. These findings suggest that the extract of BC could be a promising candidate for the development of new drugs for the treatment of various diseases related to these enzymes, such as Alzheimer's disease and other neurodegenerative disorders. However, further studies are required to evaluate the safety and efficacy of this extract by *in vivo* models and to determine the active compounds responsible for its inhibition effects. Additionally, it is important to consider traditional usage and toxicity of BC, before any clinical application. In general, this study provides a scientific basis for the traditional use of BC and highlights the importance of further research in this area to fully understand the therapeutic potential of this lichen species.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Yalçın Karagöz: Field research, material collection, extraction, manuscript preparation. **Naim Uzun**: Extraction, enzyme assays. **Bayram Alparslan**: Extraction, enzyme assays. **Murat Şentürk:** Enzyme assays, manuscript preparation.

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Research Article

Bioactive compounds of *Arctostaphylos uva-ursi* wild-growing populations from Bulgaria

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Abstract: *Arctostaphylos uva-ursi* (L.) Sprengel (bearberry), Ericaceae is a valuable medicinal plant with diuretic and uroantiseptic action which is mainly due to arbutin. In Bulgaria the species is considered as rare. Content assessment of bioactive compounds of *A. uva-ursi* leaves from three natural populations from Bulgaria was the aim of the present study. Leaf samples were collected from Pirin, Vitosha, and Rhodope Mountains. Bioactive compounds in the methanolic extracts of the samples were analysed by GC/MS. Total phenolic content was determined using Folin–Ciocalteu reagent. Arbutin, quinic acid and gallic acid were detected in the highest amounts. Catechine, 4-hydroxybenzoic acid, chlorogenic acid, triterpenes (α- and β-amyrin, uvaol and lupeol) and other primary and secondary metabolites were found, also. Differences in the content of individual compounds between samples of different origin were established. The highest total phenolic (182.98 mg GAE g^{-1}) and arbutin (8.4%) content was found in the sample from Vitosha Mountain. The presented data characterizes the profile of bioactive compounds in the Bulgarian bearberry raw material for the first time.

1. INTRODUCTION

Arctostaphylos uva-ursi (L.) Sprengel (bearberry), Ericaceae is a valuable medicinal plant that occurs in large areas in Europe, Asia, North America and Greenland. However in Bulgaria the plant is considered as rare species and is included in the Red List of Bulgarian vascular plants with the category "vulnerable" (Petrova and Vladimirov, 2009). Leaves of *A. uva-ursi* are used mainly as a diuretic, antimicrobial, anti-inflammatory and skin-whitening agent (EMA, 2016; Shamilov *et al*., 2021). The main active substances of *Arctostaphylos uva-ursi* leaves and its preparations are simple phenols (hydroquinone derivatives such as arbutin, methylarbutin and pyroside), phenolic acids (gallic and ellagic) flavonoids (myricetin, hyperoside and quercetin), iridoid glucosides (asperuloside, monotropein and unedoside (EMA, 2016; Kurkin *et al*., 2018; Shamilov *et al*., 2021). Chemical profile of the species is complemented also by the presence of corilagin (ellagitannin), picein, penta-O-galloyl-β-D-glucose, ursolic acid, tannic acid, p-

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coumaric acid, syringic acid, p-hydroxybenzoic acid, ferulic acid, caffeic acid, lithospermic acid, and proanthocyanidins (Sugier *et al*., 2021; Stefkov *et al*., 2021).

Arbutin (hydroquinone glucoside) is the main compound of *A. uva-ursi* leaves with antioxidant, anti-inflammatory, anticancer, antiparkinsonian, hypoglycemic, and antipyretic activities **(**Bhalla *et al*., 2023). The compound is used as a depigmentation agent also (Boo, 2021). The most commonly used method of arbutin content evaluation in plant material is by HPLC (Song *et al*., 2021; Stefkov *et al*., 2021). However, the GC/MS method is also determined as suitable for the analysis of this compound (Jurica *et al*., 2015). A good comparability of the data obtained by both methods was established (Lamien-Meda *et al*., 2009)

Accumulation of aubutin and other metabolites in the *A. uva-ursi* leaves depends on many factors: genetic, ontogenetic and environmental such as temperature, altitude, radiation, nature of the soil (Asensio *et al*., 2020; Sugier *et al*., 2021; Stefkov *et al*., 2021). The highest content of arbutin has been determined in the samples collected in the autumn after fructification (Stefkov *et al*., 2021). Asensio *et al*., (2020) have reported higher arbutin content in plants growing in the northern locations and at relatively higher altitudes. Sugier *et al*., (2021) have found that samples of bearberry collected from the pine forest populations contain significantly higher arbutin than those from heathlands.

Although the phytochemical profile of the species is known and analyzed in populations from different geographical regions, including Iberian Peninsula, Catalonia (Parejo *et al*., 2002; Asensio *et al*., 2020), Asiatic part of Russia (Olennikov and Chekhirova, 2013), Northern Macedonia (Stefkov *et al*., 2021), and Poland (Sugier *et al* 2021) data on the content of bioactive compounds in Bulgarian populations of the species is missing. That is why the assessment of bioactive compounds with an emphasis on the arbutin content of *A. uva-ursi* wild-growing populations in Bulgaria was the aim of the present study.

2. MATERIAL and METHODS

2.1. Plant Material

Leaf samples of *A. uva-ursi* were collected from three natural populations of Pirin, Vitosha, and Rhodope Mountains of Bulgaria at the beginning of June, 2021. The species was identified by authors (Prof. Petar Zhelev and Dr. Ina Aneva) according to Kozhuharov (1992). Voucher specimens are deposited at the Herbarium, Institute of Biodiversity and Ecosystem Research (SOM), Bulgarian Academy of Sciences, Bulgaria.

Details of the localities of the studied populations are presented at Table 1.

Table 1. Origin of the studied *A. uva-ursi* samples.

Sample code	Mountain	SOM	Geographic Coordinates Altitude m asl
AU1	Vitosha	178670	42° 31' N, 23° 16' E, 1800-1900, open areas above the upper limit of the forest
AU2	Rhodope	178671	41° 35' N, 24° 26' E, 1200-1300, sub-Mediterranean pine forests
AU3	Pirin	178672	41° 50' N, 23° 23' E, 1300-1400, sub-Mediterranean pine forests

2.2. Extraction

Methanolic extract was prepared from 100 mg powdered plant material macerated with 1 mL methanol in 2 mL Eppendorf tubes. 50 µL of 3,5 dichloro-4-hidroxybenzoic acid (1 mg/mL) were placed at the beginning of the extraction procedure as an internal standard. After 24 h of extraction at room temperature, an aliquot of 500 µL of each sample was transferred to a glass vial and was dried.

2.3. Derivatization

100 µL pyridine and 100 µL of N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were added to the dried samples of the extract (50 mg) and heated at 70 °C for 2 h. After cooling, 300 µL of chloroform were added and the samples were analyzed by GC/MS.

2.4. Gas-Chromatography-Mass Spectrometry Analysis (GC-MS)

GC/MS analysis of methanolic extracts was recorded on a Thermo Scientific Focus GC coupled with Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m x 0.25 mm x 0.25 µm) was used. The conditions of the analysis were described by Berkov *et al*., 2021. The compounds were identified by comparing their mass spectra and retention indices (RI) with standard compounds from the National Institute of Standards and Technology (NIST) and home-made MS databases. Relative quantification for metabolomics was calculated from total ion chromatogram peak area integration of single metabolite and internal standard (3,5 dichloro-4-hidroxybenzoic acid).

2.5. Quantification of Arbutin

Arbutin was quantified after the construction of a calibration curve by plotting the ratio of the peak areas of arbutin (50, 100, 150, 200, 250 and 300 µg/mL) versus that of 50 µg internal standard 3,5-dichloro-4-hydroxybenzoic acid. Arbutin content was expressed as a percentage of DW of the sample.

2.6. Total Phenolic Content

Total phenolic content of methanol extracts was determined using Folin-Ciocalteu reagent and gallic acid as standard. Methanolic extracts were diluted to a concentration of 2 mg/ mL, and aliquots of 0.200 mL were mixed with 2 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 1.8 mL of Na_2CO_3 (7.5%). After 1 h at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA) per gram of extract.

2.7. Statistical Analysis

Statistical analysis was carried out using Excel. All experiments were performed in triplicate. Results are presented as a value ± standard deviation (SD). Significant levels are defined at $p < 0.05$ as analyzed by t-test.

3. RESULTS

3.1. Gas-Chromatography-Mass Spectrometry Analysis

The GC-MS analyzes of methanolic extracts of *A. uva-ursi* samples from three populations revealed primary and secondary metabolites including phenolic acids, flavonoids, fatty acids, sterols, triterpenes, saccharides, and polyols. The results are presented in Table 2. The most abundant primary metabolites in the methanolic extracts were the monosaccharides fructose and glucose, as well as disaccharide sucrose. Arbutin and gallic acid were found in the highest amounts of the secondary metabolites. Quinic acid, catechin, triterpenes (α- and β-amyrin, uvarol) were identified as main bioactive compounds in the studied samples. GC/MS chromatograms of the extracts from the studied samples are presented in Figure1.

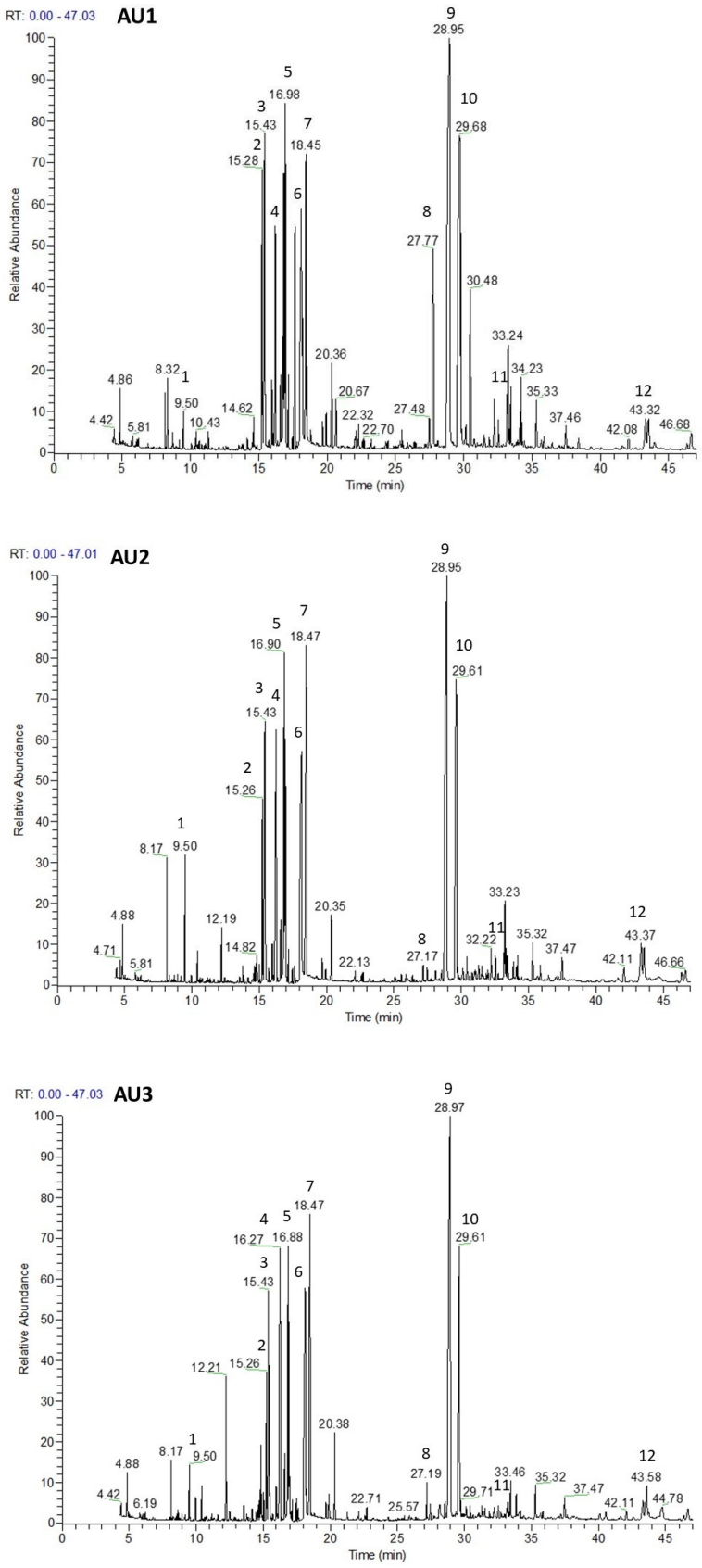

Figure 1. GC/MS chromatograms of methanolic extracts of the samples from Vitosha (AU1), Rhodope (AU2) and Pirin (AU3); 1. Hydroquinone; 2. Fructose 1; 3. Fructose 2; 4. Quinic acid; 5. Glucose; 6. Gallic acid; 7. Monosaccharide; 8. *Disaccharide*; 9. Arbutin; 10. Sucrose; 11. Catechine 12. α-Amyrin.

Quality differences in the content of individual compounds between samples of different origins were found. Monosaccharides, sugar alcohol - inositol and especially sucrose were found in significantly greater amounts in the Vitosha sample (AU1). With regard to lipid compounds, no significant quantitative differences between the samples from the different populations were found. The content of gallic acid as a major component also showed no differences between samples from the three populations. The amounts of arbutin and catechin were found to be the highest in the sample from (AU1) sample again.

Table 2. Identified compounds in the methanolic extracts of the studied samples of *Arctostaphylos uva-ursi* *

Identified compounds	RI	AU1	AU2	AU3
Phenolic compounds				
Hydroquinone	1396	63.5±29	04.6±132	559±172
4-Hydroxybenzoic acid	1637	2.7±0.8	3.2±1.2	1.3±0.3
Protocatechuic acid	1811	0.9±0.3	1.0±0.4	2.8±0.6
Quinic acid	1846	253.9±63	163.0±35	287.6±44
Syringic acid	1888	trace	0.2±0.1	0.4±0.1
4-Hydroxycinnamic acid	1934	0.2±0.1	0.2±0.1	0.4±0.1
Gallic acid	1976	628.3±86	650.4±73	652.5±105
Caffeic acid	2142	trace	trace	3.4±2
Arbutin	2561	3944.7±412	3226.6±378	3209.8±402
Catechin	2861	30.7±9	10.5±2	15.7±6.2
Lipid compounds				
Hexadecanoic acid	1929	16.5±4	19.9±5	12.5±4
Octadecanoic aicd	2132	2.9±0.6	2.7±0.8	1.1±0.4
β-Sitosterol	2614	6.0±1.6	10.7±4	4.1±2.7
β-Amyrin	3335	30.3±0.9	28.3±8.6	21.5±7.2
α-Amyrin	3382	111.6±3.5	153.5±14	76.4±18
Lupeol	3434	99.2±11	108±16	117.7±25
Uvaol	3716	60.9±2.2	41.7±9.3	50.8±14
Saccharides and polyols				
Fructose 1	1793	561.1±75	277.0±25	264.4±98
Fructose 2	1830	600.2±137	502.9±98	352.9±102
Glucose	1889	519.9±216	425.5±132	441.5±111
myo Inositol	2080	92.5±33	65±13	33.8±12
Sucrose	2712	1025.9±298	592.0±94	366.2±77

*Relative quantification for metabolomics was calculated from total ion chromatogram peak area integration of single metabolite and standart

3.2. Quantification of Arbutin and Total Phenolics

The content of arbutin in the leaves of the studied samples was determined by GC/MS and that of total phenolics by spectrophotometric assay. The results of quantitative analyses are presented in Table 3. Total phenolic content in the studied samples ranged from 137 to 182 GAE/g extract. The level of arbutin in the studied populations fluctuated from 6.8% to 8.4 %.

The highest content of arbutin and total phenols was found from the Vitosha Mountain (AU1), but a significant difference (p<0.05) among the studied samples was not found.

Table 3. Total phenol and arbutin content in the studied samples of *A. uva-ursi*.

Title	Total phenolic content [mg GAE/g extract]	Arbutin content [%, DW of the sample]
AU 1	182.98±6	8.4±2.6
AU 2	138.25±12	7.0±1.1
AU 3	137.55±12	6.8±2.3

4. DISCUSSION and CONCLUSION

Arbutin, gallic acid, quinic acid and triterpenoids were identified as the main bioactive compounds in the *A. uva ursi* leaf samples from Bulgarian populations which is consistent with previously reported data for the chemical composition of the species (Kurkin *et al*., 2018; Shamilov *et al*., 2021; Song *et al*., 2021). Among minor components considering the toxicological profile of free hydroquinone (de Arriba *et al*., 2013), it is worth noting that its content is significantly lower in the sample (AU1) from Vitosha. The established quantitative differences in the chemical profiles of the studied samples are probably determined by the local influence of environmental factors. It has been found that environmental factors such as latitude, altitude, UV-radiation, habitat types (heathlands or pine forests), the date of collection influenced on the accumulation of phenolic compounds of *A. uva-ursi* (Asensio *et al*., 2020; Stefkov *et al*., 2021; Sugier *et al*., 2021). Sugier *et al*., (2021) have reported that leaves of the species collected from the heathland population are characterized by higher total flavonoid content in comparison with the pine forest population. In the present study higher catechin (flavonoid) content was found in the samples from population (AU1) located in open areas above the upper limit of the forest than in the samples collected from population in the pine forest (AU2 and AU3). Accumulation of disaccharides is important and possibly best-known reaction of plans in response to water stress (Ingram and Bartels, 1996) furthermore Oliver *et al*., (2001) have reported that non-disaccharide compounds such as arbutin also accumulated in the condition of water deficit. The open areas of the locality (AU1) probably lead more often to water deficit and this is reflected in the higher disaccharide content in the samples from this region.

The amount of arbutin determined in the samples of the present study most closely approximates the data reported for the samples from Catalan Pyrenees, Spain (Parejo *et al*., 2002). It should be kept in mind that samples in the present study were collected at the beginning of June, and many studies reported higher arbutin content in leaves of the species collected in autumn (Parejo *et al*., 2002; Stefkov *et al*., 2021). Asensio *et al*., (2020) and Sugier *et al*. (2021) have been concluded in their comprehensive studies that *A. uva ursi* samples of populations from northern latitudes and at higher altitudes showed frequently high arbutin content. The present results are consistent with these conclusions. The sample (AU1) collected from the highest altitude had the highest arbutin content.

In conclusion, a comparative analysis of bioactive compounds of *A. uva ursi* plant material from three Bulgarian populations was done by GC/MS for the first time. The highest amounts of arbutin, total phenolics, sucrose and catechin were found in the leaves of the plant from Vitiosha Mountain. The study adds to the knowledge of the content of arbutin in bearberry leaves within the southern limits of its natural distribution. We recommend that samples be analyzed by HPLC so that the content of more polar substances can be compared.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Milena Nikolova: experimental design, chemical analysis, manuscript writing; **Ina Aneva** and **Petar Zhelev**: Collection and identification of plant material; **Strahil Berkov**: Chemical analysis; **Elina Yankova-Tsvetkova**: Conceptualization, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Research Article

Antioxidant activity, phytochemical screening and GC-MS profile of *Abies marocana* Trab.

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Abstract: The aim of this research was to explore the chemical composition and antioxidant activities of etheric extracts of *Abies marocana*. A Soxhlet apparatus was used to extract bioactive molecules from the various parts of the plant. Furthermore, the levels of antioxidant compounds were quantified, while the Gas chromatography was utilized to determine the chemical constituents of the extracted molecules. The extracts were evaluated for their antioxidant properties using the DPPH radical scavenging method and the total antioxidant capacity test. The levels of polyphenols varied across different parts of the plant, ranging from 2.474 ± 0.029 mg.g^{-1} DM in needles to 4.207 ± 0.008 mg.g^{-1} DM in twigs. Flavonoids were most abundant in needles 0.140 ± 0.001 mg.g^{-1} DM and least abundant in cones 0.069 ± 0.007 mg.g^{-1} DM. Tannins had the highest concentration in twigs 2.608 ± 0.114 mg.g^{-1} DM, followed by cones 1.948 ± 0.037 mg.g^{-1} DM and needles 1.512 ± 0.09 mg.g^{-1} DM. A chromatographic analysis revealed that 56 components were in the samples, with terpene compounds being the most abundant in the different organs. In terms of antioxidant activity, the extract derived from twigs exhibited the strongest antioxidant capacity 49.377 ± 0.371 mg EAA.g^{-1} DM, followed by cones 35.129 ± 0.084 mg EAA.g^{-1} DM and needles 13.663 ± 0.084 mg EAA.g^{-1} DM. Alternatively, the IC_{50} values for the three organs were found to be in the range of 3844 to 5047.67 µg.mL^{-1}. The results highlight the potential phytopharmaceutical value of *A. marocana* due to the presence of diverse phyto-components.

1. INTRODUCTION

The word "fir" pertains to a type of coniferous plant belonging to the Pinaceae family, specifically the genus *Abies*. Firs are easily distinguishable from other members of the Pinaceae family based on their botanical features such as their shape, bark, needles, and fragrance. In general, firs tend to grow in areas with partial shade, and they grow best in damp, nutrient-rich forest soils. They can also do well in moist sandy or clay soils, but they don't thrive in soils that are high in limestone (Mokaddem-Daroui *et al*., 2021). The genus *Abies* "fir" (Pinaceae) includes 51 species, with the majority of them distributed in the Northern Hemisphere's

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temperate and boreal zones, especially in mountainous areas such as North Africa, the Himalayas, and Türkiye (Farjon *et al*., 1989; Zheng *et al*., 1978).

Abies marocana is an indigenous tree species found in the Rif area at elevations ranging from 1400 to 2000 meters. It contributes significantly to the creation of magnificent forest ecosystems, particularly at the summit of Moroccan city Chefchaouen (Uçar & Uçar, 2014; Flora of China, 1978). *A. marocana* can be located in the bioclimatic zone of the Mediterranean that is characterized by its high levels of humidity. This particular species thrives in an environment where the average annual rainfall reaches 1500 mm, and as one ascends to an altitude of 1700 mm, the precipitation increases to 1900 mm. The stands of *A. marocana* are predominantly observed on the slopes of mountain ridges that face north and are composed of dolomitic limestone. These specific geological conditions provide an ideal habitat for the growth and development of *A. marocana* (Benabid, 1983).

Previous studies have identified various bioactive compounds, including lignans, flavonoids (Tiwari *et al*., 1980), sesquiterpenoids (Xia *et al*., 2012), triterpenoids, and tetraterpenoids (Lavoie *et al*., 2013), from *Abies* species that possess diverse biological activities (Yang *et al*., 2008; Baydar, 2006; Raldugin & Shevtsov, 1990). They have potential as active pharmaceutical ingredients in the treatment of various diseases (Gupta & Kumar, 2017; Li *et al*., 2015). The essential oils that are acquired from the seeds of this particular species are widely utilized in the practice of traditional Moroccan medicine as a means of effectively addressing and alleviating various respiratory ailments (Hmamouchi, 1999). Furthermore, it has been recently revealed that the oil derived from this species contains a significant and notable concentration of limonene, which has garnered substantial interest and intrigue from the perfume industry (Bazdi *et al*., 2006). The concentration of phytochemicals found in plants can differ based on factors such as species, age, climate, and environmental conditions. To extract these compounds, a variety of solvent systems can be utilized (Gupta & Kumar, 2017). Recently, there has been an increasing interest in natural antioxidants derived from plants, as they are believed to have the potential to improve health and prevent illnesses. These plant-based antioxidants are widely accepted by consumers and are generally considered safe for consumption (Gorinstein *et al*., 2003).

As far as we know, there haven't been any studies conducted on the physiological impacts of *Abies marocana.* Hence, this research aims to investigate the antioxidant capacity of etheric extracts derived from the aerial parts of *A. marocana* and to identify and quantify the secondary metabolites present in each organ. The study seeks to shed light on the potential health benefits and pharmaceutical applications of this plant by exploring its antioxidative properties and characterizing its chemical composition.

2. MATERIAL and METHODS

2.1. Plant Collection and Authentication

The aerial parts of *Abies marocana* were harvested in Chefchaouen, a city located in the northern region of Morocco. The harvesting took place in June at an altitude of 1785 meters on Chouihate mountain (35°11'05.6" N 5°13'47.9" W). The plant was identified at the Scientific Research Center in Rabat. To prevent the samples from deteriorating during storage, they were dried in the laboratory for three days at a temperature of 45 ± 2 °C. Once dried, they were ground using a mill and stored in opaque glass containers until needed.

2.2. Extraction Method

The technique utilized was based on the process detailed by Kuluvar *et al*. (2009), with a few adjustments. Each section of *Abies marocana*, weighing 25 g, was positioned in the Soxhlet apparatus and subjected to extraction using 250 mL of petroleum ether, with a temperature of 40 °C maintained during the 8-hour process.

Following extraction, the filtered substances were concentrated at 35 °C, and the resulting extracts were dried and stored in a refrigerator at 4 °C until they were ready for analysis. In addition, the yield of the crude extracts was calculated using the method described in (NM ISO 734: 2020), Eq. (1).

$$w = \frac{m_1}{m_0} x100\% \quad (1)$$

Where, m_o is the mass of the sample powder and m_1 is the mass of the extract.

2.3. Phytochemical Screening

Phytochemical screening was performed to identify some major groups of secondary metabolites contained in our extracts and responsible for the possible activities. The following chemical groups were identified using conventional characterization reagents: alkaloids, reducing compounds, flavonoids, polyphenols, tannins, anthocyanins, proteins, essential oils, cardiac glycosides, sterols, and triterpenes (Trease & Evans, 1989).

2.4. Determination of The Amount of Antioxidant Compounds

2.4.1. *Determination of total phenolic content (TPC)*

To determine the total polyphenol levels of the extracts, the Folin-Ciocalteu method (Cheok *et al*., 2013) was utilized. Initially, 0.5 mL of each extract was combined with 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10 in methanol) in a test tube. After 5 minutes at room temperature, 1 mL of a 7.5% aqueous sodium carbonate solution was added, and the mixture was thoroughly mixed and kept in the dark for 30 minutes. The resulting solution's absorbance at 765 nm was measured against a blank using a spectrophotometer. Additionally, a calibration curve was produced using Gallic acid as a standard under comparable conditions (Hatami *et al*., 2014).

2.4.2. *Determination of total flavonoid Content (TFC)*

The conventional method for measuring flavonoids, as described by Lamaison and Carnat, (1990), was used. To each sample, 1 mL (appropriately diluted) was added to 1 mL of $AlCl_3$ solution (2% in methanol), and the resulting mixture was left to process for 10 minutes. The blanks were prepared by substituting the extract with methanol. Afterward, the absorbance was measured at 430 nm and used to determine the flavonoid concentration using quercetin as a reference.

2.4.3. *Determination of total tannin content (TTC)*

The Vanillic acid method (Hagerma, 2002) was employed to determine the tannin content in the sample. In this process, 0.5 mL of the extract was mixed with 2.5 mL of Vanillin reagent, which is a combination of an equivalent mixture of 8% HCl in methanol. Blanks were also created by substituting the reagents with a 4% acid-methanol mixture. The test tubes were incubated at 30 °C for 20 minutes before the absorbance at 500 nm was measured. Catechin was utilized as a reference standard for comparison.

2.5. Estimation of Chemical Constituents by GC-MS

Individual extracts were characterized using an MS workstation system equipped with a BR-5ns FS capillary column (60 m x 0.32 mm ID x 0.25 µm) coupled to mass spectrometry. The carrier gas used was helium with a high purity at a rate of 1.7 $mL.min^{-1}$. The injectate temperature was 250 °C and the oven temperature was initially 40 °C and gradually elevated to 260 °C at a rate of 8 $°C.min^{-1}$. A syringe was used to withdraw extracts, which were then injected into the injector at a 40:1 split ratio. Full-scan mass spectra ranging from 40-550 AMU were collected to generate all of them. The temperature of the ion source was adjusted to 230 °C, while the quadrupole was maintained at a temperature of 150 °C. The voltage of the electron multiplier was maintained at 1100 V above self-tuning, with a solvent delay of 3 min.

The identification and characterization of these compounds in different crude extracts was based on their gas chromatographic retention times. Mass spectra were compared with Mass Spectral Library (NIST) standards. Results were expressed as percent of peak area.

2.6. Antioxidant Activity

2.6.1. *Total antioxidant capacity (TAC)*

The extract's total antioxidant capacity (TAC) was measured using the method described by Aouji *et al*. (2023). A 0.1 mL sample of extracts was combined with 1 mL of the reagent solution in a tube (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were closed and maintained for 90 minutes in a water bath at 95 °C. The absorbance of the solutions was measured at 695 nm in comparison to a blank. The contents are expressed as milligrams of ascorbic acid equivalent per gram of dry matter (mg EAA.g^{-1} DM).

2.6.2. *DPPH free radical scavenging activity*

Because of its stable free radical form and ease of analysis, DPPH (2,2-diphenyl-1-picrylhydrazyl) is the most commonly utilized substrate for evaluating antioxidant activity in a quick and direct way (McCune & johns, 2002). Subhashini *et al*. (2011) described the experimental methods utilized to investigate DPPH's radical scavenging capability.

Different quantities of the plant's etheric extracts (1 mL) were combined with 1 mL of a solution containing DPPH radicals, with the final DPPH concentration being 0.025 g.L^{-1}. After being vigorously stirred, the mixture was left undisturbed for 30 minutes before its absorbance was measured at 517 nm. Ascorbic acid was employed as the reference. The experiment was carried out three times. The percentage inhibition of DPPH radical activity was determined using Eq. (2) (McCune & johns, 2002) as shown below:

$$PI\% = \frac{A_0 - A_1}{A_0} x100 \quad (2)$$

Where, A_0 and A_1 are the absorbance of the control and extract.

2.7. Statistical Analysis

To determine the statistical significance of the findings, a one-factor analysis of variance (ANOVA) test was conducted, followed by a Tukey-test at $\alpha = 5\%$. The results were reported as mean ± standard deviation, and the significance level was defined accordingly.

3. RESULTS and DISCUSSION

3.1. The Yield of The Extracts

The acquired findings are shown in Table 1. The comparing of the yields of *A. marocana* revealed that the extraction yield for the twig and needle extracts varied from 4.212 ± 0.001% to 5.736 ± 0.001%, respectively. The cone extract, on the other hand, yielded an intermediate yield of 5.013 ± 0.001%. These results outperform those achieved by Natalia *et al*. (2020) for *Picea Abies*. The extraction yield increases with sample particle size, extraction temperature, and solvent-to-sample extraction ratio (Herode *et al*., 2023).

Table 1. Amount and percentage yield of etheric extracts from *A. marocana*.

Samples	Amount (g)	Yield (%)
Twig	1.053 ± 0.032	4.212 ± 0.001 [a]
Needle	1.434 ± 0.022	5.736 ± 0.001 [b]
Cone	1.296 ± 0.027	5.013 ± 0.001 [c]

The significant difference ($p < 0.05$) is illustrated by the letters a, b, and c.

3.2. Phytochemical Screening

The outcomes of the phytochemical screening are displayed in Table 2. The results revealed that this plant includes phenolic chemicals, terpenes, essential oils, and sterols in various organs. The needles and cones contain more flavonoids than the twigs. Free tannins, on the other side, are more abundant in the cones compared to the other aerial parts. These findings are comparable to those of Rajalakshmi *et al*. (2016), who discovered steroids, phenols, flavonoids, tannins, and saponins in *A. webbiana*. Reducing compounds, proteins, and cardiac glycosides, on the contrary, are found in modest concentrations in all parts. While alkaloids and anthocyanins are absent in the different parts of *Abies marocana*.

Table 2. Identification of bioactive compounds in etheric extracts from *A. marocana.*

Compounds group	Twig	Needle	Cone
Polyphenols	+++	+++	+++
Flavonoids	++	+++	+++
Free tannins	++	++	+++
Sterols and terpenes	+++	+++	+++
Alkaloids	-	-	-
Essential oils	+++	+++	+++
Reducing compounds	+	+	+
Proteins	+	+	+
Cardiac glycosides	++	++	++
Anthocyanins	-	-	-

(-) Absent; (+) Present; (++) Abundant; (+++) So abundant

3.3. Determination of The Amount of Antioxidant Compounds

In accordance with Nunes *et al*. (2012), it is possible to predict the antioxidant capacity of a matrix by determining the total concentration of polyphenols. The results of the analyses conducted are presented in Table 3. The total phenolic content (TPC) of *A. marocana*'s etheric extracts was determined in terms of Gallic acid equivalent. The TPC was found to differ significantly, ranging from 2.475 ± 0.029 mg GAE.g^{-1} DM in the needle extract to 4.207 ± 0.008 mg GAE.g^{-1} DM in the twig extract. The cone extract had an estimated intermediate concentration of 3.661 ± 0.032 mg GAE.g^{-1} DM.

Table 3. Total phenolic, flavonoid and tannin content of *A. marocana.*

Samples	Twig	Needle	Cone
TPC (mg GAE.g^{-1} DM)	4.207 ± 0.008 ^{a}	2.475 ± 0.029 ^{b}	3.661 ± 0.032 ^{c}
TFC (mg EQ.g^{-1} DM)	0.088 ± 0.003 ^{a}	0.140 ± 0.001 ^{b}	0.068 ± 0.007 ^{c}
TTC (mg EC.g^{-1}DM)	2.608 ± 0.114 ^{a}	1.511 ± 0.094 ^{b}	1.948 ± 0.037 ^{c}

The significant difference ($p < 0.05$) is illustrated by the letters a, b, and c

The total flavonoid content (TFC) was measured in terms of Quercetin equivalent. The TFC varied from 0.068 ± 0.007 to 0.140 ± 0.001 mg EQ.g^{-1} DM. The flavonoid content found in needles is consistent with their protective role against the harmful effects of solar radiation and the prolonged exposure that they receive (Gehin *et al*., 2006). The results revealed a significant variation in the TTC, with the twig extract containing the highest amount 2.608 ± 0.114 mg EC. g-1 DM, followed by the cone 1.948 ± 0.037 mg EC.g^{-1} DM and needle extracts having lower

content 1.511 ± 0.094 mg EC.g^{-1} DM. These results are similar to those obtained for *Picea Abies* (TPC = 3.21 mg GAE.g^{-1}, TFC = 0.62 mg EQ.g^{-1}, and TTC = 0.84 mg TAE.g^{-1}) (Zeppetzauer *et al*., 2021).

Various factors, including geographical and climatic conditions, plant maturity, and shelf life, may significantly influence phenolic compound concentrations (El Hazzat *et al*., 2015; Merouane *et al*., 2014; Bouzid *et al*., 2010).

3.4. Estimation of Chemical Constituents by GC-MS

The chemical components, including their respective retention time (RT), and concentration (%), are outlined in Table 4. 28 components were identified in the etheric extract of twigs representing 92.98% of the total chemical composition. Its main components are (12Z)-abienol (21.35%), Abietic acid (3.90%) and Octacosanol (3.54%). Fatty acids and their derivatives (51.32%) and terpene compounds (43.35%) were the most abundant fraction of this extract. Vitamin A derivatives were also present in considerable quantities, namely retinol and retinol acetate (1.50 and 0.89% respectively).

However, the GC-MS analysis of the needle extract identified six primary components, which were Dotriacontan-1-ol (24.92%), delta-Cadinene (15.47%), caryophyllene (7.48%), D-Limonene (7.01%), Neophytadiene (4.07%), and alpha-pinene (3.70%). Sesquiterpene hydrocarbons made up the majority of the components, accounting for 48.65%. These findings are consistent with those of Alejandro *et al*. (1992), who had previously identified a group of natural diterpenoids, in the hydrocarbon fraction of a hexanoic extract of *A. marocana* needles. The GC-MS analysis also detected Phytol, which has been linked to beneficial properties (Sun *et al*., 2020; Banjare *et al*., 2017). In addition to its antimicrobial properties, squalene also possesses antioxidant characteristics. (Bhattacharya *et al*., 2021; Ugoeze *et al*., 2020).

The GC-MS analysis of cone extract revealed that it contains 26.52% monoterpenes, with the primary compounds being D-Limonene (21.55%), 1-Dotriacontanol (18.97%), Caryophyllene (7.11%), Methyl dehydroabietate (6.79%), Retinol (6.01%), and α-Humulene (3.09%). Wajs *et al*. (2015) investigated the volatile composition of *Abies alba* Mill cone scales and found that α-pinene was the primary constituent of the cone scale oil.

These results suggest that *A. marocana* has potential applications in various pharmaceutical industries.

Table 4. Phytocomponents identified in the etheric extracts of *A. marocana* by GC-MS.

Name of the compounds	Twig		Needle		Cone	
	RT	%	RT	%	RT	%
α-pinene	11.551	14.95	11.220	3.70	11.234	2.00
Tricosane	-	-	12.970	0.12	-	-
(-)-β-Pinene	13.718	5.69	-	-	-	-
Mesitylene	-	-	14.493	2.62	-	-
β-Myrcene	14.525	0.71	-	-	-	-
3-Carene	-	-	15.197	2.55	15.209	0.90
D-Limonene	16.561	15.05	16.268	7.01	16.583	21.55
Verbenol	-	-	-	-	19.755	1.22
α-Copaene	-	-	28.505	1.35	28.530	1.09
Behenic acid	-	-	-	-	28.774	0.36
(-)-β-Copaene	-	-	-	-	29.436	0.65
caryophyllene	30.810	0.60	30.857	7.48	30.952	7.11
(-)-Germacrene D	32.752	0.56	31.137	1.41	31.170	1.95
α-Humulene	-	-	31.950	3.35	32.020	3.09
cis-Muurola-4(15),5-diene	-	-	33.061	1.77	32.186	0.26
γ-Muurolene	-	-	32.602	1.04	32.651	1.16

Table 4. *Continues*.

α-Muurolene	-	-	33.344	2.51	33.372	1.12
δ-Cadinene	-	-	34.026	15.47	-	-
Cubenene	-	-	-	-	34.380	0.48
D-Guaiene	-	-	35.892	0.48	-	-
α-Selinene	-	-	-	-	36.985	1.07
τ-Muurolol	-	-	37.588	5.70	-	-
τ-Cadinol	-	-	-	-	37.577	2.26
α-Cadinol	-	-	37.918	2.97	37.955	1.40
Ylangenol	-	-	38.484	0.34	-	-
Neophytadiene	-	-	42.713	4.07	-	-
Phytol	-	-	42.837	0.33	-	-
Bicyclo[9.3.1]pentadeca-4,14 diene	46.232	0.40	-	-	-	-
Palmitic acid	46.949	1.62	45.090	1.85	46.608	2.42
Margaric acid	48.233	0.88	-	-	-	-
(12Z)-abienol	50.373	18.71	-	-	-	-
Oleic acid	50.917	6.04	51.020	0.99	51.193	2.57
Stearic acid	52.282	0.99	52.906	0.96	51.492	1.81
Methyl dehydroabietate	53.927	5.25	-	-	52.603	6.79
Methyl abietate	54.927	2.25	-	-	-	-
Squalene	55.968	0.71	52.422	1.36	-	-
Linoleic acid	-	-	51.447	0.65	-	-
dehydroabietic ester	-	-	55.570	1.76	-	-
Abietic acid	57.076	3.41	-	-	57.094	8.38
Tridecenoic acid	57.319	0.20	-	-	-	-
Retinol	57.692	1.32	-	-	58.022	6.01
7-oxo-dehydroabietic acid, methyl ester	58.378	0.95	-	-	-	-
Retinol, acetate	58.552	0.78	-	-	58.709	0.14
Tetracosamethyl-cyclododecasiloxane	59.054	1.07	-	-	-	-
Eicosanal	-	-	59.451	0.64	-	-
1-Heptacosanol	60.708	1.59	-	-	-	-
1,4-Benzenedicarboxylic acid dimethyl ester	61.347	0.81	-	-	-	-
cis-9-Octadecenal	62.638	3.10	66.143	1.81	-	-
Dotriacontan-1-ol	-	-	69.820	24.92	69.534	18.97
Not identified	**-**	**12.36**	**-**	**0.79**	**-**	**5.24**

3.5. Total Antioxidant Capacity

The total antioxidant capacities were expressed as mg ascorbic acid equivalent per g of dry extract (mg EAA.g^{-1} DM). Figure 1 shows that the total antioxidant capacity of plant extracts varies significantly depending on the plant part used. The extracts obtained from twigs showed a higher antioxidant capacity (49.377 ± 0.371 mg EAA.g^{-1} DM) compared to cones and needles (35.129 ± 0.084 and 13.663 ± 0.084 mg EAA.g^{-1} DM, respectively). Thus, the high antioxidant capacity of *Abies marocana* extracts suggests the abundant presence of bioactive antioxidant compounds. Several studies have suggested that flavonoid and polyphenolic compounds are the primary contributors to the phosphomolybdate scavenging activity of medicinal plants (Oueslati *et al*., 2012; Khan *et al*., 2012; Sharififar *et al*., 2009; Negro *et al*., 2003).

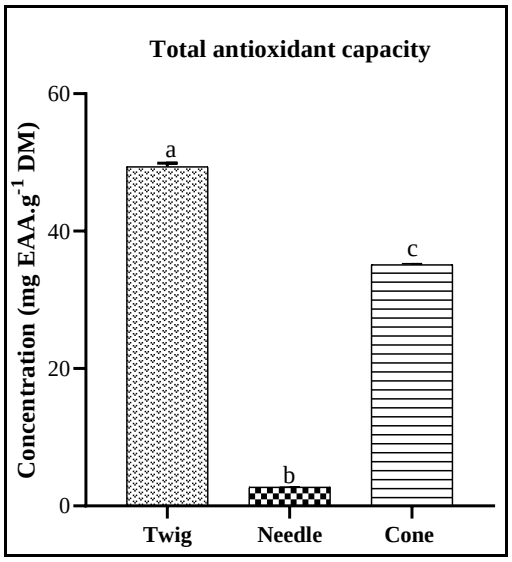

Figure 1. Variation in total antioxidant capacity in etheric extracts of *A. marocana*. The significant difference ($p < 0.05$) is illustrated by the letters a, b, and c.

3.6. DPPH Free Radical Scavenging Activity

Because of its ease and speed, the DPPH free radical scavenging activity is the preferred method for evaluating the antioxidant potential of natural and synthetic compounds (Angeli *et al*., 2021). Figure 2 illustrates the results obtained from the DPPH assay of the different extracts and ascorbic acid.

Extracts that were analyzed were compared based on their ability to scavenge DPPH free radicals, using the IC_{50} values, which serve as a quantifiable measure to determine the concentration level necessary to inhibit the activity of 50% of the free radicals. These free radicals, in themselves, serve as a reliable and trustworthy indicator of the overall effectiveness and efficiency of the antioxidants present within the extracts being evaluated.

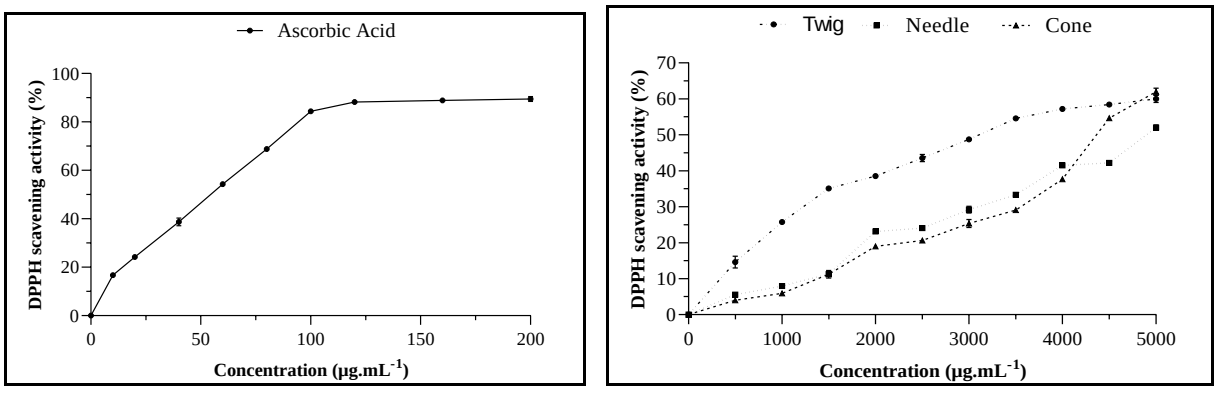

Figure 2. DPPH free radical scavenging activity of etheric extracts of *Abies marocana* and ascorbic acid.

Both ascorbic acid and the extracts were evaluated against each other, and it was found that the twig and cone extracts (3844 ± 55.95 and 4496 ± 31.00 µg. mL^{-1} respectively) had higher anti-radical activity than the needle (5047.67 ± 83.69 µg.mL^{-1}). Ascorbic acid, with the lowest IC_{50} value of 41.86 ± 0.49 µg.mL^{-1}, demonstrated the most significant free radical scavenging activity compared to the other extracts. This implies that the extracts derived from *A. marocana* possess remarkable capabilities in safeguarding cells from the detrimental effects caused by free radicals. This inhibition of DPPH activity may be due to the transfer of electrons from the

plant's phytoconstituents (Chukwuma *et al*., 2020). Therefore, the extracts' high DPPH scavenging activity could be attributed to the transfer of electrons from the polyphenols' various phenolic rings present in the extracts (Batool *et al*., 2019; Ishola *et al*., 2018).

Several studies have established that DPPH reduction is closely associated with the amount of phenolic compounds present (Boadi *et al*., 2021; Aryal *et al*., 2019; Sethi *et al*., 2020). This finding indicates the antioxidant ability of the three extracts.

4. CONCLUSION

In this study, *Abies marocana* was investigated, and its three parts were analyzed for bioactive compounds using phytochemical and GC-MS techniques. Terpenoids, polyphenols, fatty acids, and vitamin A derivatives were among the identified compounds and could be studied for their anticancer, anti-inflammatory and other potential therapeutic properties.

The highest antioxidant activity was found in the etheric extract of the twigs, which was attributed to the presence of retinol, phenolic compounds, and unsaturated fatty acids. The findings indicate that *Abies marocana* is rich in biologically active substances and should be recognized as a significant plant for phytopharmaceutical purposes.

This finding deserves to be elucidated by further in-depth studies on clinical trials to investigate the efficacy and safety of *Abies marocana* extracts in the treatment of specific diseases, and to develop new methods for extracting and purifying bioactive compounds from *Abies marocana*.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Malak Zirari: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Marouane Aouji**: Methodology, Analysis and interpretation. **Meryem Zouarhi**: Design, Visualization. **Ahmed Dermaj**: Materials, Resources. **Hamid Erramli**: Materials, Resources. **Driss Hmouni**: Methodology, Supervision, and Validation. **Nouredine El Mejdoub**: visualization, editing the original draft.

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Research Article

Effects of selenium on structure of the essential oil isolated from *Satureja hortensis* L. (Lamiaceae) under the cadmium stress

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Abstract: One of the heavy metals which cause severe environmental pollution and biochemical changes in plants is Cadmium (Cd) while Selenium (Se) acts as an important anti-stress agent in plants. In the present research, the glasshouse experiment was conducted to examine the effect of Cd and Se on structure of the essential oil isolated from the savory plant, *Satureja hortensis* L. To do so, the plants were polluted with different Cd levels including 0 (the control), 75, 100, and 150 µM. Moreover, Se was used in the form of sodium selenite salt in concentrations of 0 (control), 10, 20, and 40 µM. The results show that carvacrol was the main constituent in most of the essential oil analyses, except for one of them (0 µM of Cd×10 µM of Se). Furthermore, differences among minor constituents in most of treatments were not significant. Therefore, these results indicate the role of Cd and Se in the compositional changes of *S. hortensis* essential oil.

1. INTRODUCTION

Trace pollutants such as cadmium (Cd) are toxic to humans, animals, and plants. They enter the environment through human activities. This chemical compound is easily absorbed by root and transported inside the plant, where it is toxic for living cell even at very low concentrations (Gallego *et al*., 2012). Plants affected by cadmium are disturbed in photosynthesis, nutrition, and water balance. Several cellular interactions indicate the toxicity by Cd. Metals bind to enzymes ligands, so their toxic effects are mainly determined by interaction with those when enzyme inhibition is probably due to the masking of catalytically active groups (Saidi *et al*., 2014). Several studies have indicated that Cd generates oxidative stress either by causing oxygen free radical production and reactive oxygen species (ROS) or by declining enzymatic and non-enzymatic antioxidants activity due to its high affinity towards sulfur-containing peptides and protein (Wang *et al*., 2014).

Selenium (Se) as an essential element for humans and animals is also useful for plants (Pezzarossa *et al*., 2012). Se has not been confirmed as an essential micronutrient in higher

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plants, but several studies have shown that relatively low concentrations of it can have beneficial effects on plant growth under normal and stressed conditions. Se acts as an antioxidant agent and can increase plant tolerance (Hawrylak-Nowak *et al*., 2014). Several studies have suggested that Se can limit production of ROS and participate in their quenching through both direct and indirect mechanisms. Three possible mechanisms have been proposed for Se to scavenge ROS (Feng *et al*., 2013). In the first mechanism, superoxide anions are dismutated into H_2O_2 without involvement of superoxide dismutase. Secondly, its presence in seleno compounds, quenching superoxide anions, and hydroxyl radicals has been proven. The third mechanism involves increasing antioxidative enzyme activity (Handa *et al*., 2019).

Secondary metabolites are derivatives of primary metabolites that are produced by plants when their physiological processes change (Ashraf *et al*., 2018). Plant growth and survival are significantly improved by the secondary metabolites under different environmental stresses, making them important metabolic molecules (Zandalinas *et al*., 2018). Under natural conditions, plants are susceptible to biotic and abiotic stresses. Secondary metabolites are synthesized by plants in response to these environmental stresses, so they can counteract the adverse effects of both biotic and abiotic stresses. Thus, stresses in the environment strongly influence the production of plant secondary metabolites (Raduisene *et al*., 2012).

Essential oils make up a significant part in aromatic plants. When a plant is under stress, essential oil is generally considered remnants of its main metabolic processes (Mohtashami *et al*., 2018). Essential oils are made in medicinal and aromatic plants and known as the secondary metabolites. They are used in pharmaceutical, food, and cosmetic industries, due to their antimicrobial, anti-inflammation, anti-spasmodic, sedative, carminative, and appetizer properties (Hajhashemi *et al*., 2003; Mohtashami *et al*., 2021). The genus *Satureja* (the family Lamiaceae) comprises 200 species of herbs and shrubs which are widely distributed in the Mediterranean regions. Savory plant is used as an antiseptic for the digestive system as it improves blood pressure and reduces cough (Mohtashami *et al*., 2021). The summer savory, *Satureja hortensis* L. (Figure 1) has medicinal and food values due to its prominent essential oil compounds such as carvacrol and thymol (Alizadeh *et al*., 2020). It is very important to investigate the changes of secondary metabolites in medicinal plants treated with different chemical compounds. Based on this, the present research was designed. We investigated the changes in structure of the essential oil from this medicinal plant under Cd and Se treatments, which is important from different aspects.

Figure 1. The medicinal plant, *S. hortensis*.

2. MATERIAL and METHODS

2.1. Research Design and Treatments

The present research was conducted during 2017 in the greenhouse of Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran under a factorial

experiment in the form of a completely randomized design (CRD) under three replications. The main goal was to investigate the effect of Cadmium (stress agent) and Selenium (anti-stress factor) on the essential oil structure of *S. hortensis*. Cadmium chloride ($CdCl_2$) and Sodium Selenite salt were obtained from Merck and Sigma Companies, Germany, respectively. Soil contamination was performed with different doses of $CdCl_2$ (0, 75, 100, and 150 mM) and Sodium Selenite salt (0, 10, 20, and 40 µM) under the Field Capacity (FC) moisture. The soils were exposed to wet/dry cycles for 4 months as approximate natural conditions for a long-term contamination. Finally, the seeds of *S. hortensis* were planted in the contaminated soil and monitored until seedling emergence. In the control, no chemical compounds were applied (Azizi *et al*., 2021).

2.2. Essential Oil Isolation

The aerial parts of savory plant which contained the most essential oil content were collected from each treated sample and the control plants. Then, they completely dried under the room temperature (about 25°C) in shadow for about a week. Then, the dried samples were powdered by an electric mill and 50 g of each was added to 500 ml of the distilled water. Finally, their essential oils were separated by a Clevenger apparatus in a temperature of 100 °C for 4 h (Figure 2). The water in each essential oil was removed by Na_2So_4 and the pure essential oils were stored at 4°C in special sealed vials (dark glass bottles) until the chemical analysis by GC-MS (Asadi *et al*., 2018, 2019).

Figure 2. The essential oil isolation from *S. hortensis*.

2.3. Chemical Analysis of The Isolated Essential Oils

Quantitative and qualitative analyses of the essential oils were done by the Agilent Technology 7890B gas chromatography-mas spectroscopy (made in USA) with the following specifications:

- Column: HP-5MS
- Long: 30 m
- Diameter: 0.25 mm
- Film thickness: 0.25 mm.
- Analysis temperature: 350°C

Determining of ranges was done by mass data bank, coates index, and retention time. At the end, each compound from the essential oil was identified by extent pattern compared to the valid libraries (Adams *et al*., 2001).

2.4. Statistical Analysis

The present study was repeated three times for all treatments and uninfected plants. Then the data obtained were checked for normality. Finally, the normalized data (if needed) were analyzed by using one-way ANOVA and their means compared by Tukey's test (p<0.05) with SPSS software version 20.

3. RESULTS

3.1. Treatment of Cd Concentrations with 0 µM of Se

Treatments of Cd (0, 75, 100, and 150 µM) with 0 µM of Se were investigated on the structure of *S. hortensis* essential oil (Table 1). About all treatments, Carvacrol was determined as the main constituent. By treatment of 0 µM×0 µM (Cd×Se), differences among most of the constituents were significant ($F_{17, 36}$= 10.16). Under treatment of 75 µM×0 µM (Cd×Se), carvacrol, γ-terpinene, and benzene had high percentages in the essential oils structure, respectively ($F_{17, 36}$= 4.90). Also, with 100 µM×0 µM (Cd×Se) treatment, limonene showed the lowest percentage compared to the others ($F_{17, 36}$= 14.16). Finally, about 150 µM×0 µM (Cd×Se) treatment, most of differences were significant when octatriene being absent in the essential oil structure ($F_{17, 36}$= 7.93).

Table 1. The effects of different Cd concentrations with 0 µM of Se on the structure of *S. hortensis* essential oil.

Compound	Cadmium × Selenium			
	0 µM × 0 µM	75 µM × 0 µM	100 µM × 0 µM	150 µM × 0 µM
α-Pinene	1.03 ± 0.15 ^{efg}	1.04 ± 0.07 ^{e}	1.15 ± 0.04 ^{ef}	1.21 ± 0.02 ^{e}
α-Terpinene	4.14 ± 0.37 ^{d}	4.37 ± 0.05 ^{d}	4.94 ± 0.12 ^{d}	4.99 ± 0.35 ^{d}
α-Thujene	1.32 ± 0.11 ^{ef}	1.31 ± 0.08 ^{e}	1.52 ± 0.09 ^{e}	1.51 ± 0.04 ^{e}
ß-Bisabolene	0.07 ± 0.03 ^{g}	0.22 ± 0.07 ^{e}	0.36 ± 0.16 ^{f}	0.32 ± 0.05 ^{ef}
ß-Myrcene	1.34 ± 0.14 ^{e}	1.26 ± 0.11 ^{e}	1.50 ± 0.11 ^{e}	1.48 ± 0.17 ^{e}
ß-Pinene	0.37 ± 0.02 ^{efg}	0.40 ± 0.01 ^{e}	0.49 ± 0.02 ^{ef}	0.46 ± 0.05 ^{ef}
γ-Terpinene	19.92 ± 0.86 ^{b}	19.66 ± 0.40 ^{b}	21.43 ± 0.42 ^{b}	23.42 ± 0.49 ^{b}
Benzene	7.98 ± 0.09 ^{c}	6.24 ± 0.52 ^{c}	7.16 ± 0.65 ^{c}	6.70 ± 0.06 ^{c}
Camphene	0.18 ± 0.06 ^{efg}	0.04 ± 0.02 ^{e}	0.07 ± 0.03 ^{f}	0.06 ± 0.00 ^{f}
Carvacrol	60.00 ± 0.40 ^{a}	62.59 ± 0.78 ^{a}	58.71 ± 0.48 ^{a}	56.84 ± 0.63 ^{a}
Carene	0.09 ± 0.05 ^{g}	0.07 ± 0.07 ^{e}	0.09 ± 0.02 ^{f}	0.03 ± 0.03 ^{f}
Caryophyllene	0.26 ± 0.02 ^{efg}	0.32 ± 0.08 ^{e}	0.27 ± 0.03 ^{f}	0.28 ± 0.03 ^{ef}
Cyclohexen	0.06 ± 0.11 ^{efg}	0.20 ± 0.02 ^{e}	0.08 ± 0.04 ^{f}	0.25 ± 0.09 ^{ef}
Limonene	0.06 ± 0.16 ^{g}	0.46 ± 0.31 ^{e}	0.03 ± 0.03 ^{f}	0.53 ± 0.42 ^{ef}
Octatriene	0.06 ± 0.03 ^{g}	0.08 ± 0.05 ^{e}	0.05 ± 0.02 ^{f}	0.00 ± 0.00 ^{f}
Phellandrene	0.45 ± 0.14 ^{efg}	0.34 ± 0.03 ^{e}	0.34 ± 0.04 ^{f}	0.39 ± 0.04 ^{ef}
Sabinene	0.13 ± 0.02 ^{fg}	0.08 ± 0.05 ^{e}	0.08 ± 0.04 ^{f}	0.20 ± 0.04 ^{ef}
Thymyl acetate	0.35 ± 0.06 ^{efg}	0.03 ± 0.03 ^{e}	0.04 ± 0.04 ^{f}	0.13 ± 0.08 ^{ef}

The values in each column with different letters show significant differences (Tukey's test, p<0.05).

3.2. Treatment by Cd Concentrations with 10 µM of Se

Treatments by Cd (0, 75, 100, and 150 µM) with 10 µM of Se were investigated on *S. hortensis* essential oil (Table 2). About 0 µM×10 µM (Cd× Se), carvacrol and camphene had the highest and lowest percentage in total, respectively ($F_{17, 36}$= 9.32). By treatment of 75 µM×10 µM (Cd×Se), carvacrol had the highest percentage and carene being the lowest ($F_{17, 36}$= 13.28). Moreover, about 100 µM×10 µM (Cd× Se), carvacrol and carene had the highest and lowest percentage in comparison to the others ($F_{17, 36}$= 5.21). Finally, by treatment of 150 µM×10 µM (Cd×Se), carvacrol, γ-terpinene, and benzene showed high percentages in the essential oil structure, respectively ($F_{17, 36}$= 17.18).

Table 2. The effects of different concentrations of Cd with 10 µM of Se on the structure of *S. hortensis* essential oil.

Compound	Cadmium × Selenium			
	0 µM × 10 µM	75 µM × 10 µM	100 µM × 10 µM	150 µM × 10 µM
α-Pinene	1.33 ± 0.12 ^{ef}	1.24 ± 0.02 ^{d}	1.31 ± 0.09 ^{d}	1.23 ± 0.02 ^{d}
α-Terpinene	1.08 ± 0.15 ^{efg}	4.63 ± 0.06 ^{c}	5.22 ± 0.17 ^{c}	5.11 ± 0.44 ^{c}
α-Thujene	1.43 ± 0.10 ^{e}	1.43 ± 0.05 ^{d}	1.52 ± 0.08 ^{d}	1.55 ± 0.04 ^{d}
ß-Bisabolene	4.44 ± 0.13 ^{d}	0.22 ± 0.07 ^{d}	0.50 ± 0.12^{d}	0.29 ± 0.08 ^{d}
ß-Myrcene	6.09 ± 0.58 ^{c}	1.28 ± 0.05 ^{d}	1.54 ± 0.14 ^{d}	1.59 ± 0.03 ^{d}
ß-Pinene	19.82 ± 0.15 ^{b}	0.42 ± 0.02 ^{d}	0.48 ± 0.05 ^{d}	0.54 ± 0.07 ^{d}
γ-Terpinene	62.18 ± 0.49 ^{a}	20.57 ± 0.40 ^{b}	22.04 ± 0.23 ^{b}	23.43 ± 0.17 ^{b}
Benzene	0.44 ± 0.03 ^{fg}	4.87 ± 0.69 ^{c}	6.04 ± 1.03 ^{c}	5.62 ± 0.68 ^{c}
Camphene	0.05 ± 0.03 ^{g}	0.03 ± 0.01 ^{d}	0.09 ± 0.02 ^{d}	0.14 ± 0.00 ^{d}
Carvacrol	0.23 ± 0.12 ^{g}	62.97 ± 0.91 ^{a}	58.65 ± 0.90 ^{a}	58.15 ± 1.59 ^{a}
Carene	0.06 ± 0.03 ^{g}	0.10 ± 0.06 ^{d}	0.03 ± 0.03 ^{d}	0.08 ± 0.04 ^{d}
Caryophyllene	0.14 ± 0.06 ^{g}	0.32 ± 0.00 ^{d}	0.32 ± 0.00 ^{d}	0.30 ± 0.02 ^{d}
Cyclohexen	0.35 ± 0.02 ^{g}	0.20 ± 0.03 ^{d}	0.28 ± 0.06 ^{d}	0.20 ± 0.09 ^{d}
Limonene	0.17 ± 0.08 ^{g}	0.16 ± 0.08 ^{d}	0.33 ± 0.23 ^{d}	0.21 ± 0.12 ^{d}
Octatriene	0.22 ± 0.04 ^{g}	0.16 ± 0.01 ^{d}	0.13 ± 0.00 ^{d}	0.03 ± 0.03 ^{d}
Phellandrene	0.09 ± 0.06 ^{g}	0.34 ± 0.03 ^{d}	0.41 ± 0.01 ^{d}	0.40 ± 0.04 ^{d}
Sabinene	0.10 ± 0.10 ^{g}	0.16 ± 0.09 ^{d}	0.19 ± 0.02 ^{d}	0.18 ± 0.04 ^{d}
Thymyl acetate	0.54 ± 0.08 ^{efg}	0.12 ± 0.06 ^{d}	0.05 ± 0.05 ^{d}	0.10 ± 0.00 ^{d}

The values in each column with different letters show significant difference (Tukey's test, p<0.05).

3.3. Treatment by Cd Concentrations with 20 µM of Se

Treatments by Cd (0, 75, 100, and 150 µM) with 20 µM of Se were investigated on the constituents of *S. hortensis* essential oils (Table 3). About all essential oils, carvacrol was tha major constituent. On treatment of 0 µM×20 µM (Cd× Se), carvacrol and octatriene showed the highest and lowest percentages than the others ($F_{17, 36}$= 8.27). Moreover, the treatment of 75 µM×20 µM (Cd× Se), carvacrol, γ-terpinene, and α-terpinene showed high percentages in the essential oil structure ($F_{17, 36}$= 5.64).

Table 3. The effects of different Cd concentrations with 20 µM of Se on the structure of *S. hortensis* essential oil.

Compound	Cadmium × Selenium			
	0 µM × 20 µM	75 µM × 20 µM	100 µM × 20 µM	150 µM × 20 µM
α-Pinene	1.03 ± 0.15 ^{d}	1.34 ± 0.07 ^{e}	1.29 ± 0.01 ^{e}	1.40 ± 0.03 ^{de}
α-Terpinene	4.84 ± 0.19 ^{c}	4.51 ± 0.06 ^{c}	5.35 ± 0.37 ^{c}	4.96 ± 1.08 ^{c}
α-Thujene	1.44 ± 0.09 ^{d}	1.46 ± 0.08 ^{e}	1.55 ± 0.07 ^{de}	1.75 ± 0.07 ^{de}
ß-Bisabolene	0.38 ± 0.12 ^{d}	0.30 ± 0.03 ^{e}	0.49 ± 0.08 ^{e}	0.38 ± 0.07 ^{e}
ß-Myrcene	1.45 ± 0.13 ^{d}	1.35 ± 0.07 ^{e}	1.77 ± 0.03 ^{de}	1.84 ± 0.13 ^{de}
ß-Pinene	0.45 ± 0.03 ^{d}	0.47 ± 0.02 ^{e}	0.56 ± 0.00 ^{e}	0.67 ± 0.03 ^{e}
γ-Terpinene	20.87 ± 0.28 ^{b}	21.16 ± 0.74 ^{b}	22.51 ± 0.32 ^{b}	23.27 ± 0.10 ^{b}
Benzene	4.31 ± 0.70 ^{c}	3.41 ± 0.40 ^{d}	3.78 ± 0.81 ^{cd}	2.92 ± 0.40 ^{d}
Camphene	0.18 ± 0.07 ^{d}	0.14 ± 0.02 ^{e}	0.11 ± 0.01 ^{e}	0.14 ± 0.03 ^{e}
Carvacrol	62.59 ± 0.89 ^{a}	62.85 ± 0.62 ^{a}	59.59 ± 1.93 ^{a}	59.08 ± 1.44 ^{a}
Carene	0.18 ± 0.03 ^{d}	0.12 ± 0.02 ^{e}	0.10 ± 0.01 ^{e}	0.22 ± 0.10 ^{e}
Caryophyllene	0.33 ± 0.07 ^{d}	0.37 ± 0.02 ^{e}	0.38 ± 0.02 ^{e}	0.42 ± 0.06 ^{e}
Cyclohexen	0.38 ± 0.03 ^{d}	0.17± 0.04 ^{e}	0.18± 0.04 ^{e}	0.18± 0.0.0 ^{e}
Limonene	0.25 ± 0.15 ^{d}	0.70 ± 0.22 ^{e}	0.40 ± 0.23 ^{e}	0.81 ± 0.09 ^{e}
Octatriene	0.12 ± 0.00 ^{d}	0.21 ± 0.02 ^{e}	0.17 ± 0.00 ^{e}	0.24 ± 0.03 ^{e}
Phellandrene	0.41 ± 0.03 ^{d}	0.44 ± 0.06 ^{e}	0.42 ± 0.02 ^{e}	0.50 ± 0.06 ^{e}
Sabinene	0.19 ± 0.08 ^{d}	0.25± 0.08 ^{e}	0.28± 0.11 ^{e}	0.24± 0.05 ^{e}
Thymyl acetate	0.21 ± 0.12 ^{d}	0.22 ± 0.05 ^{e}	0.22 ± 0.01 ^{e}	0.29 ± 0.05 ^{e}

The values in each column with different letters show significant differences (Tukey's test, p<0.05).

By treatment of 100 µM×20 µM (Cd× Se), carvacrol and carene showed the highest and lowest percentage in comparison to the other constituents, respectively ($F_{17, 36}$= 7.87). Finally, by treatment of 150 µM×20 µM (Cd× Se), carvacrol had the highest when camphene showed the lowest percentage in the essential oil ($F_{17, 36}$= 6.73).

3.4. Treatment of Cd Concentrations with 40 µM of Se

Treatments of Cd (0, 75, 100, and 150 µM) with 40 µM of Se were investigated on the constituents of *S. hortensis* essential oil (Table 4). About all, carvacrol was the major constituent. By treatment of 0 µM×40 µM (Cd× Se), carvacrol showed the highest percentage when camphene being the lowest compared to the others ($F_{17, 36}$= 20.21). Moreover, under the treatment of 75 µM×40 µM (Cd× Se), carvacrol and carene showed the highest and lowest percentages ($F_{17, 36}$= 8.06). About 100 µM×40 µM (Cd× Se) treatment, carvacrol, γ-terpinene, and α-terpinene showed high percentages, respectively ($F_{17, 36}$= 8.15). Finally, by treatment of 150 µM×40 µM (Cd×Se), carvacrol had the highest percentage, while sabinene showed the lowest percentage compared to the others ($F_{17, 36}$= 10.52).

Table 4. The effects of different treatments of Cd with 40 µM of Se on composition of *S. hortensis* essential oil.

Compound	Cadmium × Selenium			
	0 µM × 40 µM	75 µM × 40 µM	100 µM × 40 µM	150 µM × 40 µM
α-Pinene	1.16 ± 0.11 ^{ef}	1.41 ± 0.07 ^{d}	1.42 ± 0.08 ^{efg}	1.35 ± 0.03 ^{ef}
α-Terpinene	4.54 ± 0.13 ^{c}	4.84 ± 0.06 ^{c}	5.57 ± 0.15 ^{c}	5.37 ± 0.27 ^{c}
α-Thujene	1.51 ± 0.11 ^{e}	1.52 ± 0.08 ^{d}	2.04 ± 0.34 ^{def}	1.76 ± 0.07 ^{ef}
ß-Bisabolene	0.32 ± 0.03 ^{g}	0.32 ± 0.02 ^{d}	0.41 ± 0.10 ^{gh}	0.37 ± 0.02 ^{ef}
ß-Myrcene	1.48 ± 0.11 ^{e}	1.52 ± 0.13 ^{d}	2.11 ± 0.11 ^{de}	1.91 ± 0.04 ^{e}
ß-Pinene	0.53 ± 0.01 ^{fg}	0.56 ± 0.02 ^{d}	0.72 ± 0.03 ^{gh}	0.66 ± 0.05 ^{ef}
γ-Terpinene	20.50 ± 0.41 ^{b}	21.40 ± 1.42 ^{b}	22.64 ± 0.26 ^{b}	23.24 ± 0.53 ^{b}
Benzene	3.65 ± 0.26 ^{d}	3.53 ± 0.34 ^{c}	2.96 ± 0.63 ^{d}	3.35 ± 0.12 ^{d}
Camphene	0.18 ± 0.04 ^{g}	0.17 ± 0.04 ^{d}	0.22 ± 0.06 ^{h}	0.12 ± 0.08 ^{f}
Carvacrol	63.27 ± 0.26 ^{a}	61.11 ± 1.08 ^{a}	58.65 ± 0.42 ^{a}	59.38 ± 1.19 ^{a}
Carene	0.25 ± 0.08 ^{g}	0.18 ± 0.00 ^{d}	0.17 ± 0.02 ^{g}	0.25 ± 0.05 ^{f}
Caryophyllene	0.43 ± 0.03 ^{fg}	0.40 ± 0.02 ^{d}	0.39 ± 0.01 ^{gh}	0.39 ± 0.03 ^{ef}
Cyclohexen	0.23± 0.08 ^{g}	0.28± 0.01 ^{d}	0.21± 0.10 ^{h}	0.18± 0.06 ^{f}
Limonene	0.57 ± 0.31 ^{fg}	0.71 ± 0.20 ^{d}	1.01 ± 0.39 ^{gh}	0.32 ± 0.17 ^{f}
Octatriene	0.19 ± 0.03 ^{g}	0.22 ± 0.02 ^{d}	0.16 ± 0.02 ^{g}	0.17 ± 0.02 ^{f}
Phellandrene	0.44 ± 0.01 ^{fg}	0.52 ± 0.09 ^{d}	0.57 ± 0.07 ^{gh}	0.46 ± 0.05 ^{ef}
Sabinene	0.19± 0.08 ^{g}	0.38± 0.14 ^{d}	0.38± 0.14 ^{h}	0.11± 0.03 ^{f}
Thymyl acetate	0.30± 0.07 ^{g}	0.20 ± 0.01 ^{d}	0.19 ± 0.09 ^{h}	0.25 ± 0.04 ^{f}

The values in each column with different letters show significant differences (Tukey's test, p<0.05).

4. DISCUSSION and CONCLUSION

Very limited studies have been carried out on the effects of Se and the other heavy metals on the secondary metabolites of *S. hortensis*. Mumivand *et al*., (2011) studied the changes in *S. hortensis* essential oil under calcium carbonate and nitrogen treatments and found their effect on the difference in essential oil composition as significant. The GC-MS results showed that *S. hortensis* constituents changed by relative percentage of carvacrol, γ-terpinene, and β-bisabolene. The above results were different from ours despite different treatment, because we observed certain changes in the secondary metabolites of this medicinal plant. Karimi *et al*., (2013) studied the effects of Cd on *S. hortensis* and reported that arsenic, Cd, and mercury were observed in artichoke and savory root in comparison to aerial parts. Futhermore, artichoke showed higher heavy metals absorption as bioaccumulation factor and transfer efficiency from root to stem than savory plant. Accordingly, artichoke showed higher accumulation capacity

than savory. Based on this, extraction of metals by artichoke can be applied to clean the soils from heavy metals pollution. Heavy metals have different effects on secondary metabolites and this subject has been proven in different studies. This issue should be considered by researchers in soils contaminated with these metals.

Ashraf *et al*., (2018) reported that plants are exposed to abiotic stresses such as fertilizers, soil type and its composition, high temperatures, light intensity, lack of access to water, and salinity that can affect their life. Plants need specific amounts of abiotic components while lack or excess of them causes changes in biosynthesis of secondary metabolites and determine plants growth and development. Also, concentration of them in plants increases in response to environmental stresses such as temperature, lack of nutrients, wounds, and UV rays. Plants also show changes in concentration of phenols due to the lack of effective substances in their nutrition. In the present study, significant changes in the secondary metabolites were observed by application of Se and Cd treatments, which is in line with the results of our study. The effect of organic fertilizers on the essential oil of *S. hortensis* was studied by Esmailpour *et al*., (2018) while the highest and lowest essential oil content were obtained in plants treated with vermicompost 30% and unwashed mushroom compost, respectively. Also, the main compounds were determined as carvacrol and γ-terpinene. The highest level of them was observed in plants grown on 40% and 20% substrates including washed spent mushroom, respectively. In our study, the role of organic fertilizers was not investigated, but this is a new aspect that should be considered in future. Despite the application of different treatments, the main constituents of the essential oil from this medicinal plant has not changed, which confirms our results.

Azizollahi *et al*., (2019) studied Cd accumulation in *S. hortensis* and reported that its main constituent was carvacrol, which showed suitable values under treatment by this heavy metal. *S. hortensis* can also be considered as an invaluable alternative crop for contaminated soils by Cd. Besides, due to suitable potential of Cd accumulation in the root, it can be a suitable tool for phytostabilization purposes. Finally, Memari-Tabrizi *et al*., (2021) investigated foliar spraying of silicon nanoparticles in reducing cadmium stress on *S. hortensis* essential oil and reported the dominant compounds were carvacrol, γ-terpinene, p-cymene, and thymol. Their results are somewhat consistent with ours. The type of treatment was different, which affected the results to some extent.

About the other plants, Manquián-Cerda *et al*., (2016) studied the effect of Cd in concentrations of 50 and 100 μM on antioxidant potential and phenolic compounds accumulation in *Vaccinium corymbosum* L. (Ericaceae) in the form of DPPH activity in which free-radical of DPPH interacts with an odd electron to yield a strong absorbance at 517 nm. Furthermore, the production of phenolic compounds was significantly affected under Cd stress. The highest amount of chlorogenic acid was produced in blueberry plants at higher levels of Cd. It has been suggested that antioxidant activity of the plant had a positive correlation with phenolic compounds. The results of their research are consistent with ours regarding the effect of Cd stress on the amount of phenolic compounds. In another study, Handa *et al*., (2019) evaluated the effect of Se on biochemical factors and secondary metabolites in *Brassica juncea* L. (Brassicaceae) under chromium stress conditions. The co-application of Se and Cr led to increasing of total phenolic, flavonoid, and anthocyanin content in plants grown inside Cr-amended soils. According to their findings, Se has a similar effect on secondary metabolites under heavy metal stress as we have found this in our study as well.

In another study conducted by Elguera *et al*., (2013), the effect of Se and Cd on the phenolic compounds of *Lepidium sativum* L. (Brassicaceae) was investigated when quantity of phenolic compounds increased under the treatment of these two elements. Azizi *et al*., (2021) investigated the effects of foliar application by Se on morphological and physiological indices

of *S. hortensis* under Cd stress and found that Se spray reduced the toxic effects of Cd stress on it by increasing proline, stimulating enzymes, and limiting leakage in cell membrane. Also, Se foliar application under Cd stress conditions improved chlorophyll and reduced Cd accumulation in the root. Overall, their study showed that Se foliar application can reduce Cd toxicity and improve growth under different levels of Cd and another heavy metals. Although, changes in secondary metabolites were not investigated in their study, the role of Se has been effective in reducing negative effects of Cd and it was consistent with the present research. Karimi *et al*., (2022) investigated Se- and Silicon-mediated recovery of *Satureja mutica* Fisch. chemotypes subjected to drought stress followed by rewatering and concluded that no separation was observed in savory chemotypes in response to foliar Se and silicon applications. However, plants treated with Se showed a decrease in proline accumulation under drought stress conditions. Based on their results, *S. mutica* was a valuable medicinal plant resistant to drought especially in areas with low rainfall and can be introduced into the agricultural systems. In our studies, we observed certain changes in the essential oil of this medicinal plant under Se and Cd treatments. However, plant species was different in two studies and this important issue is very effective in changing the results.

In conclusion, this research indicated that treatment by Se and Cd made obvious effects on *S. hortensis* essential oil although major constituents were not changed due to their high differences with the others. These studied recommend improving the biological position of *S. hortensis* plants under Cd stress and Se as its anti-stress agent. For this, the authors of this article encourage other researchers to examine the essential oils structure from the other medicinal plants under treatment of various agents. They hope that it will be possible to determine the conditions in which the highest number of useful compounds with the highest percentage can be produced.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Iraj Azizi: Investigation, Methodology, Supervision, and Validation. **Mohammad Asadi**: Resources, Visualization, Software, Formal Analysis, and Writing Original draft.

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Research Article

Effect of Na, Mg, Ca chloride salts on mineral element, proline and total protein contents in rice (*Oryza sativa* L.) grown *in vitro*

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Abstract: In this study, the effects of different types and concentrations of salts on local Siverek rice plant (*Oryza sativa* L.) grown *in vitro* were investigated in terms of mineral elements (K, Ca, P, Mg, Na, Fe, Cu, Zn, Mn, Mo, Co), proline, and total protein content. Sterilized seeds were planted in hormone-free and salt-free MS medium. After one week, the seedlings were subjected to different concentrations of NaCl, $CaCl_2$, and $MgCl_2$ salts (0, 30 mM, 90 mM) in order to evaluate the effect of salinity on plant growth and development. In response to salt stress, a decrease in nutrient elements was observed for all three types of salt compared to the control group, which can be attributed to disruptions in ion balance. Changes in element levels generally showed varying levels of increase or decrease depending on both the type and concentration of the salt and these changes were statistically significant. The increase in proline level was found to be directly proportional to the changes in the amounts of Ca, Mg, K, and Na elements. Both total protein and proline content showed the lowest values for all salt concentrations with $CaCl_2$, while the highest values were obtained with NaCl. In conclusion, the changes in the level of mineral elements, total protein, and proline content levels, which decrease or increase in different ratios, depending on the type and concentration rising of the salt, are associated with the varying tolerance of the plant to different types of salts.

1. INTRODUCTION

Soil salinity is one of the environmental stresses worldwide adversely affecting plant growth and productivity. Approximately one billion hectares of land are affected by salt stress globally (Fageria *et al*., 2012), and this number is increasing every year. The significant impact of high salinity on crop growth and development is of great concern, as it can affect agricultural productivity on more than 20% of the global cultivated land (Botella *et al*., 2005; Fahad *et al*., 2019; Dramalis *et al*., 2021).

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Nutrient depletion, disruption of soil aggregates, and impairment of plant growth and development are among the main effects of soil salinization (Kordrostami *et al*., 2017). Salinity impedes plant growth through three main principles; namely, 1) water deficiency (water stress), 2) ion toxicity, and 3) intracellular fluid due to imbalances in ion transport disruption of the mineral balance (Marschner, 1995; Akyol *et al*., 2020). Osmotic stress results from reduced water availability due to salt stress, which leads to impaired nutrient transport efficiency, including macro elements such as nitrogen (N), phosphorus (P), potassium (K), and calcium (Ca). This, in turn, leads to nutrient deficiency and ion toxicity (Razzaq *et al*., 2020). Salinity exerts diverse stresses on crops, leading to imbalances in nutrient uptake, toxic ion accumulation, and exposure to oxidative and osmotic stress (Wang *et al*., 2012; Singh *et al*., 2018). When ions expand in the soil, plants are exposed to salt stress. These ions accumulate at higher levels, leading to a decrease in essential ions within the plants. Consequently, this disrupts the relationship between the plant and water by inducing conditions resembling drought, causing osmotic stress. This stress factor is accountable for the reduction in stomatal conductance and the activities of photosynthetic enzymes, ultimately triggering the generation of reactive oxygen species (ROS) in plants (Hasanuzzman *et al*, 2021). ROS have the potential to harm cellular components such as cell membranes, proteins, lipids, and genetic materials (DNA and RNA). Additionally, they may trigger programmed cell death (Kumar *et al*, 2020). While the primary response mechanism to osmotic degradation is recognized, the exact molecules involved and their specific roles are yet to be fully elucidated. The cytosolic Ca++ level increases in response to osmotic imbalance by unknown sensors, which is sensed by SOS2 SOS3 (salt overly sensitive) protein complex. SOS2-SOS3 complex activates the SOS1 protein by phosphorylate. SOS1 functions as a Na^+/H^+ antiporter protein located in the cell membrane, facilitating the expulsion of Na ions from the cell. SOS2 can also regulate the activity of NHX1 (Na/H antiporter) and V-ATPase (vacuoler type ATPase) antiport independently of SOS3. It can be regulated by SCaBP (SOS liked Ca^{++} bindings protein), which targets the tonoplast. Salinity can lead to an increase in ABA (abscisic acid) accumulation, which is activated by ABI1 and ABI2, negatively affecting the SOS system and NHX1 (Shanker, 2011).

Rice (*Oryza sativa* L.) is one of the most important food sources, and there are still countries that cover a considerable part of their energy requirements from this plant or its derivatives. Salinity stress affects rice in several ways, causing changes in the physiological and morphological structures of cells, as well as affecting the synthesis of certain biochemicals (Kumar *et al*., 2012; Liu *et al*., 2010; Nam *et al*., 2012; Pani *et al*., 2012; Rajendran *et al*., 2009; Wang *et al*., 2013; Orcan *et al*., 2017; Mondal *et al*., 2018). Saline soils lead to ion imbalances, which negatively affect both the growth and yield of rice plants and their nutrient content, thus affecting the overall quality of the crop (Rao *et al*., 2013).

The studies on salt stress in rice generally focus on the effects of NaCl, and there is a lack of research on the effects of $CaCl_2$ and $MgCl_2$ salts. In addition, there are no studies on the content of mineral elements in local Karacadağ rice (Siverek population) under the influence of NaCl and various salts. Therefore, the aim of this study was to investigate the effects of different salt types (NaCl, $CaCl_2$, and $MgCl_2$) and concentrations (0, 30, 90 mM) on the content of important micro- and macro-mineral nutrients such as potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), molybdenum (Mo), and cobalt (Co), as well as on the total protein and proline amounts in the local Karacadağ rice (Siverek population) under salt stress.

2. MATERIAL and METHODS

2.1. Seed Sterilization

The Karacadağ rice variety, which was obtained from local growers near Diyarbakır, Türkiye, served as the plant material in this study. Prior to the experiments, the rice seeds underwent a 30-second soak in 70% ethanol, followed by optimal surface sterilization through a 60-minute soak in 5% NaOCl.

2.2. *In vitro* Conditions

MS medium was prepared with MS main solution, MS-1, MS-2, complex chelator, vitamin mixture, and B1 vitamin containing thiamine. The nutrient media were formulated by combining 30 g of sucrose and 5,458 g of agar with 1liter of distilled water. The medium was adjusted to pH 5,7 by using acid and base. The prepared medium was autoclaved at 121^0C and 1 atm pressure for 25 minutes. The final solution was transferred to a Magenda GA-7 (purchased from Merck KGaA, Darmstadt, Germany) culture vessel in portions of approximately 50-60 mL.

2.3. Plant Growth

The sterilized seeds were planted in magendas containing hormone-free and salt-free MS medium to germinate. After 1 week of germination, in order to evaluate the effect of salinity stress on plant growth and development, the seedlings were transferred to a culture vessel containing separately NaCl, $CaCl_2$ and $MgCl_2$ salts at different concentrations (control, 30 mM, 90 mM). Rice seedlings were grown in salt-free medium for 3 weeks as a control and under salt stress conditions. At the end of the 3-week growth period, the green part and the roots of the *in vitro* grown plants were harvested.

2.4. Determination of Mineral Element Contents

Harvested and dried samples were weighed. To determine elemental contents, weighed samples were digested in 7mL nitric acid (65% HNO_3) and 1mL hydrochloric acid (37% HCl) using a microwave digester. Digested samples were adjusted to 15mL by adding extra pure water. The samples were then analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) to quantify the elements.

2.5. Determination of Proline Content

In accordance with the technique outlined by Bates *et al*. (1975), proline determination was conducted. The samples were subjected to protein precipitation by sulphosalicylic acid treatment, followed by centrifugation, and the supernatant was then transferred to a fresh tube. After reacting the supernatant with glacial acetic acid and ninhydrin reagent, the reaction was stopped by cooling the tubes on ice. The proline products were subsequently extracted with toluene by vortex mixing, and the absorbance of the toluene phase was recorded at 518 nm. Proline concentrations were computed using a set of proline standards (0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 mM).

2.6. Determination of Total Protein Content

For protein extraction, leaves were treated with an extraction buffer comprising 0.01 M Tris-HCl, 10% glycerol, 5% PVP, and 1% Triton X 100 at pH 6.8. Subsequently, the total protein content of the extracts was quantified according to the Bradford method (Bradford, 1976).

2.7. Statistical Analysis

Triplicate experiments were conducted for all measurements, and the data were reported as mean ± standard deviation (SD). Statistical analysis was carried out using SPSS 20.0 for Windows (SPSS Inc., Chicago, USA), with the significance of differences tested using one-way analysis of variance (ANOVA) and Duncan test.

3. RESULTS

3.1. Mineral Element Contents

Under saline conditions, the reduction in the osmotic potential of the soil solution leads to a reduction in the water potential and thus a reduction in water uptake by plants. It is known that this situation is caused by direct toxic effects of Na and similar cations present in the environment and by the reduced uptake of K, Ca and N, leading to disturbances in the ionic balance (İnal *et al*., 1995; Yakıt & Tuna, 2006). The changes in mineral contents in the Siverek rice population exposed to NaCl, $CaCl_2$, and $MgCl_2$ salts at concentrations of 0, 30, and 90 mM are shown in Table 1. High Na levels in the soil solution can reduce the uptake and mobility of K by inhibiting its transport to the growth zone, leading to a decrease in growth quality of both vegetative and reproductive organs (Dionisio & Tobita, 2000). Ghoulam *et al*. (2002) on sugar beet, Lacerda *et al*. (2002) on sorghum and Essa (2002) on soybean reported an increase in Na content in leaves and roots due to increased Na concentration, while the content of cations such as Ca and K decreased under NaCl salt stress. In our study, the Siverek rice treated with NaCl at all concentrations showed a decrease in the elements Ca, K and Mg. In varieties of *Medicago sativa* (alfaalfa), an increase in the concentration of NaCl led to a decrease in the contents of Ca and Mg. (Bhattarai *et al*., 2022). A decrease in Ca content was observed in rice varieties with varying levels of salt tolerance in response to salt stress, while the change in K content in the leaves of the treated plants was not statistically significant (Thu *et al*., 2017). Mokabel *et al*. (2022) reported that using the microbiome to alleviate the effects of salt stress in Solanum melongena (eggplant) resulted in a significant reduction in Ca and K levels compared to the control group. In addition, they observed a decrease in Mg content in plants treated with 200 mM NaCl, but this decrease was not statistically significant. These studies emphasize the importance of understanding how individual plant species respond to salt stress and mineral nutrient content. Bernstein *et al*. (2017) reported that 30 mM NaCl stress in basil (*Ocimum basilicum*) slowed plant growth, decreased Ca and K concentrations, and increased Na concentration. Similarly, in lentil (*Lens esculenta* Moench) treated with different chlorine salts ($CaCl_2$, $MgCl_2$, and NaCl), increasing concentrations led to a decrease in germination rate, N, Ca, Mg, and K content, while increasing Na content (Karaman & Kaya, 2015). Çalışkan *et al*. (2017) subjected basil (*Ocimum basilicum* L.) to salt stress using different concentrations of NaCl, $CaCl_2$ and $MgCl_2$ (between 1 and 8 ds/m). The lowest root weight was obtained at the highest concentration for each salt type, and the highest root and leaf weight were observed in the control group. The importance of salt stress levels for basil plant development was emphasized by the researchers. In our study, there was a decrease in the amounts of K and Mg elements under $CaCl_2$ salt stress and a decrease in Ca and K elements under $MgCl_2$ stress compared to the control group. The decrease in element quantities under both types of salt stress can be attributed to a disturbance of the ionic balance.

Due to the competition between Na and especially cationic elements such as K and Ca, the balance of Na^+/K^+ and $Na^+/Ca^{++}$is quickly disturbed. This situation leads to K and/or Ca deficiency, which hinders osmoregulation and enzyme activation and has a negative effect on plant metabolism. Studies have found a positive correlation between the amount of K and Ca in leaves and the increase in plant resistance under saline conditions, as well as a direct relationship between high K^+/Na^+ and Ca^{++}/Na^+ ratios and salt tolerance (Vicente *et al*., 2004; Martinez-Atienza *et al*., 2007; Deinlein *et al*., 2014). In our study, the highest K^+/Na^+ ratio was observed in the treatment with $CaCl_2$, $MgCl_2$ and NaCl, respectively, at low salt concentrations compared to the control. At high salt concentrations, the group treated with $MgCl_2$ exhibited the highest K^+/Na^+ ratio. Therefore, it can be concluded from our study that Ca and Mg containing salt solutions, although not as strong as NaCl, still had some negative effects on the macro-element content in rice.

Table 1. Mineral content of elements in Siverek rice exposed to different salt types and concentrations (µg/g)*

Mineral Element	Control	NaCl		$CaCl_2$		$MgCl_2$	
	0 mM	30 mM	90 mM	30 mM	90 mM	30 mM	90 mM
Ca	831.64 ± 16.99^{c}	472.84 ± 9.40^{f}	509.49 ± 10.2^{e}	2928.65 ± 59^{b}	5687.50± 113.68^{a}	194.93± 3.90^{g}	647.48± 13.01^{d}
K	3529.06 ± 63.52^{a}	1678.15 ± 30.2^{f}	2324.59 ± 41.84^{b}	1793.84 ± 32.27^{d}	1134.37± 20.41^{g}	1691.71± 30.43^{e}	2011.57± 36.21^{c}
Mg	54.42 ± 2.16^{e}	18.99 ± 0.74^{g}	44.89 ± 1.79^{f}	131.28 ± 5.24^{c}	60.48± 2.48^{d}	441.20± 17.64^{b}	575.65 ± 22.92^{a}
Na	143.27 ± 3.57^{c}	195.55 ± 4.87^{b}	670.11 ± 16.74^{a}	89.35 ± 2.22^{f}	110.96± 2.75^{d}	103.52± 2.57^{e}	147.82± 3.67^{c}
P	489.32 ± 22.98^{b}	319.80 ± 14.95^{g}	553.88 ± 26.12^{a}	340.97 ± 15.98^{f}	433.97± 20.35^{c}	375.46± 17.62^{e}	389.51± 18.28^{d}
Fe	96.91 ± 3.84^{c}	80.04 ± 3.28^{d}	112.61 ± 4.44^{b}	24.07 ± 0.96^{g}	57.38± 2.28^{f}	67.79 ± 2.68^{e}	139.95± 5.56^{a}
Zn	27.85 ± 0.13^{b}	29.34 ± 0.14^{a}	24.32 ± 0.12^{c}	21.58 ± 0.01^{d}	27.04± 0.13^{b}	27.24± 0.13^{b}	23.89± 0.11^{c}
Co	0.293 ± 0.01^{e}	0.296 ± 0.01^{e}	0.31 ± 0.02^{d}	0.44 ± 0.02^{c}	0.51± 0.02^{a}	0.30± 0.01^{d}	0.46± 0.02^{b}
Cu	8.48 ± 0.12^{g}	10.21 ± 0.14^{e}	19.34 ± 0.20^{a}	9.36 ± 0.12^{f}	13.12± 0.15^{c}	12.67± 0.13^{d}	15.25± 0.18^{b}
Mn	4.36 ± 0.13^{g}	36.77 ± 1.28^{a}	9.04 ± 0.30^{f}	16.67 ± 0.57^{d}	11.59± 0.42^{e}	30.52± 1.06^{b}	17.20± 0.60^{c}
Mo	3.74 ± 0.18^{a}	1.43 ± 0.07^{e}	0.99 ± 0.04^{g}	1.38 ± 0.06^{f}	1.59± 0.07^{d}	2.24± 0.11^{b}	1.79± 0.08^{c}

Note: Different letters (a-g) in each line indicate significant differences by ANOVA and Duncan's test compared to the control group.

Manganese (Mn) acts primarily as a cofactor in important metabolic processes related to photosynthesis, lipid biosynthesis, and oxidative stress in plants. When Mn is deficient, plant growth and yield decrease, and sensitivity to various stress factors such as pathogens, salinity, and frost damage increases (Socha & Guerinot, 2014; Sevilmiş *et al*., 2020). On the other hand, Mn ions have been found to play a role in ageing-related processes, regulation of protein synthesis and chlorophyll synthesis, although these aspects have not been extensively studied (Leidi *et al*., 1991). During transport, Mn competes with other cations as many carriers are used to transport the different cations. This competition can have a negative effect on Mn uptake (Schimansky, 1981; Kacar & Katkat, 2010). Scagel *et al*. (2017) studied the growth and development of basil plants under stress induced by $CaCl_2$ and NaCl. The researchers reported that while Mn uptake is increased by NaCl treatment, it is reduced by $CaCl_2$ treatment. In their study Bhattarai *et al*. (2022) applied NaCl salt stress to different varieties of *Medicago sativa* plants and found an increase in Mn content in all tested cultivars with increasing NaCl concentration. In the present study, an increase was observed in Mn content in all salt types and concentrations applied compared to the control group, but this increase varied according to salt type and concentration. In the control group, the Mn content was measured at 4.36 µg/g, while in the 30 mM salt treatments it was 36.77 µg/g for NaCl, 30.52 µg/g for $MgCl_2$ and 16.67 µg/g for $CaCl_2$. For the 90 mM salt treatments, it was found to be 17.20 µg/g for $MgCl_2$, 11.59 µg/g for $CaCl_2$ and 9.04 µg/g for NaCl.

The importance of molybdenum (Mo) arises from its presence in the structure of nitrogenase and reductase enzymes and the role of the nitrogenase enzyme in the vital activity of microorganisms that fix elemental N from the atmosphere and convert it to N in the soil. Considering this role of Mo, a study was conducted to determine the contribution of Mo fertilization to the N content of chickpea plants using different doses of Mo (0, 0.05, 0.10, 0.15, and 0.20 ppm). Considering the total N gain, the highest value was reached at 0.15 ppm Mo, and the further the dose deviated from this value, the lower the N gain became. Mo deficiency had a negative effect on root formation, resulting in underdeveloped green parts of the plant, and consequently low N gain from the soil (Vural & Müftüoğlu, 2012). In our study, Mo content was found to be lower in all treatments than in the control, with the highest decrease in NaCl and the lowest in $MgCl_2$.

In a study by Zhang *et al*. (2022), the content of certain elements was measured in different rice varieties exposed to different NaCl concentrations as salt stress. The P content increased with increasing salt concentration, although this increase was statistically different depending on the rice variety. Similarly, stress studies conducted on different plants have also recorded varying levels of increase and decrease in P content depending on the species and concentration (Bhattarai *et al*., 2022; Mokabel *et al*., 2022). In our study, as the salt concentration of NaCl and CaCl2 increased, the P content increased and was found to be higher than the control at 90 mM NaCl concentration. However, for $MgCl_2$, there was a varying increase or decrease in P content depending on the concentration, and overall, there was a decrease compared with the control at all concentrations. The P content in all treatments was statistically different.

Furthermore, Malkoç and Aydın (2003) reported that an increase in salt dose generally resulted in a decrease in the content of N, P, K, Ca, Mg, Fe, Mn, Zn, and Cu depending on the variety of maize (*Zea mays* L.) and bean plants. Zeiner *et al*. (2022) applied 200 mmol/L NaCl to different varieties of brassica plants grown in a hydroponic system. The researchers reported a decrease in Fe content in the white cabbage variety compared to the control, while there was no change in the Kale and Chinese cabbage varieties. In our study, the Fe content increased with the rising concentration in all three types of salts, and it was higher than the control at 90 mM $MgCl_2$.

The Cu content was higher than the control in all salt treatments, with a more pronounced increase observed in NaCl and $MgCl_2$ compared to $CaCl_2$. In terms of Zn, the highest increase was observed at 30 mM NaCl compared to the control (27.858 µg/g). For Co, particularly in $MgCl_2$, the Co content increased with an increase in concentration and it was higher than in the control. In the present study, these elements showed varying degrees of increase and decrease depending on the concentration and type of salts.

3.2. Proline Content

Under stressful conditions, plants synthesize and accumulate organic substances like proline within the cell, which plays a significant role in maintaining membrane integrity and osmotic balance (Tuna & Eroğlu, 2017). Proline amino acid not only contributes to osmotic regulation but also plays a role in adjusting cytosolic pH, preserving the integrity of cellular structures and proteins and activating enzymatic processes under stress conditions (Büyük *et al*., 2012). Therefore, in the present study, the proline content of the samples was examined in comparison with the control in response to different concentrations and types of salt stress (Figure 1).

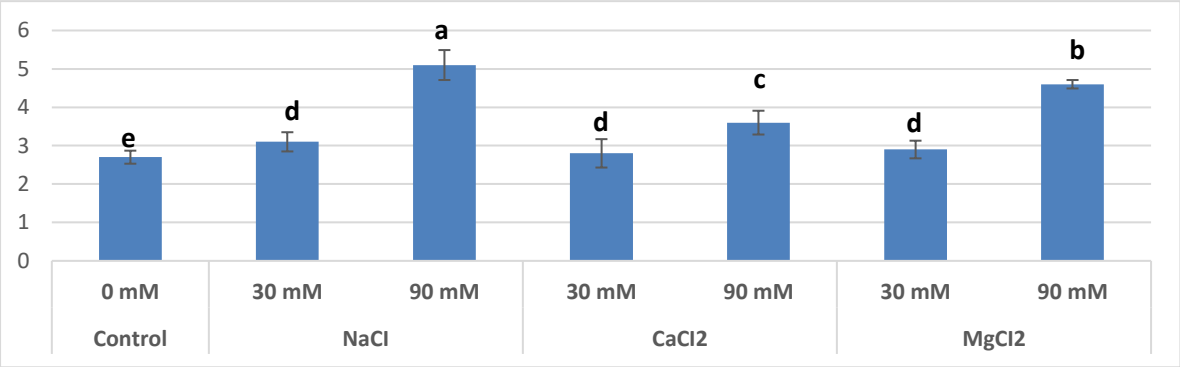

Figure 1. Effect of different salinity stress on proline content (mM/g).

Vertical bars indicate ± SE. Letters indicate significant differences compared with control group.

The study conducted by Özden *et al*. (2011) reported an increase in proline levels in grape (*Vitis vinifera*) plants exposed to salt stress. Similarly, in our study, when compared to the control group, the proline content showed a consistent increase with the rising concentration of each salt type.

The study by Özcan *et al*. (2000) also reported an increase in proline and Na concentrations, while K content decreased in plants under salt stress. Abdelhamid *et al.* (2013) reported that proline treatment through foliar spraying increased P and K concentrations while decreasing Na concentration under salt stress to bean (*Phaseolus vulgaris* L.) plants. Suleiman *et al.* (2023) studied native desert plant species under saline conditions and found that proline accumulation increased with increasing salinity level. In our study, the lowest proline level was obtained from the 30 mM $CaCl_2$ treatment, while the highest values were obtained from the 90 mM NaCl treatment, when compared to the control group. Considering the literature and our study, it can be concluded that the decrease in proline levels is directly related to changes in Ca, Mg, Na, and K element levels, which vary depending on the type of salts used. This could be evidence that the plant has developed a tolerance or self-protective mechanism against stress.

3.3. Total Protein Content

Secondary effects of salinity include impaired protein functionality and reduced chlorophyll content due to limited photosynthesis (Zhu, 2001; Elhindi *et al*., 2017; Kaya & İnan, 2017). Data on the total protein content of extracts from the Siverek rice population exposed to three types of salts (NaCl, $CaCl_2$, and $MgCl_2$) at different concentrations (30 and 90 mM) compared to the control (0 mM) are presented in Figure 2.

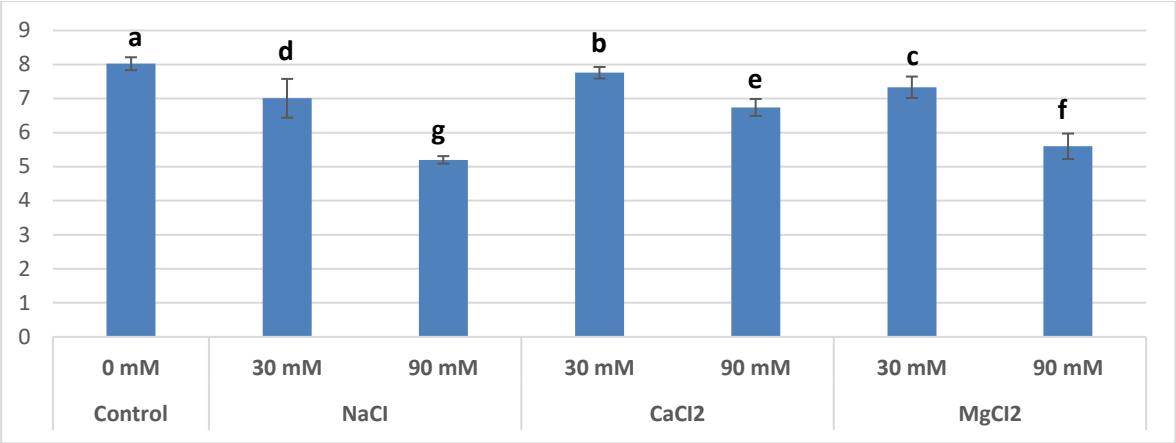

Figure 2. Effect of different salinity stress on total protein content (mg/g).

Vertical bars indicate ± SE. Letters indicate significant differences compared with control group.

Rajakumar (2013) investigated the effect of salinity (NaCl) on seed germination and some biochemical cursors of rice (*Oryza sativa* L.) under *in vitro* conditions. Higher NaCl concentrations resulted in increased proline accumulation and reduced total protein content in the study and it was found that NaCl had a negative effect on seed germination and total biomass. In our study increasing salt concentration resulted in rising proline content and reducing total protein content when compared with the control group. Among all treatments, the lowest decrease in total protein content was observed in $CaCl_2$ treatment, while the highest was observed in NaCl treatments. Demir and Kocaçalışkan (2001) found that soluble protein content decreased in salt-sensitive bean (*Phaseolus vulgaris* L.), and Karaman and Kaya (2015) reported a similar decrease in lentil under NaCl treatment, which is consistent with our findings.

4. DISCUSSION and CONCLUSION

In the present study, the mineral element contents, total protein, and proline levels were investigated for the first time in the Siverek rice population under different types/concentrations of salt. It is concluded that the reason for changes in cations may be relevant with their concentration and competition with each other. A decrease in Ca, Mg, and K levels during NaCl treatment or a decrease in Na, Mg, and K levels during $CaCl_2$ treatment may suggest evidence for it.

The changes in mineral elements involved in biochemical and enzymatic activities are remarkable. Mn plays a crucial role as a micronutrient in supporting the growth and development of plants. The subcellular Mn amount to maintain Mn-dependent metabolic processes such as ROS scavenging, and photosynthesis is mediated by a multitude of transport proteins from diverse gene families (Alejandro *et al*., 2020). Mn level increased in all treatments, but total protein content decreased in the current study. This change in manganese may be linked to other metabolic events related to the stress response other than protein content. Cu acts as a cofactor in proteins engaged in electron transfer reactions and serves as a vital micronutrient for plant growth (Burkhead *et al*., 2009). It can be concluded that similar changes in both Cu and Mn levels may considerably affect the generation of ROS due to their role in the electron transport system.

Salt stress studies focusing on NaCl have usually led to insufficient information on other chloride compounds, sulphates, nitrates, etc. and to confusion of information. Therefore, understanding the effects of different types of salts on plants and the complex responses of plants to these effects remains an area of interest. Further studies on salt stress, supported by molecular investigations, could identify genes that are activated in response to stress caused by different types of salts. Therefore, this study is essential for understanding the stress caused by

NaCl and other chloride compounds in rice and may shed light on the complex responses of plants to different types of salts.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

All authors carried out the experiments, conducted the data analysis, and wrote and approved the final version of the manuscript.

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Review Article

Current review of biodegradation and detoxification strategies for zearalenone contaminated food and feed

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Abstract: Mycotoxins are toxic metabolites produced by fungi that may cause serious health problems in humans and animals. Zearalenone is an estrogenic mycotoxin produced by *Fusarium* species that leads to huge economic losses in the food industry and livestock husbandry. Contamination of food and feed with zearalenone has reproductive problems, carcinogenicity, immunotoxicity, and other cytotoxic effects. At present, microorganisms and enzymes derived from microbial strains have been widely used for the degradation of zearalenone in food and feed. Researchers have developed biodegradation of zearalenone by the use of microbial and their enzyme derivatives, which offers harmless products and is environmentally friendly. Development of recombinant enzymes improves enzymatic detoxification of zearalenone to a non-toxic product without damaging the nutritional content. This review summarizes biodegradation and detoxification strategies of zearalenone using microorganisms and enzyme derivatives to nontoxic products.

1. INTRODUCTION

Mycotoxins are naturally occurring toxic secondary metabolites of some microscopic filamentous fungi (Liu *et al.*, 2022). Mycotoxins produced mainly by some fungal species belonging to *Alternaria*, *Aspergillus*, *Fusarium,* and *Penicillium* genera pose health threats to humans and animals (Greeff-Laubscher *et al.*, 2020). Mycotoxins contamination of foods and feeds is a current global issue and causes huge economic losses to animal husbandry (Navale & Vamkudoth, 2022). More than 400 different types of mycotoxins have been identified so far, with different levels of toxicity (Arroyo-Manzanares *et al.*, 2021). Among all mycotoxins, Aflatoxins B1, Zearalenone, Ochratoxin A, Patulin, and Trichothecenes have received particular attention due to their severe health outcomes in both humans and animals, which can range from acute to severe and chronic intoxications in both humans and animals (Ahn *et al.*, 2022; Nahle *et al.*, 2022).

Bouajila et al. (2022) reported that zearalenone contaminate feeds like corn, wheat, barley, sorghum, rice, and other grains have a variety of toxic effects on humans and animals (Jia *et al.*, 2022). Zearalenone (ZEN) is a potent non-steroidal oestrogen mycotoxin biosynthesized

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via the polyketide pathway and could bind to estrogen receptors, subsequently activating estrogen response elements in animals (Singh & Kumari, 2022; Yli-Mattila *et al.,* 2022).

Zearalenone (ZEN) consumption causes hypoestrogenism in animals and interferes with the expression of estrogen and organ function (Gajęcka *et al.,* 2021). It could reduce the nutritional value of feed, damage the growth and health of livestock and poultry, and cause huge economic losses to livestock production. However, some animals, like chickens, show strong resistance to the toxicity of ZEN. ZEN can also cause abortion, infertility, stillbirth, and other reproductive effects on animals (Yadav *et al.,* 2021; Jia *et al.,* 2022).

In humans, ZEN has a chronic toxicity effect and stimulates the growth of mammary gland cells that might be involved in breast cancer (Ropejko *et al.,* 2021). There is a report that shows ZEN has immunotoxin, hepatotoxic, hematotoxicity, and reproductive toxic effects like reducing fertility, vaginal prolapse, and causing vulvar swelling (Jia *et al.,* 2022). The degradation of zearalenone toxicity is commonly done by the use of physical, chemical, and biological approaches. Zearalenone is heat-stable and shows great resistance to conventional degradation methods (Kabak *et al.,* 2006; Wu *et al.,* 2021). However, physical and chemical degradation destroys nutritional structure, decreases palatability of the feed, and causes pollution to the environment (Guan *et al.,* 2021). Biological degradation has great specificity and degrades zearalenone completely without producing harmless products (Xu *et al.,* 2022).

Recently, numerous studies have focused on degradation through biological approaches by using microorganisms including bacteria, yeast, and fungi, and microorganisms' enzymes to remove zearalenone from food sources (Luo *et al.,* 2020; Nahle *et al.,* 2022). The development of genetic engineering technology in the advancement of recombinant proteins improves the enzymatic degradation of zearalenone (Guan *et al.,* 2021). This review aims to discuss the biological degradation of ZEN through microorganisms and enzymes developed in recent years.

2. Degradation of Zearalenone by Microorganisms

Microbial degradation occurs when microorganisms (bacterial and yeast) secrete their metabolites or enzymes during their growth and development process. Microorganisms can directly adsorb targeted toxins or reduce toxins of interest to impede the production of mycotoxins (Feng *et al.,* 2020; Xu *et al.,* 2022). Many studies have reported on the biodegradation of ZEN using microorganisms. They show high specificity and eco-friendliness in decreasing the possibility of ZEN toxicity from food and feed (Song *et al.,* 2021). A variety of non-pathogenic microbes like probiotics, *Bacillus*, *Saccharomyces*, and *Lactobacillus* species have a high capability to detoxify feeds contaminated with zearalenone because they follow standards like safe to be used and possess detoxifying ability without forming bad odour or taste in the feeds (Wang *et al.,* 2019; Zhu *et al.,* 2021).

Many studies reveal the detoxification of zearalenone using probiotics, including yeast, *Bacillus,* and lactic acid bacteria, as they are involved in the adsorption of ZEN and preventing its absorption by animals (Hathout & Aly, 2014). Various bacteria, yeasts, and fungi can convert ZEN to alpha and beta zearalenol (Cho *et al.,* 2010). Among *Bacillus* strains, *B. licheniformis, B. subtilis, B. natto,* and *B. cerues* were those found to have the highest detoxification effect on zearalenon in food and feed (Wang *et al.,* 2019). *Bacillus pumlius* ANSB01G is also reported to degrade ZEN in the feed of animals (Xu *et al.,* 2022). According to Xu *et al.* (2016) *B. amyloliquefaciens* ZDS-1 has ZEN degrading ability in screened colonies.

Probiotics are a great choice for biodegradation of ZEN in the food industry because it shows health benefits for humans and animals. Most lactic acid bacteria [LABs] are considered as safe probiotics in the food industry. It is reported that *Lactobacillus* strains have a potential role in degrading ZEN from fermented food products (Średnicka *et al.,* 2021). *Lact. paracasei*, and *Lc.*

lacti have the ability to remove ZEN in aqueous food solutions (Kabak *et al.*, 2006). There is a report that shows zearalenone can be degraded from PBS buffer solution by *Lact. acidophilus* CIP 76.13T by a bioremediation range of 57% (Ragoubi *et al.*, 2021).

There is a report that shows *B. licheniformis* CK1 has good probiotic properties and can degrade ZEN more than 90% after 36 hours of incubation in the contaminated corn meal medium by ZEN (Hsu *et al.*, 2018). Other strains of bacteria called *Saccharomyces cerevisiae* also have high ZEN degradation abilities as described in Table 1. There is a report that shows *S. cerevisiae* isolated from grapes can degrade ZEN (Rogowska *et al.*, 2019).

Saccharomyces cerevisiae isolated from silage has biodegradation properties and can degrade up to 90% of ZEN in two days (Keller *et al.*, 2015). According to Harkai *et al.* (2016) the bacteria *Streptomyces rimosus* (K145, K189) can degrade ZEN in liquid media. Wang et al. (2018) also investigated whether a *Lysinibacillus* strain isolated from chicken large intestine digest is capable of degrading zearalenone. Degradation of zearalenone by microorganism was illustrated in Table 1.

Table 1. *Recent research that shows microorganisms used for the degradation of zearalenone (ZEN).*

Food source or media used	Strain	ZEN concentration	Degradation range	References
Liquid LB medium	*Streptomyces rimosus* [K145, K189]	1 µg mL-1	100%	(Harkai *et al.*, 2016)
Feed	*Bacillus licheniformis* CK1	1.20 ± 0.11, 0.47 ± 0.22 mg/kg	Can degrade ZEN	(Fu *et al.*, 2016)
Liquid chromatography-tandem mass spectrometry and thin layer chromatography	*Candida parapsilosis*	20 µg/mL	Decreased by 97%	(Pan *et al.*, 2022)
Potassium phosphate buffer	*Lact. plantarum* 3QB361	2 µg/mL	82%	(Møller *et al.*, 2021)
Aqueous solution	*Lact. plantarum* BCC 47723	0.2 µg/mL	0.5%–23%	(Adunphatcharaphon *et al.*, 2021)
Culture medium/liquid food /solid-state fermentation	*Bacillus subtilis* *Bacillus natto*	20ug/mL, 1 mg/kg, 20 µg/mL	Culture mdium [100% and 87%], liquid food [65% and 73%], SSF [75% and 70%]	(Ju *et al.*, 2019)
Nutrient broth	*Bacillus subtilis, Candida utilis, Aspergillus oryzae*	1 µg/mL	[92.27- 95.15]%	(Liu *et al.*, 2019)
Malting wheat grains with bacterial suspension	*P. acidilactici*	19.5–873.7 µg/L	38.0%	(Juodeikiene *et al.*, 2018)
LB medium and simulated gastric fluid [GSF]	*Bacillus cereus* BC7	10 mg/L	100% and 89.31%	(Wang *et al.*, 2018)
Corn meal medium	*B. licheniformis* CK1	5 µg/mL	73%	(Hsu *et al.*, 2018)
Culture medium	*Bacillus pumilus* ES 21	17.9 mg/mL	95.7%	(Wang *et al.*, 2017)
MRS broth	*Lactobacillus rhamnosus*	200 µg/mL	Showed the highest adsorption [68.2%]	(Vega *et al.*, 2017)
MRS broth	*Lactobacillus plantarum* ZJ316	5 mg/L	highest ZEA degradation ability	(Chen *et al.*, 2018)
The LB medium	*Acinetobacter calcoaceticus*	5 µg/mL	85.77%	(Deng *et al.*, 2021)
HPLC-TOF-MS and NMR	*B. subtilis* Y816	40 mg/L	Transform of ZEN within 7 hour	(Bin *et al.*, 2021)
Cell suspensions on MRS agar	*Lb.fermentum* 2I3, *Lb.reuteri* L26, *Lb.plantarum* L81, *Lb.reuteri*, *Lb.plantarum* CCM 1904,	0.01 ppm	[57.9—100]%	(Harčárová *et al.*, 2022)
Cell suspensions on MRS agar	*Bacillus subtilis* CCM 2794	0.01 ppm	11.7 %	(Harčárová *et al.*, 2022)

3. Degradation of Zearalenone by Enzymes

Recent advancements in genetic engineering technology have attracted researchers' attention towards recombinant enzymes to degrade mycotoxins in food and feed with high efficiency. The attainment and cloning of recombinant enzyme genes lead to the safe expression of genes in microbes, which has become a novel progress in molecular modification for ZEN degradation (Azam *et al.*, 2019; Xu *et al.*, 2022).

Enzymatic degradation has wide advantages over microbial degradation because it can perform biodegradation with high efficiency, lower cost, reproducibility, and homogenous performance (Loi *et al.*, 2017; Liu *et al.*, 2022). A bacterial strain of *E. coli*, *S. cerevisiae*, and *Pichia pastoris* has been reported to remove ZEN from the culture medium (Wang *et al.*, 2020). Gao et al. (2022) identify and describe the activity of the ZEN degrading enzyme from *Exophiala spinifera*, ZHD_LD. Recently, microbial strains which can degrade ZEN have been isolated, and subsequently genes like ZHD101, ZLHY-6, and ZEN-jjm, as well as ZHD518 have been cloned (Cheng *et al.*, 2010). ZHD101 is one of the recombinant enzymes derived from *Clonostachys rosea* that degrades ZEN (Yang *et al.*, 2017). Wang et al. (2018) reported that the lactonohydrolase Zhd518 enzyme in *E. coli* has high biodegrading ability against ZEN in food and feed industries. A study that shows RmZHD, a ZEN hydrolyzing enzyme from *Rhinocladiella mackenziei*, has the ability to degrade ZEN (Zhou *et al.*, 2020).

Recombinant Prx (peroxiredoxin), a cloned gene from *Acinetobacter* sp. SM04 expressed in *E. coli*, has the ability to degrade ZEN in the presence of hydrogen peroxide (Yu *et al.*, 2012). It has been reported that laccase enzymes that are found in bacterial and yeast cells have the ability to degrade mycotoxins (Bi *et al.*, 2018). Song *et al.* (2021) show the laccase gene obtained from the fungus *P. pulmonarius* has an enzymatic property to degrade zearalenone when it is expressed in the *Pichia pastoris* X33 yeast strain by producing recombinant protein as shown in Table 2.

Studies have shown that laccase enzymes are considered as an effective zearalenone toxicity antidote. Furthermore, *Pleurotus eryngii* laccase enzyme can degrade aflatoxin B_1, ochratoxin A, zearalenon, and other mycotoxins (Wu *et al.*, 2021). A gene ZENC, zearalenone lactonase gene from *Neurospora crassa*, is expressed in *P. pastoris*. It had a maximal enzyme activity when fermented using high density fermatation at pH 8 and a temperature of 45 °C. Furthermore, ZENC was also found to be effective in ZEN containing feed materials with a high degradation rate (Guo *et al.*, 2020).

Garcia et al. (2018) also reported that the peroxidase enzyme has the ability to degrade zearalenone concentrations. According to the study, a fusion of multifunctional recombinant enzymes ZHDCP with genes of ZEN hydrolases and carboxypeptidases has the ability to detoxify zearalenone in 2 hours at pH and temperature of 35 °C (Azam *et al.*, 2019). The degradation of zearalenone by enzyme is discussed in (Table 2).

Table 2. *Enzymatic degradation of zearalenone (ZEN).*

Enzymes name	Source	Expression System	Degrading properties	References
Peroxiredoxin	*Acinetobacter* sp. SM04	*S. cerevisiae*	Optimal activity at pH 9.0, 80 ^{0}C and H_2O_2 concentration of 20 mmol/L Thermal stable, alkali resistance	(Tang *et al.*, 2013)
Lactone hydrolase ZHD	*Gliocladium roseum*	*P. pastoris*	Enzyme activity in flask fermentation was 22.5 U/mL and specific activity of 4976.5 U/mg· Maximum enzyme activity of the supernatant was 150.1 U/mL in 5-L fermenter	(Xiang *et al.*, 2016)
Cb ZHD	*C. rosea*	*Cladophialophora bantiana*	Optimal enzyme activity at temperature 35 °C and pH 8	(Hui *et al.*, 2017)
Lactonohydrolase	*Clonostachys rosea*	*Lactobacillus reuteri Pg4*	Not affect cell growth, acid and bile salt tolerance	(Yang *et al.*, 2017)
Lactonohydrolase Zhd518	*Clonostachys rosea*	*E. coli*	Activity of 207.0 U/mg with optimal temperature 40 ^{0}C and pH 8.	(Wang *et al.*, 2018)
Lactonase	*Neurospora crassa*	*P. pastoris*	Optimal activity at pH 8.0 and 45^{0}C, stable at pH 6.0–8.0 for 1 h at 37 ^{0}C, Maximal enzyme activity at 290.6 U/mL in 30-L fermenter	(Guo *et al.*, 2020)
Lactonehydrolase ZENC	*Neurospora crassa*	*P. pastoris*	99.75% of ZEN [20 µg/mL] was degraded at pH 8.0, 45 °C for 15 min	(Guo *et al.*, 2020)
Fusion ZHDCP enzyme	*C. rosea B.amyloliquefaciens* ASAG	*E. coli*	100% degradation rate at pH 7 and 30 ^{0}C	(Azam *et al.*, 2019)
ZLHY-6	*Pichia pastoris*	*P. pastoris* GSZ	low nutrient loss safe removal of ZEN	(Chang *et al.*, 2020)
lac2	*Pleurotus pulmonarius*	*P. pastoris* X33	Lac2-ABTS and Lac2-AS degrade ZEN at optimum pH 3.5 and temperature 55 ^{0}C of recombinant *Lac2*	(Song *et al.*, 2021)
Lactonohydrolase	*Trichoderma aggressivum*	*E. coli* BL21	With superior pH stability, the surface exhibit ZHD-P retained 80% activity	(Chen *et al.*, 2021)
ZPF1	*C. rosea* fused with *Phanerochaete chysosporium*	*Kluyveromyces lactis* GG799	ZEN degraded up to 46.21% ±3.17%	(Xia *et al.*, 2021)
DyP	*Streptomyces thermocarboxydus* 41291	*E. coli* BL21	ZEN was degraded slightly by StDyP	(Qin *et al.*, 2021)
Ase	*Acinetobacter* Sp	*E. coli* BL21	Degraded 88.4% of ZEN [20 µg/mL]	(Tang *et al.*, 2022)

3. CONCLUSION

The severe impact of zearalenone on animals and humans' health, present in contaminated food and feed, has received global attention. Many approaches have been established for the removal of ZEN. Biodegradation is considered the safest approach because it degrades toxins without residual toxic substances. Recent research shows the development of recombinant microorganisms and recombinant enzymes to detoxify ZEN in foods and feeds. However, the health impacts of recombinant enzymes are not clearly described. Currently, biodegradation of zearalenone is laboratory-based. The commercial scale of biodegradation needs further studies. Further interdisciplinary studies concerning gene cloning, genetic modification of microorganisms, and the development of recombinant enzymes are promising approaches for safe zearalenone degradation.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

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Review Article

Physiological and Biochemical Responses to Heavy Metals Stress in Plants

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Abstract: Heavy metal (HM) toxicity is a severe abiotic stress that can cause significant harm to plant development and breeding, posing a challenge to sustainable agriculture. Various factors, including cellular toxicity, oxidative stress, osmotic stress, imbalance in the membrane, and metabolic homeostasis cause negative impacts on plant molecular, physiology and biochemistry. Some heavy metals (HMs) are essential micronutrients that play important roles in various plant processes, while excessive amounts can be harmful and have negative impacts on plant growth, metabolism, physiology, and senescence. Phytotoxicity with HMs and the deposition of reactive oxygen species (ROS) and methylglyoxal (MG), can lead to lipid peroxidation, protein oxidation, enzyme inactivation, DNA damage, and harm to other vital components of plant cells. Generally, HM toxicity as environmental stress led to response of plant with different mechanisms, first, the stimulus to external stress, secondly all signals transduction to plant cell and finally it beginning to find appropriate actions to mitigate the adverse stress in terms of physiological, biochemical, and molecular in the cell to survive plant. The purpose of this review is to better understand how plants respond physiologically and biochemically to abiotic HM stress.

1. INTRODUCTION

Heavy metal (HM) is referred to elements with a high atomic weight and a density at least five times greater than that of water (Bindu *et al*., 2021). HMs are non-biodegradable, meaning they persist in the environment for over 20 years (Hadia-e-Fatima, 2018). Through the environmental stresses, among the most significant stresses having adverse effects on growth and development is HM stress. Additionally, the biochemical and physiological responses of plants are affected by HM toxicity (Hafeez *et al*., 2023) which can manifest through oxidative damage, ionic imbalance, osmotic stress and metabolic imbalances in cells (Hoque *et al*., 2021). Certain HMs such as zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), cobalt (Co), molybdenum (Mo), and nickel (Ni) are vital for the growth of a plant but could be toxic if present in excess. On the other hand, cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As) are severely harmful to plants' growth and not essential to them (Chibuike & Obiora, 2014). Soil contamination by HMs can happen due to natural and anthropogenic activities. The primary

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source of pollution is the parent material, which releases these metals into the soil, both natural and human activities contribute to HM contamination in soil (Choudhary *et al*., 2022). However, human activities such as mining, industrial processes, fertilizers, pesticides, fossil fuel, sewage irrigation, can release these elements into the environment, leading to pollution of water and soil (Hafeez *et al*., 2023). Plants are able to absorb HMs from the soil through their roots. This can lead to the depot of HMs in plant tissues, making the plant toxic to animals and humans that consume them (Iqbal *et al*., 2020).

2. THE EFFECT OF HEAVY METALS ON PLANTS

Various physiological processes in plants, including photosynthesis, seed germination, and the remobilization and accumulation of seed reserves during germination and growth, can be adversely affected by HMs. As a result of these negative effects, the production of crops may be reduced (Shahid *et al*., 2014). In different regions of the world, HMs have been discovered especially in agriculture fields including Pb, Cd, Ni, Co, Fe, Zn, chromium (Cr), As, silver (Ag), and platinum (Pt) group (Rahman *et al*., 2023). The primary defense system of plants against high concentrations of these contaminants is the production of ROS. According to Figure 1 showed that the production of ROS by HMs which are the major sites in the chloroplast, peroxisomes, mitochondria, moreover in the cell wall, plasma membrane, and endoplasmic reticulum, they can cause oxidative stress in plants. HM stress leads to stomatal closure, triggers the photorespiratory pathway, increases ROS productions such as hydroxyl radicals (OH^-), superoxide anion radicals (O_2^-), and hydrogen peroxide (H_2O_2), these substances adversely affect the antioxidant system's functionality and hinder the normal operation of electron transport chains in plants. The production of ROS may trigger lipid peroxidation, which further deteriorates the cell membrane integrity and function. (Das & Roychoudhury, 2014; Emamverdian *et al*., 2015; Kärkönen & Kuchitsu, 2015; Hoque *et al*., 2021). In addition, ROS, which are detrimental to plant growth and survival, are produced in excess by exposing plants to high concentrations of HMs and can affect the synthesis of secondary metabolites in plants. Secondary metabolites are compounds produced by plants that are not essential for their growth and survival, but play significant roles in the plant's defense mechanisms, attract pollinators, or deter herbivores for instance flavonoids and carotenes can tolerate when they are confronted with metal stress (Khare *et al*., 2020; Karakas, 2020; Pehlivan Karakas & Bozat, 2020).

Generally, plants produce a variety of secondary metabolites from primary metabolites, such as carbohydrates, lipids, and amino acids, which are divided into different groups according to their chemical makeup and functional groups. Under normal conditions, there is a balance between the production and detoxification of ROS in plants whenever this balance is interrupted, the defense mechanisms of plants against stress situations effect heavily, their chemical composition changes and produces some secondary metabolites, including, phytoalexins, alkaloids, hydrocarbons, terpenes, flavonoids, phenolic chemicals. (Shahid *et al*., 2014). These chemical compounds play a crucial role in protecting plants from biotic and abiotic stress (Anjitha *et al*., 2021). It has been noted that the putrescine (Put) level declined in sunflower leaf disks treated with Cd^{2+} or Cu^{2+} when treated with them, sunflower leaf disc demonstrated a discernible reduction in spermidine (Spd) content but no change in spermine (Spm) levels. It has been demonstrated that Cd^{2+} and Cu^{2+} increase the yields of secondary metabolites such as shikonin (Mizukami *et al*., 1977; Groppa *et al*., 2003). Vanillic acid accumulated more in *Zea mays* after exposure to metals like Cu, Pb, and Cd (Kısa *et al*., 2016). The effects of HM toxicity on secondary metabolites and the metal concentration can really vary based on the plant species, the type of metal, and other circumstances. Some of these secondary metabolites, including phenolics and flavonoids, have direct antioxidant properties that can scavenge ROS (Maleki *et al*., 2017). The impact of arsenic on the synthesis of artemisinin (a sesquiterpenoid) in *Artemisia annua* under stress was studied, upregulation of

genes involved in artemisinin production increased artemisinin accumulation in soil culture and hydroponic systems (Rai *et al*., 2011). Studying on *Zea mays* leaves exposed to Zn stress showed an increase in anthocyanins, indicating that cyanidin might interact with Zn^{2+} by taking on two electrons and producing a cyanidin-Zn complex (Janeeshma *et al*., 2021). Moreover, phytoalexins are secondary metabolites with antimicrobial properties that plants synthesize in response to fungal attacks. Additionally, they can also be induced by other elicitors. Studies have shown that metal adaptation can affect biotic stress responses in plants and activate defense mechanisms such as the secretion of phytoalexins and phenolic compounds. Studying about *Silene paradoxa* from different soil types including copper mine soil, serpentine soil, and non-contaminated soil, all plants under the different treatments faced a pathogen. The population from the copper mine soil showed an increase in the production of phytoalexins due to the activation of mitogen-activated protein kinase (MAPK) cascades. This activated protective systems, resulting in the production of phytoalexins and other phenolic molecules in the plant (Martellini *et al*., 2014). In the other hand, in plant biotechnology, nanoparticles can be utilized as efficient abiotic elicitors to stimulate the manufacture of secondary metabolites. They have the ability to boost the emergence of genes that are in the production of secondary metabolites (Hatami *et al*., 2019), because of elicitation with TiO_2 nanoparticles, *Salvia officinalis* was shown to contain higher levels of monoterpenes, flavonoids, essential oils, and total phenols (Ghorbanpour, 2015) and similar treating *Artemisia* suspension culture with nano-Co resulted in a significant increase in artemisinin production, boosting it by 2.25 times compared to the control (Ghasemi *et al*., 2015). When exposed to HMs, the photosynthetic system of plants becomes impaired due to interactions with the light-harvesting complex II protein, resulting in changes to its shape. Maleva et al., 2012 and a similar study by (Li *et al*., 2012) have demonstrated that the level of chlorophyll has decreased by Cu, Mn, Cd, Ni, and Zn in *Elodea densa*.

The photochemical activity of photosystem II (PS II) also decreased as a result, which is responsible for converting light energy into chemical energy and in addition to chlorophyll, carotenoid level decreased as well as the quantum yield of PS II in *Thalassia hemprichii*. In addition to impacting the light-dependent reactions, HMs reduce the assimilation of carbon dioxide (CO_2) by either inhibiting the activity of RUBP carboxylase or by reacting with the thiol group of RUBISCO, an enzyme involved in CO_2 fixation HMs also induce senescence in plants by triggering the production of ethylene, which in turn activates the jasmonic acid signaling pathway (Khare *et al*., 2020). Plants have various ways of detoxifying metals, including triggering the activity of multiple antioxidants, sequestering and compartmentalizing metals internally, binding them to cell walls, producing osmoprotectants, transporting metal ions, and chelating them with low molecular weight organic acids, within the cell. Additionally, if the concentration of HMs in the soil is with high concentration, they may compete with the transport of essential nutrients, and metal-chelating molecules or rhizosphere microorganisms may be necessary to immobilize the metal ions in the soil to avoid competition for nutrient transport (Yaashikaa *et al*., 2022). In some cases, HM exposure can induce the production of secondary metabolites that act as defense mechanisms, such as phytochelatins (PCs) and metallothioneins (MTs), which are peptides that chelate and detoxify HMs in cytosol and putting them in the vacuole or another subcellular structure (Clemens, 2001). However, in other cases, HM exposure can lead to a reduction in the synthesis of secondary metabolites, resulting in decreased plant growth, reduced seed production, and decreased resistance to diseases and pests. Abiotic stress signals, such as toxic metals, may trigger genes involved in the biosynthesis of secondary metabolites too (Anjitha *et al*., 2021; ul Hassan *et al*., 2019). Two groups of plants can collect toxic HMs known as hyperaccumulators and accumulators (Niu *et al*., 2021; Pasricha *et al*., 2021; Sharma and Kumar, 2021) and reported from Brassicaceae and Phyllanthaceae families about 721 species of them recognized as hyperaccumulators plants

(Reeves *et al*., 2018). Hyperaccumulator plants play an essential role in the remediation of HM-contaminated soil by effectively extracting and mitigating the detrimental impacts of these metals. According to (Shahi, 2002) showed that the affordability of lead phytoextraction is feasible when plants have the capability to accumulate more than 1% of lead (Pb) in their shoots. *Sesbania drummondii*, a leguminous shrub that grows in the wild, has been taken into consideration in this study. Shoot concentrations of Sesbania plants grown in modified Hoagland's solution containing 1 g Pb $(NO_3)_2$/L were > 4% Pb. The absorption was increased by 21% by EDTA (100 M) in the media containing 1 g Pb $(NO_3)_2$/L. Detailed examination using transmission electron microscopy and X-ray microanalysis revealed the localization of Pb granules in the plasma membrane, cell wall, and vacuoles of root sections.

This study demonstrates that S. drummondii prepares the criteria for a hyperaccumulator. However, several methods have been developed for eliminating HMs from polluted soil, utilizing physical, chemical, and biological techniques. The technique of soil replacement as a physical method involves the utilization of uncontaminated soil to substitute the polluted soil, which helps to lower the concentration of pollutants in the soil which is adequate for small-scale soil contamination and also thermal desorption involves heating contaminated soil using methods such as steam, microwave, or infrared radiation. This process converts the pollutants into a volatile form, which can then be collected using a vacuum negative pressure or carrier gas. Ultimately, this method is used to remove HMs from the soil (Haritash, 2023). Chemical methods used for soil remediation, such as soil washing, flushing, solidification/stabilization, vitrification, and electro kinetic remediation. Soil washing, which involves using fresh water, solvents, or other liquids, even gases to wash contaminated soil (Sidhu, 2016). The other method belongs to biological approach includes both microbial remediation and phytoremediation, as well as a combination of both methods (Haritash, 2023). In fact, phytoremediation is an eco-friendly and economical method that involves using plants and their associated microorganisms in the soil to either bind, break down, or store metal contaminants in both soil and water (Pasricha *et al*., 2021), while microbes are unable to directly break down or eliminate transforming HMs into harmless forms, they can still impact the mobility, bioavailability, and transformation of these metals in the soil by modifying the physical and chemical properties of the pollutants. Several processes lead to the interaction of HMs with microorganisms such as bioaccumulation, bioassimilation, biosorption, biotransformation and bioleaching. During bioaccumulation, solutes are transported through the cell membrane into the cell cytoplasm from outside of the microbial cell, where metals are sequestered. Biotransformation can alter the HM's chemical form through various methods, including methylation/reduction and dealkylation/oxidation, which can change the mobility, toxicity, and bioavailability of the HM (Peng *et al*., 2018). One of the most significant processes in microbial remediation is biosorption, in which HMs bind to anionic functional groups on the surface of cells and in extracellular polymers. HMs are immobilized because of this binding process, which involves covalent bonding and electrostatic interactions between metal ions and the cell surface (Haritash, 2023).

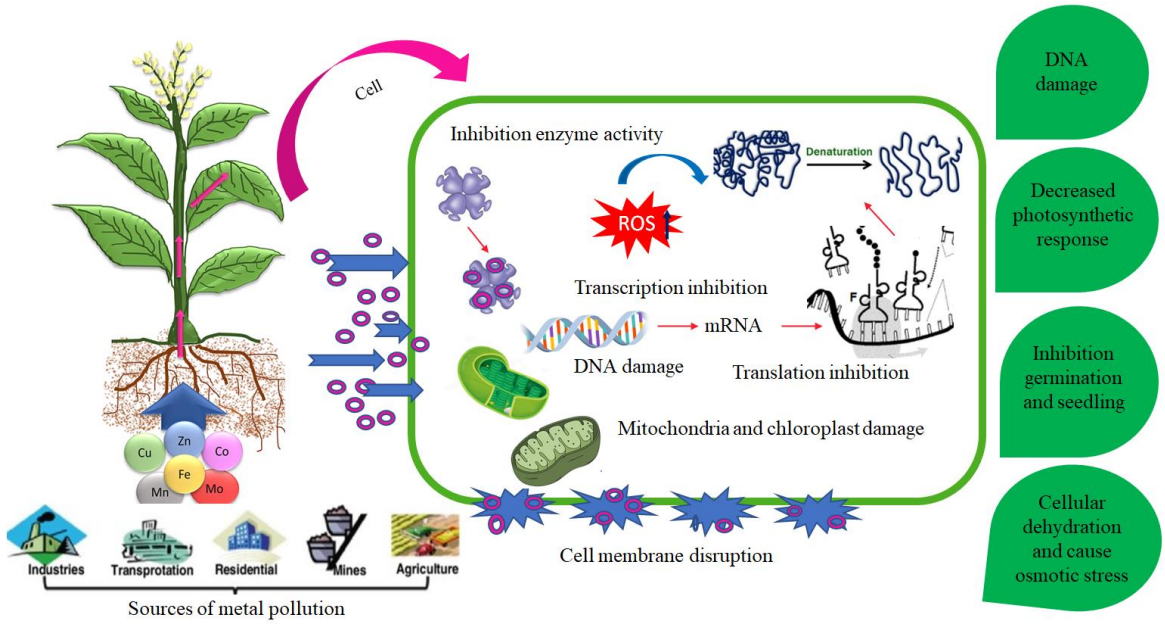

Figure 1. Toxic consequence of entering HM into a cell that led to inhibition of cell division and finally make death of cell.

3. RELATION BETWEEN HM TOXICITY AND OSMOREGULATION

HMs also impact plant water relations and the availability of water in soils, which can lead to decreased water uptake and the onset of various stress conditions. When soils are contaminated with HM, the osmotic potential in the soil decreases relative to the cell sap within the root system. As a result, metal ions can accumulate to levels that severely limit water uptake by plants and cause osmotic disturbances (Rucińska-Sobkowiak, 2016). This can result in stunted root growth, decreased root mass, and reduced root cell elongation, impaired secondary growth and reduced capacity of water uptake by the plant (Mashabela *et al*., 2023). A research study investigated the effects of combined osmotic stress and exposure to cadmium (Cd^{2+}) on the roots of *Brachypodium* seedlings. The study found that these a few points were responsible for inhibiting seedling growth and causing significant changes in plant physiology and phenotype, such as reduced relative water content, plant height, and primary root length (Chen *et al*., 2018). In terms of osmoregulation, HMs can cause changes in the water balance of the plant, so that the concentration of solutes in the root cells will be increased. This can lead to dehydration and decreased water uptake by the roots. Moreover, the imbalance in osmotic pressure can lead to oxidative damage, which occurs due to alterations in the electron transport chain and reduced mitochondrial respiration. This results in the overproduction of detrimental free radicals and ROS which damage to various cellular structures, including those involved in transpiration, photosynthesis, and DNA/RNA synthesis, leading to reduced plant growth, development, fertility, or even death (Dumanović *et al*., 2021). Additionally, the overproduction of ROS can lead to oxidative stress characterized by the loss of cellular membrane construction and function due to lipid peroxidation (Mashabela *et al*., 2023).

3.1. Plant Response and Adaptation for Tolerance to HM Toxicity Related to Osmotic Condition

Some of the key plant responses and adaptations for tolerance to HM toxicity and osmotic stress include HMs can be sequestered within the plant cell to minimize their toxicity by binding to specific compounds, such as phytochelatins, or by being compartmentalized within the vacuole. Antioxidant defense is another response to HMs that can generate ROS which can cause oxidative damage to plant cells. To counter this, plants have evolved a series of antioxidant

defense mechanisms such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) enzymes activate that help to neutralize ROS and prevent oxidative damage and also, they can activate signaling pathways in the plant, such as the calcium signaling pathway, the ROS signaling pathway, and the abscisic acid (ABA) signaling pathway. These pathways help to coordinate the plant's response to stress. In osmoregulation in response to osmotic stress, plants can adjust the osmotic potential of their cells by regulating the accumulation of solutes such as compatible solutes and proline. This helps to prevent water loss from the cells and maintain turgor pressure. Changes in gene expression, HM toxicity, and osmotic stress can cause changes in gene expression in the plant, leading to the activation of stress-responsive genes that are involved in the defense against HM toxicity and osmoregulation (Mashabela *et al*., 2023). The recognition of these stimuli initiates a signaling pathway known as mitogen-activated protein kinases (MAPKs), which includes three kinases namely MAPKs, MAPK, and NAPKINs. They are a family of serine/threonine protein kinases that have been shown to play a role in the response to HM toxicity and osmotic stress. (Mashabela *et al*., 2023). This cascade involves the phosphorylation and activation of several MAPKs, leading to the activation of downstream target proteins that are involved in the plant's response to stress (Cargnello *et al*., 2011). Additionally, MAPKs have also been shown in the regulation of root plasticity, which is an important mechanism by which plants overcome HM toxicity. For instance, Ye *et al*. (2013) found that Cd exposure activated MPK6 in *Arabidopsis* plants, which in turn activated caspase-3-like enzymes and caused programmed cell death (PCD) as a defense mechanism against stress (Jalmi *et al*., 2018). Following exposure to Cu and Cd, *Arabidopsis* was used to demonstrate the role of MPK3 in the response to metals (Dos Reis *et al*., 2018). Exogenous protectants refer to substances that are applied externally to plants to help them overcome the damage caused by HM toxicity. These protectants can be used to mitigate the effects of HM toxicity by either chelating (binding) the HMs to prevent their toxicity or by providing a source of energy and nutrients to the plant. Activation of Ca^{2+} signaling by plants leads to changes in gene expression, accumulating the stress hormone abscisic acid, altering development, and increasing stress tolerance in response to osmotic stress (Chen *et al*., 2020). Osmotic stress stimulation induces a rapid increase in Ca^{2+} cyst in the roots of *Arabidopsis* seedlings, indicating that cytosolic calcium is implicated in the osmotic stress response (Huang *et al*., 2017). There are some commonly used exogenous protectants in mitigating HM-induced damages: Chelating agents such as EDTA (Ethylene Diamine Tetra Acetic acid) and citric acid, can be used to chelate HMs, making them less toxic to the plant. By binding HMs, chelating agents prevent their uptake by the plant and reduce their toxicity. Antioxidants, such as ascorbic acid, can help to neutralize ROS generated by HMs and prevent oxidative damage to plant cells. Salicylic acid is a naturally occurring plant hormone that can be applied to induce the expression of stress-responsive genes and enhance the antioxidant defense mechanisms of the plant. Calendula (*Calendula officinalis*) cultivated in calcareous soils enriched with Cd (50-100 mg/kg) did not exhibit any physiological indications of Cd toxicity in plants treated with exogenous citric acid at levels of 0.05 and 0.1 mM (Vega *et al*., 2022). Citric acid was demonstrated to be a more effective phytoremediation agent in the same study when compared to other chelators as tartaric acid and ethylenediaminetetraacetic acid (EDTA). Additionally, citric acid forestry settings have been used to evaluate -mediated tolerance to HM toxicity (Saffari & Saffari, 2020). For instance, the presence of 20 mM citric acid increased the mobility, bioavailability, and distribution of Cd in the basket willow *(Salix viminalis*), as well as the amount of chlorophyll, the rate of photosynthetic respiration, and other parameters of the plant (Vega *et al*., 2022). Compatible solutes can accumulate in plant cells to help regulate the osmotic potential of the cells and mitigate the effects of osmotic stress caused by HMs and nutrient sources, such as phosphorous and nitrogen, in a mechanism where molecules of a ligand are attached to ions of the core metal through a coordination bond in a

cyclic or ring structure, a group of low molecular weight chemical molecules provides tolerance to these pollutants (Barceló & Poschenrieder, 1999) such as proteins like phytochelatins (PCs) and metallothioneins (MTs) or amino acids like proline and histidine. The MTs proteins are involved in the uptake and transport of HMs into and within the plant. The addition of MTs considerably raised Cd tolerance and decreased Cd concentration in the leaves of tomatoes (*Solanum lycopersicum* L. cv. Hezuo 903) as demonstrated by decreased growth inhibition, photoinhibition, and electrolyte leakage. MTs decreases Cd uptake and mitigates toxicity in plants (Li *et al*., 2016). Arnao and Hernández-Ruiz, (2009) shown that melatonin can also be absorbed by the leaves in addition to the roots, as a result, they noticed that incubating *Hordeum vulgare* L. (barley) leaves with rising exogenous melatonin concentrations in the growth medium caused an accumulation of indole in the leaves that were dose-dependent.

4. HM TOXICITY AND PHYTOHORMONES

HM toxicity can significantly alter the hormonal balance of plants, leading to changes in growth, development, and stress responses. Figure 2 showed that hormones play crucial roles in regulating various physiological and biochemical processes in plants, and the effects of HM toxicity on hormones can be diverse and complex. The major phytohormones associated with HM stress are discussed below:

Auxin, a key hormone involved in plant growth and development, is known to be affected by HM toxicity. HMs can disrupt the transport and metabolism of auxin, leading to alterations in the patterns of cell division, elongation, and differentiation. Numerous observations showed that the auxin endogenous status in shoot and root tissues were variably affected, demonstrating both a synergetic and antagonistic relationship between HM stress and auxin level (Yuan & Huang, 2016). Auxin production and signaling are largely dependent on the several auxin-regulatory genes that are increased in response to the harmful effects of HMs, according to several molecular-based studies (Rahman *et al*., 2023). For instance, Under Cd stress, it was observed that *Arabidopsis* triple mutant (ddc) plants demonstrated improved IAA status than wild-type ones. The transcriptomic-based analysis showed that ddc mutants mostly decrease the methylation genes (MES7 and 17) and auxin biosynthesis genes (YADOKAR11; YDK1, GH3.3, and GH3.17). In comparison to Wild - type plants, ddc mutants were reported to have an instantaneous overexpression of IAOX (indole-3-acetaldoxime), which is connected to auxin biosynthesis genes (CYP71A13 and NIT2). As an alternative, the AUX/IAA gene family, which controls the regulation of auxin, was suppressed in ddc plants, improving phytohormonal signal transmission and increasing HM resistance (Pacenza *et al*., 2021).

Abscisic acid (ABA) is another hormone that is involved in regulating plant stress responses. Various plants subjected to HMs have shown elevated endogenous ABA concentrations, including *Helianthus annuus* L., *Lactuca sativa*, *Populus canescens*, *Tritium aestivum*, *Oryza sativa*, *Cucumis sativus*, and *Solanum* (Pacenza *et al*., 2021). ABA biosynthesis gene OsNCED4 was shown to be overexpressed in a transcriptomics-based study on Cd-stressed rice, highlighting the critical function of ABA in reducing Cd toxicity (Tandon *et al*., 2015). Another recent study looked at the role of ABA in protecting Cd-stressed *Sedum alfredi*, and the findings showed that ABA foliar treatment along with Cd improved ABA endogenous status by upregulating the expression of ABA biosynthesis enzymes (NCED, AAO, and ZEP) and downregulating ABA inhibitor (ABA 8-hydroxylase) expression. The outcomes of the study showed that ABA is essential for lowering Zn toxicity by altering genes involved in Zn absorption and detoxification(Tao *et al*., 2021). Critical genes involved in the laboratory's lead (Pb) absorption, transportation, and excretion were increased by ABA when applied topically (Shi *et al*., 2019). Cytokinin, gibberellins, jasmonic acid and ethylene are other hormones known to be affected by HM toxicity. Cytokinin, for example, plays important roles in regulating cell division and elongation, and exposure to HMs such as cadmium can decrease

their levels, leading to reduced cell division and elongation. that contribute to the developmental alterations brought on by osmotic stress. Due to an increase in JA content in wild type rice compared to a mutant with JA biosynthesis disrupted, it has been demonstrated that intense jasmonates (JA) can make rice more resistant to osmotic stress (Tang *et al*., 2020).

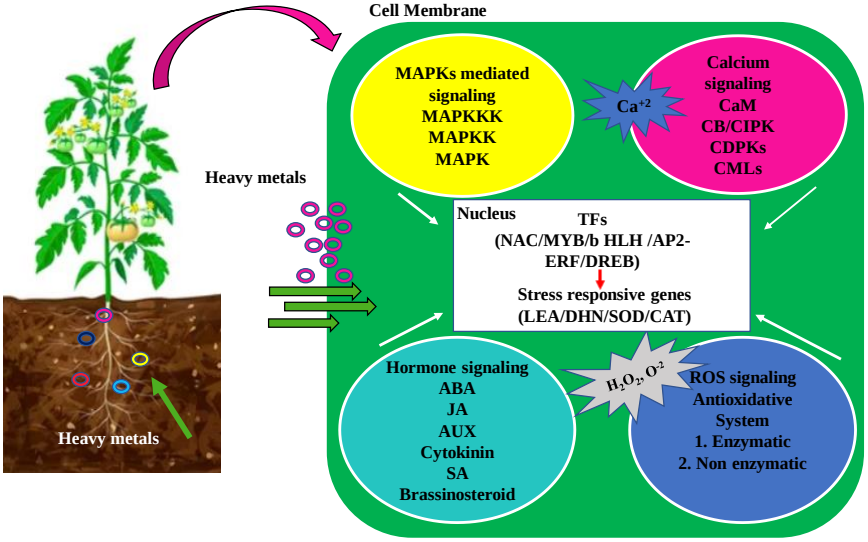

Figure 2. HM stress signaling cascade in plants that mediate signal transduction to improve genes of expression to stress response.

5. MACRO AND MICRONUTRIENT OF METAL-BASED FOR PLANTS

The function of important enzymes and the control of metabolic processes including redox homeostasis, metabolism, DNA synthesis, and photosynthesis depend on proper levels of metal-based micro and macro nutrients like Cu, Zn, Co, Ni, Fe, Cr, Mn, I, and Se. However, HMs in excess show toxicities that can be dangerous. For convenience, some recent study about reaction of plants against HM is given in Table 1.

5.1. Zinc Effects on Plants

Zinc is an essential micronutrient for plants, playing a crucial role in various plant processes, such as growth and development, photosynthesis, and defense mechanisms. However, when present in large quantities, zinc can harm plants and have negative impacts. At low concentrations, zinc is involved in the activation of several enzymes and transcription factors, which regulate plant growth and development. Zinc also plays a role in the regulation of the hormone auxin, which is involved in the control of cell elongation and division *(Broadley et al*., 2007). According to Tsonev and Cebola Lidon (2012) Zn concentrations in unpolluted soils are normally lower than 125 ppm, and in plants growing in these soils, this metal concentration varies between 0.02-0.04 mg/g dry weight. The bioavailability of Zn in soil solution increases at low pH, while organic ligands and hardness cations such as Ca^{2+} decrease Zn availability (Pedler *et al*., 2004). At higher concentrations, zinc can become toxic to plants and lead to various physiological and biochemical changes. For example, high levels of zinc can lead to oxidative stress, which can cause damage to cellular membranes and result in the accumulation of reactive oxygen species. Additionally, zinc toxicity can also disrupt the plant's ion balance, leading to changes in osmotic potential and water uptake. Zinc and cadmium's phytotoxicity are observed in plant species such *Phaseolus vulgaris*, *Brassica juncea* and tobacco (Cakmak & Marschner, 1993; Prasad & Hagemeyer, 1999), which exhibit reduced growth, metabolism, and oxidative damage (Tkalec *et al*., 2014).

Table 1. Tolerance of plant growth reaction to HMs/metalloids in soil.

Plant	Heavy metal	Plant response and related parameter	References
Sedum hybridum 'Immergrunchen'	Cd	Higher concentrations (200 mg/kg) significantly damaged the plant.	Guan *et al*., 2022
Althaea rosea *Abutilon theophrasti*	Cd	The root, shoot, and plant height of both plants were significantly impacted by cadmium stress. Tolerance Index (TI) was 1.	Wu *et al*., 2018
Helianthus annuus	Pb	Without significantly affecting the production of biomass, the plant demonstrated considerable metal accumulation capability at all concentrations (0-250 mg/kg).	Al-Jobori and Kadhim, 2019
Lavandula angustifolia	Ni	Plant grew well up to 40 mg/kg Ni of soil	Barouchas *et al*., 2019
Zinnia elegans	Cr	The plant grew up to a Cr stress of 50 mg/kg.	Panda *et al*., 2020
Hydrangea	Pb	Under any Pb treatments (0-1600 mg/ kg), no apparent evidence of heavy metal toxicity was seen in the plant.	Ma *et al*., 2022
Euphorbia pulcherrima	Hg, Zn, As, Pb,Cr,Cu	Zn, Pb, and Cu were significantly accumulated in the roots of the plant, while Hg was removed and transported in the leaves. Additionally, the plant was discovered to be tolerant to As and Cr.	Xiao *et al*., 2021
Mirabilis jalapa	Cd	The plant effectively eliminated Cd, As, and Pb from contaminated soil without facing with any negative consequences from phytotoxins.	Li *et al*., 2022
Pteris cretica, *Spinacia oleracea*	As	The significant effect of 100 mg/kg as treatment on the analyzed parameters such as chlorosis, growth process inhibition, oxidative stress. *S. oleracea* is an As-root excluder while *P. cretica* is an As-hyperaccumulator.	Zemanová *et al*., 2021
Cherry radish (*Raphanus sativus* var. *sativus* Per s. 'Viola')	As	The metabolism of free amino acids (AAs), phytohormones, and antioxidative metabolites changed as a result of the rising As content in tubers and rising soil pollution. The majority of the changes were seen in environments with significant As pollution (20 and 100 mg/kg).	Pavlíková *et al*., 2023
Pleioblastus pygmaeus	As	Due to the high production of reactive oxygen species (ROS) components and induction of cell membrane peroxidation, at 150 and 250 M, the plant growth was considerably affected.	Emamverdian *et al*., 2023
Eucalyptus nitens	As	Roots can accumulate to levels ranging between 69.8 and 133 µg/g for plants treated with 100 and 200 µg/mL As and leaves between 9.48 µg/g (200 As) and 15.9 µg/g (100 As) without apparent morphological damage and toxicity symptoms. The As effects on the uptake and translocation of Ca, Fe, K, and Zn revealed two contrasting interferences.	Ramalho *et al*., 2023
Arabidopsis thaliana	*Al*	Growth inhibition, ROS increase, lipid peroxidation	Kochian *et al*., 2015

Phaseolus vulgaris and *pea* plants have demonstrated that Cd and Zn change the catalytic efficiency of enzymes (Romero-Puertas *et al*., 2004; Somashekaraiah *et al*., 1992; van Assche & Clijsters, 1983) reported zinc levels in polluted soil have been measured to be between 150 and 300 mg/kg (de Vries &., 2007; Warne *et al*., 2008). The toxicity of zinc to plants limits the growth of roots and shoots (Malik *et al*., 2011). Zinc toxicity can also cause the yellowing of young leaves, which can spread to old leaves after long-term exposure to high concentrations

of zinc in the soil (Ebbs & Kochian, 1997). The toxicity of zinc to plants restricts the growth of roots and shoots and can also result in young leaves getting yellow, which spreads to older leaves with prolonged exposure to excessive concentrations of zinc in the soil (Ebbs & Kochian., 1997*; Malik et al*., 2011). However, hydrated Zn^{2+} and Fe^{2+} ions have equal radii, the chlorosis may be caused in part by an induced iron deficit (Marschner, 1986). Additionally, excessive zinc can cause plant shoots to be deficient in copper and manganese. The emergence of a purplish red color in leaves, which is attributed to phosphorus insufficiency, is another typical result of Zn toxicity (Lee *et al*., 1996).

5.2. Cadmium Effects on Plants

The amount of cadmium (Cd) in agricultural fields should not exceed 100 mg/kg *(Salt et al*., 1995) because this may damage plants and induce symptoms including chlorosis, growth inhibition, blackening of the root tip, and death (Guo *et al*., 2008; Mohanpuria *et al*., 2007). According to studies, Cd affects the way plants absorb, transport, and use a variety of nutrients, including water, Ca, Mg, P, and K. It also decreases the absorption and transport of nitrate from the root to the bud, which inhibits the function of nitrate reductase in the bud (Hernandez *et al*., 1996). Although it has been observed that Cd interacts with water balance, the toxicity of this metal will impact the permeability of the plasma membrane, resulting in a decrease in water content (Costa & Morel, 1994). Additionally, causes changes in chloroplast metabolism due to the inhibition of chlorophyll biosynthesis and decreased activity of CO_2 fixation enzymes, as well as changes in membrane function by inducing lipid peroxidation (Fodor *et al*., 1995; Raziuddin *et al*., 2011). Cd accumulation in the tobacco plant was demonstrated to enhance oxidative damage and reduce catalase and superoxide dismutase activity (Islam *et al*., 2009). Noticed in previous research, various HM-stressed plants were found to have decreased mitosis content, which led to accelerated root development. For instance, disrupting the extracellular matrix's (ECM) normal function led to an overproduction of H_2O_2 (up to 116%), which consequently led to a 77% reduction in the roots of wheat plants grown in a 200 M Cd hazardous environment (Howladar *et al*., 2018). In other study showed that seeds of sweet basil (*Ocimum basilicum* L.) treated to various amounts of cadmium also had lower germination rates (Fattahi *et al*., 2019). Moreover, *Coriandrum sativum* seed germination was significantly inhibited by soil with a Cd concentration of 50 mg/kg (Sardar *et al*., 2022). Pollution with cadmium significantly decreased the amounts of N, Ca, Mg, and P in the roots and shoots of alfalfa (Zhang *et al*., 2019). Cd toxicity reduced the amounts of Cu, Mg, Fe, and K in *Trifolium repens* L. plant shoots, but significantly increased the Ca content. However, when compared to control plants, Cd stress significantly decreased the Ca, Mg, and Fe levels in roots while increasing the concentration of K and Cu (Hafeez *et al*., 2023). In finding of (El Rasafi *et al*., 2020) while increasing intercellular CO_2 concentration, cadmium stress dramatically decreased net photosynthetic ratio, stomatal conductance, transpiration ratio, chlorophyll a, b, and total chlorophyll content. Similar to this, (Kaya *et al*., 2020) observed that under 0.10 mM Cd stress, wheat plants significantly reduced their chlorophyll a, b concentration as well as their photosystem II (Fv/Fm) maximal photochemical efficiency. When compared to their respective control plants, Rahul and Sharma (2022) reported that Cd stress (500 M) caused H_2O_2 and MDA content to accumulate more in castor *(Ricinus communis)* genotypes S1 and S2, whereas a nonsignificant change was seen in genotypes T1 and T2.

5.3. Chromium Effects on Plants

Germination is the first physiological activity in plants, the rate of seed germination in a medium containing chromium could demonstrate a plant's resistance to such a metal *(Peralta et al*., 2001). while according to (Rout *et al*., 2000) revealed that the germination rate of barnyard grass (*Echinochloa colona*) seedlings declined to 25% after adding 20 ppm Cr. Hexavalent chromium, present in the soil in high concentrations (500 ppm), can cause kidney

bean (*Phaseolus vulgaris)* germination rates to drop by 48% (Dreyer Parr & Taylor, 1982). In addition, (Peralta *et al*., 2001) investigated the germination and growth of alfalfa seeds (*Medicago sativa* cv. Malone) increased by 40 ppm Cr (VI) in a contaminated environment. Other research showed that with 20 and 80 ppm Cr, respectively, sugarcane bud germination was reduced by 32-57% (Jain *et al*., 2000). It is possible that Cr Amylase activity's inhibitory effect and subsequent sugar transfer to the embryonic axis are the causes of the lower seed germination under Cr stress (Zeid, 2001). Alternatively, protease activity rises with the Cr treatment, which may possibly be a factor in the decreased germination of Cr-treated seeds (Zeid, 2001). HMs in trees and crops have a well-documented effect on root formation (Tang et al., 2020). According to (Prasad *et al*., 2001) Cd and Pb were the most toxic to new root primordia in *Salix viminalisis*, whereas Cr had the most significant impact on root length. Cr stress is one of the most significant elements influencing the generation of plant-related pigments like anthocyanin and chlorophyll during photosynthesis. It has been demonstrated that chromium in the radish plant interferes with nutrient translocations and metabolic activity (Tiwari *et al*., 2013). Boonyapookana *et al*, (2002) presented changes in the metabolic pool to induce the production of biochemically related metabolites, which may provide tolerance or resistance to Cr stress with phytochelatins and histidine, act as a direct reaction to Cr stress, or produce other metabolites such as glutathione and ascorbic acid that may harm the plants. Additionally, studies on transgenic *Arabidopsis thaliana* seedlings revealed that Cr increases the expression of the low phosphate (Pi) inducible reporter genes AtPT1 and AtPT2, which are both involved in photosynthesis. The outcome showed that seedlings exposed to Cr had primary-root development 60% reduced. Root growth fully resumed because of increased Pi supply to the seedlings, which also resulted in a reduction in Cr content. Moreover, alterations in the amounts of auxin-inducing genes and auxin transporters expressed by MPK6 are associated with changes in the differentiation of root meristems caused by low phosphorus levels (López-Bucio *et al*., 2014). Therefore, Supplements containing Pi and sulfate can be utilized in management plans for Cr-contaminated soil.

5.4. Lead Effects on Plants

It is known that lead (Pb) has negative impacts on a plant's morphology, growth, and photosynthetic activities. In addition, Pb stress causes plants to do oxidative damage to proteins, lipids, and nucleic acids when too many very damaging ROS build up (Shahzad *et al*., 2018). Pb also prevents the germination of *Spartina alterniflora* and Hefei pine seeds (Nakos, 1979). There is a hypothesis that germination inhibition may result from lead interfering with essential enzymes. It is also noteworthy, Pb severely hampered root and stem elongation, as well as leaf expansion, in *Allium* species (Gruenhage & Jaeger, 1985) as well as in barley (Juwarkar & Shende, 1986). When maize plants were seedlings, they significantly reduced the fresh and dry weight of their branches and roots when exposed to Pb toxicity. The morphological properties of maize seedling root tissues changed as a result of Pb stress because roots are more sensitive to metal stressors. These alterations demonstrated increased central cylinder diameter, cortex thickness, and endodermis thickness to 20%, 19%, and 53%, respectively. However, Pb toxicity did not cause changes in the diameters of the metaxylem and protoxylem. (Zanganeh *et al*., 2021). Additionally, 100-200 ppm of Pb distributed to potted sugar beet plants resulted in chlorosis and decreased development (Hewitt, 1953). Similarly, low lead concentrations of 5 ppm severely inhibited lettuce and carrot root growth (Baker, 1972). The inhibitory effect of Pb^{2+} may derive from changes in the metabolic pathways that impact on growth and development (Sharma & Dubey, 2005). High amounts of Pb can also conflict with enzyme function, disrupt water balance, change membrane permeability, and ultimately interfere with mineral nutrition (Sharma & Dubey, 2005; Sinha *et al*., 1988). Lead also has an effect on the process of photosynthesis by slowing down the activity of aminolevulinic acid dehydratase (ALAD) (Cenkci *et al*., 2010). According to a study by (Zhu *et al*., 2009) lead

hyperaccumulation in *Brassica* species caused a reduction in growth. In a recent investigation, it was found that coriander plants (*Coriandrum sativum* L.) cultivated in soil contaminated with Pb (at levels of 0, 500, 1000, and 1500 mg/kg of soil) demonstrated a marked increase in MDA content, with the highest concentration recorded at 1000 mg/kg Pb, followed by a decrease at higher levels. Additionally, flavonoids were found to be higher under Pb stress at the 1500 mg/kg Pb concentration. The study revealed that the enzymatic activities of SOD and POD exhibited a significant increase when exposed to 1000 mg/kg of Pb but decreased at 1500 mg/kg Pb. Conversely, CAT activity increased at 500 mg/kg Pb but declined at higher concentrations. Additionally, a decrease of 15% in vitamin C content was observed under 1500 mg/kg Pb. On the other hand, at 500 mg/kg Pb, there was a noteworthy increase of 93% in anthocyanin, which a higher concentration of Pb decreased when compared to witness group (Fatemi *et al*., 2021).

5.5. Arsenic Effects on Plants

Arsenic (As) toxicity can interfere with the normal functioning of plant cells by disrupting the normal flow of ions and other essential nutrients. At higher concentrations, As can cause oxidative stress, leading to the formation of reactive oxygen species and the subsequent damage of cellular elements like lipids, proteins, and DNA (Gunes *et al*., 2009). As toxicity can also interfere with the normal functioning of the plant's hormonal balance, leading to changes in growth and development. Three primary forms of this metal can be found in soils and water, namely arsenite [As (III)] and arsenate [As (V)], with the latter being the most prevalent form in soil (Garg & Singla, 2011). Additionally, there are methylated versions of this metal, including monomethylarsinic acid (MMA) and dimethylarsinic acid (DMA) (Angulo-Bejarano *et al*., 2021). Plant species absorb As by utilizing phosphate transporters and nodulin 26-like intrinsic aquaporin (NIP) channels (Pommerrenig *et al*., 2020). As a result, arsenic can disrupt many metabolic pathways in plants, leading to diminished germination, growth, development and crop production. Studies have found that As can affect starch metabolism enzymes, resulting in decreased seed germination (Zia *et al*., 2017). As is an analog of P that competes forth the same uptake carriers in the root plasmalemma of plants. Interestingly, in the *Holcus lanatus* L. plant, the short-term uptake kinetics of the high-affinity arsenate transport system were examined in excised roots of arsenate-tolerant and non-tolerant genotypes, it showed that there is a single gene encoding suppressed P/As transport as an arsenate-tolerant (Meharg & Macnair, 1992). The lack of induction of the synthesis of the arsenate (phosphate) carrier may be the cause of the low rate of arsenate uptake across a wide range of different root phosphate statuses. On the other hand, several research (Barker, 1972) showed that arsenate effects on carrots, lettuce, spinach, and sunflowers resulted in a loss in growth, an increase in the generation of stress biomarkers, and a decrease in photosynthetic pigments (Bergqvist *et al*., 2014; Yadav *et al*., 2014). A recent study by Wu et al. (2020) revealed that the exposure of rice plants to As resulted in a notable decline in seed germination percentage. The impact of arsenic stress on plants has been found to have a negative effect on their photosynthetic pigments, as reported by (Bali & Sidhu, 2021) For instance, a important reduction was observed in the content of Chl a, Chl b, and Chl a/b ratio in wheat plants. Additionally, As was discovered to have an adverse effect on various gas exchange attributes in wheat plants, including transpiration and photosynthetic rates, and water use efficiency, and internal CO_2 concentration decline in Rubisco activity and the maximum efficiency of photosystem (PS) II in rice plants under As stress (Ali & Perveen, 2020; Khan *et al*., 2021).

6. THE MECHANISM OF TOXIC HMs ACTIVITY IN PLANT CELLS

The toxicity of HMs has manifested in many ways when they are accumulated in plant cells (Figure 3). Plants have developed different mechanisms to maintain a balance, including ROS signaling, an upregulated antioxidant defense system, biosynthesis of root exudates, binding of HM to the cell wall, sequestration, and compartmentation. Higher plant species detoxify metal-

trigerred ROS through the activation of enzymatic antioxidant systems, such as SOD, POD, CAT, and non-enzymatic antioxidants such as phenolic compounds (Xu *et al*., 2020; Pehlivan Karakas *et al*., 2022). In addition, MTs, low molecular weight, and cysteine-rich metal binding proteins play a significant biochemical and physiological role in metal homeostasis and protect plants from oxidative damage through ROS scavenging and sequestration of HM (Chaudhary *et al*., 2018). Moreover, plants also activate chaperones that protect and repair proteins and assist MTs in the sequestration and detoxification of metal ions (Haap *et al*., 2016). Two types of HMs include redox such as Co, Cu, Fe, and Cr, and elements containing Al, Zn, and Cd, which belong to inactive redox groups. The capability of HMs to form strong chemical bonds with oxygen, nitrogen, and sulfur atoms is another significant mechanism of HM toxicity. The free formation enthalpy of HM products and their poorly soluble ligands are connected to this binding affinity. These properties allow HMs to bind to cysteine residues and thereby inactivate enzymes. The oxidation and cross-linking of protein thiols, the suppression of essential membrane proteins like H^+-ATPase, or modifications in the composition and fluidity of membrane lipids are other ways that HMs affect membranes (Meharg & Macnair, 1992). According to previous studies, there are three potential causes of HM toxicity, HMs target structural, catalytic, and transport sites of the cell through direct interaction with proteins because of their propensities for thionyl-, histidyl-, and carboxyl groups. On the other hand, stimulation of ROS, MG is produced through auto-oxidation and the Fenton reaction. In addition, the antioxidant defense system and glyoxalase system modification also showed displacement of essential metal ions from specific binding sites, leading to the function change (Sharma and& Dietz, 2009).

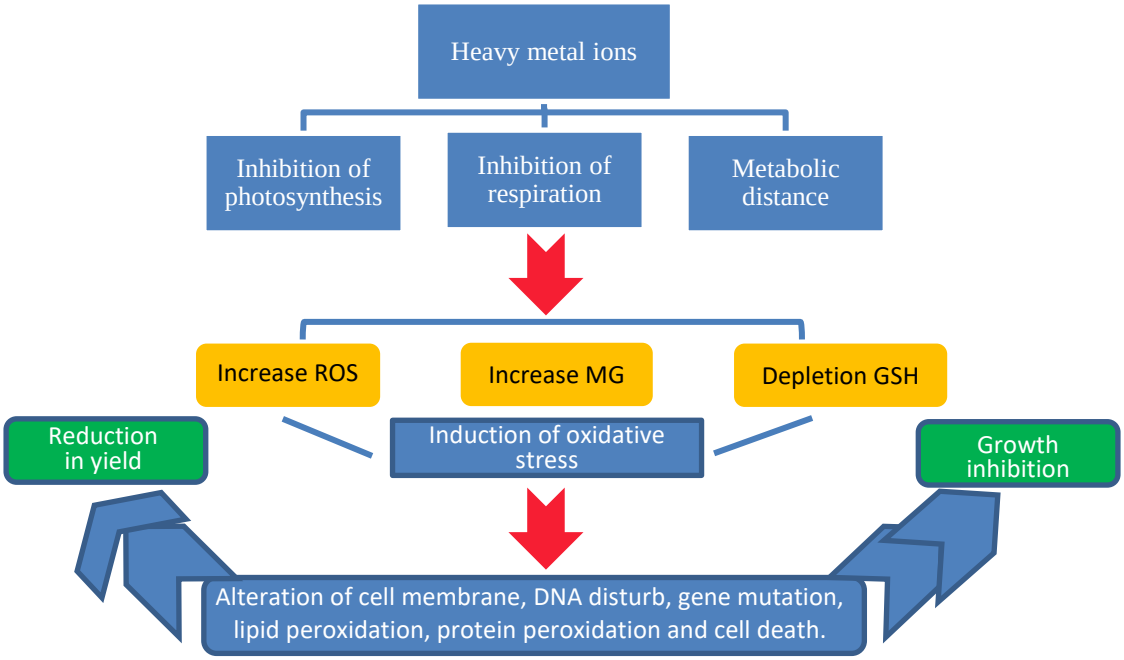

Figure 3. In response to HM stress the potential processes of ROS-induced damage in vulnerable plants.

7. CONCLUSION

HM toxicity is a significant challenge for plants because numerous human activities result in the excessive release of HMs into the environment, creating a significant risk to environmental integrity and agricultural productivity indeed, it poses a risk to the protection of the food chain. Due to the struggle with HM toxicity problem, plants have evolved several mechanisms for detoxification, defense, and adaptation. These mechanisms involve changes at the molecular, biochemical, and physiological levels, including, metal transporters, activate enzymatic and

non-enzymatic antioxidants, and produce phytohormones led to the boost antioxidative defense system by activating some biosynthetic genes. Furthermore, the most important mechanisms responsible for phytohormone induced HM toxicity tolerance in plants contain upregulated glutathione (GSH) biosynthesis genes, osmoregulation, and modulation of metal transporter gene. In recent decades, much progress has been made in understanding the molecular mechanisms underlying HM stress tolerance in plants. By using omics-based approaches and biotechnology, researchers have been able to identify novel genes, metabolic pathways, and signaling networks involved in HM stress tolerance in plants. Future advances in plant science, biotechnology, and genomics are anticipated to deepen the comprehension of the mechanisms underlying the ability of plants to withstand HM stress. In pollution regions of HM, finding new strategies for improving HM stress tolerance in agriculture would lead to increased food security and sustainability. The development of efficient, low-cost technologies for detoxifying HMs from contaminated soils will also be critical for the long-term health of the environment and the populations that depend on it.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Sarah Jorjani: Investigation, Visualization, Writing – original draft. Fatma Pehlivan Karakaş: Supervision, Methodology, Software, Formal Analysis, Validation, and Writing – review and editing.

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