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Phytochemical Screening and Evaluation of the Anxiolytic and Cytotoxic Potential of the Leaves and Stem of *Ocimum basilicum* Linn. var. *pilosum* (Willd.) Benth.

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Abstract

This research investigated the qualitative screening of phytochemicals, anxiolytic and cytotoxic potentials of crude methanol extracts of the leaves (MEL) and stem (MES) of *Ocimum basilicum* Linn. var. *pilosum (Willd.)* Benth is a new variant of sweet basil herb. For the solvent extraction process, methanol was selected due to its ability to solubilize both polar and non-polar plant constituents. Phytochemical analysis was carried out by using the established methods. The anxiolytic activity was assessed on Swiss albino mice using the hole board apparatus, and cytotoxicity was measured using the brine shrimp lethality assay. The current results showed that MEL and MES contained several active secondary metabolites, including alkaloids, glycosides, flavonoids, phenols, tannins, triterpenes, and resins. MEL (200, 400 mg/kg) and MES (400 mg/kg) were found to be significantly ($P < 0.001$) effective anxiolytic agents as compared to the negative control group. In the case of cytotoxicity activity, MEL showed an LC_{50} value of 244.45 µg/ml (standard 0.16 µg/ml), indicating cytotoxic potential compared to the negative control. Thus, the findings revealed that MEL and MES were excellent anxiolytic agents with a moderate cytotoxic potential.

Keywords: *Ocimum basilicum*, phytochemicals, anxiolytic and cytotoxicity

Introduction

Anxiety is a mood disorder that can manifest as tremors, perspiration, tense muscles, and high heart rate with other motor and autonomic symptoms[1]. Individuals with anxiety disorders also frequently have co-occurring conditions like depression, misuse of drugs, asthma, and cardiovascular disease[2]. This disorder can be treated with a variety of techniques, such as cognitive behavioral therapy, anxiety management, exposure therapy, and medication[3]. Herbal medicine, as well as complementary and alternative medicine (CAM), are commonly used by people who have anxiety and mood disorders nowadays. Benzodiazepines are still the main medications which are used for the treatment of anxiety[4]. Compared to conventional pharmacotherapies like benzodiazepines, many psychotropic herbal remedies are relatively safe and have fewer adverse effects such as drowsiness, dependence, and withdrawal issues[5,6].

Cancer is the most frequent and most fatal disease in modern age[7]. In 2015, there were over 17.5 million new cancer cases and 8.7 million deaths from cancer worldwide, making it the most significant cause of death[8]. A few examples of how biodiversity and conventional medical knowledge have produced valuable lead compounds for cancer chemotherapy are the vinca alkaloids (vincristine and vinblastine), taxols (paclitaxel and docetaxel), camptothecin and etoposide[9,10]. Several chemotherapeutic drugs are currently being utilized to treat cancer; however, the issues of selective toxicity and severe side effects

persist. Therefore, the search for novel anticancer medication leads is a necessity.

Plants are the potential source of various biologically active secondary metabolites exhibiting significant pharmacological properties. Plants are still used for primary healthcare in approximately 85% of the world's population for their safety, efficacy, and cost effectiveness[11] and as a drug discovery resource. It has been estimated that 80% of the synthetic drugs have been synthesized from medicinal plants[12]. Basil *(Ocimum basilicum)* is an annual aromatic spicy herb belonging to the Lamiaceae family of 220 genera and about 4000 species. It has been cultivated for its aroma and medicinal uses in the tropical parts of Asia, Africa, Central and South America[13]. A few variants of the species *O. basilicum* have been identified. Various scientific investigations for their phytochemical, pharmacological and morphological features were published in the current scientific literature[14,15]. *Ocimum basilicum* Linn. var. *pilosum (Willd.)* Benth. is one of those discovered variants that has not yet been studied for their possible pharmacological properties. It was an ornamental culinary herb used in folk medicine to treat colds, as a sedative, for soothing nerves, reducing heat, clearing heartburns, removing toxins, and making perfume[16,17]. A study demonstrated the significant antifungal properties of the essential oils extracted from this plant against a few pathogenic fungi[18]. Although different species of this plant have been studied for several biological effects, this variety is yet to be studied extensively. Therefore, this research investigated a comparative outlook of the phytochemicals between its leaves and stem extracts and the in vivo anxiolytic and in vitro cytotoxic effects of the extracts.

Materials and Methods

Solvents and chemicals

Methanol (Merck, Germany) was used for the extraction process. Other analytical and laboratory-grade reagents, such as Wagner's Reagent, Potassium iodide, Copper sulphate, Ferric chloride, Potassium sodium tartrate, Sodium hydroxide, Hydrochloric acid, Sulfuric acid, Alpha naphthol, Acetic anhydride, Chloroform, Acetone, Glacial acetic acid, etc., were used for phytochemical screening.

Plant collection and authentication

The entire plant was collected in December 2023 from the Bandarban district, Chattogram division, Bangladesh. Then, the plant was identified as *Ocimum basilicum* Linn. var. *pilosum (Willd.)* Benth. by an experienced taxonomist and Professor in the Department of Botany at the University of Chittagong. The leaves and stem of the plant were taken for analysis.

Plant material preparation and extraction

The leaves and stem of the plants were thoroughly washed with clean water and then chopped into little pieces. They were sun-dried for seven days in a semi-shed, followed by pulverization into a powder using a grinder machine in the Research Laboratory of the Department of Pharmacy, University of Chittagong. The powder portion of the leaves (936 gm) and stem (787.50 gm) were soaked in 3L of pure methanol separately. It was maintained at room temperature for around 15 days with frequent shaking. Filter cloth and Whatman's filter paper were used to filter the solution, and the filtrate was concentrated under reduced pressure at 60°C using a rotary evaporator (Stuart, UK) in the Research Laboratory, Department of Pharmacy, University of Chittagong. The plant extracts were stored at 4°C in a small, sterile container. Following the established protocols, these extracts were then used for phytochemical screening and evaluation of anxiolytic and cytotoxic effects.

Qualitative evaluation of phytochemicals

Qualitative phytochemical analysis of MEL and MES were carried out using protocols mentioned elsewhere[19,20].

In vivo pharmacological studies

Experimental animals

Swiss Albino male mice weighing 25–30 g of the age of 4-5 weeks were used for the hole board test. These were procured from the Bangladesh Council of Scientific and Industrial Research Laboratories (BCSIR) in Chittagong, Bangladesh. For adaptation, animals were housed in polypropylene cages in the animal house for one week under standard environmental conditions (T: 25 ± 2°C, RH: 55–65%, and 12 h light/12 h dark cycle). Food and water were withheld from the animals 12 hours before and during the studies to maintain a steady rate of hydration. Every ethical approach to the use of experimental animals was thoroughly examined.

Experimental design

For testing of anxiolytic activity, animals were randomly chosen and placed in groups designated as Group I – VI (five mice in each group). To prepare 400 mg/kg and 200 mg/kg dose, 120 mg/kg and 60 mg/kg of each of the extracts were measured and triturated in a unidirectional manner by adding 1% Tween-80 to a volume of 3 ml. The suspensions were thoroughly mixed with a vortex for stabilization. Group I received a control treatment (1% tween-80 at 10 ml/kg), whereas Group II received the standard treatment (diazepam 1.0 mg/kg). Groups III, IV, V, and VI were designated for MEL 200 and 400 mg/kg, MES 200 and 400 mg/kg respectively.

Evaluation of anxiolytic activity

The hole-board device was made of a (40×40) cm wooden board with sixteen holes positioned equally distanced with a diameter of 3 cm. The board was set 15 cm above a table. At least one hour before testing, mice were brought to the dimly lighted laboratory. Thirty minutes after the injection with the control or the test samples, the animals were placed individually in the center of the board, facing away from the observer, and head dip numbers were recorded for 30, 60, 90, and 120 minutes. If both eyes slipped into the hole, a head dip was noted[21,22].

Evaluation of in vitro cytotoxic activity

Using the brine shrimp lethality test[23], the cytotoxic activity of this plant was assessed.

Preparation of seawater

Sterile artificial seawater was prepared using 38 g of pure NaCl salt dissolved in 1L distilled water, which was then filtered to obtain a clean solution. The pH of the solution was adjusted to 8.0 using 1M NaOH.

Hatching of brine shrimps

Brine shrimps (*Artemia salina)* were hatched using brine shrimp eggs in a small tank filled with the prepared seawater. Brine shrimp eggs were added to one edge of the tank and covered with a lid. Shrimps in this tank were hatched under constant aeration for 48 hours to give mature shrimp named nauplii. The perforated dam led the hatched shrimps to the bulb placed on the tank. Ten living nauplii were transferred to test tubes containing 5 ml of seawater using a Pasteur pipette.

Procedure

A stock solution was made by dissolving 5 mg of the plant extracts in 1 ml of pure DMSO (dimethylsulfoxide) and then making the volume 5 ml with seawater. Different sample concentrations (1000, 800, 500, 300, and 100 µg/ml) were prepared by serial dilution with seawater. Then, these solutions were transferred into test tubes, each containing 10 brine shrimp nauplii and 5 ml of saltwater. The test tubes were maintained at room temperature for 24 h under the light, and surviving nauplii were counted using a magnifying glass[24,25]. Vincristine sulfate solution was used as a positive control. The data were processed in a simple program (Microsoft Excel 2021) to estimate the LC_{50} values. The percent mortality of brine shrimp was calculated with the formula -

[% mortality = Number of brine shrimp dead/Number of brine shrimp introduced ×100]

Statistical analysis

Data were presented as Mean ± SEM (Standard Error of Mean). The data were statistically analyzed by using one-way ANOVA, followed by post-hoc Dunnett's "t" test and Tukey test with the Statistical Package for Social Science (SPSS, Version 16.0, IBM Corporation, NY). Statistical significance was determined at $p<0.001$

compared to the control group. Graphs were created with the Graph Pad Prism Data Editor for Windows, Version 5.0 (Graph Pad Software Inc., San Diego, CA).

Results and Discussion

Qualitative evaluation of phytochemicals

The phytochemical screening of MEL and MES revealed the presence of a mixture of secondary metabolites. These findings might serve as a direction for future research using advanced techniques for characterizing the secondary metabolites of this plant that could be linked to various pharmacological actions[26,27,28]. The results are demonstrated in Table 1.

Evaluation of anxiolytic activity

Depression and anxiety-related diseases are worldwide psychiatric ailments[29], and the causes behind these diseases are increasing exponentially with modernization. To examine the anxiety index in an animal model, the head-dipping behavior needs to be analyzed to change the animal's emotional state. This effect can be obtained by using a Hole-board test apparatus. This protocol indicates that an increase in head-dipping behaviour may reflect the expression of an anxiolytic state. The results of the current investigations are tabulated in Table 2. This study

Table 1. Qualitative analysis of phytochemical studies of crude methanol extract of *O. basilicum* var. *pilosum* leaves and stem

Phytochemicals	Reagent used	MEL	MES
Alkaloid	Wagner's test	+	+
Carbohydrate	Molish's test	+	+
Glycoside	Sodium hydroxide test	+	+
Saponin		–	–
Steroid	Liebermann Burchard's test	–	–
Phenol	$FeCl_3$ test	+	+
Tannin	$FeCl_3$ test	+	+
Flavonoid	Zinc Hydrochloric acid reduction test	+	–
Reducing sugar	Fehling's Reagent test	+	–
Protein	Biuret test	–	–
Tri-terpene	Liebermann-Burchard's test	+	+
Fixed oil & fat	NaOH Reagent test	–	–
Cardiac glycoside	Keller-Kiliani test	–	–
Anthraquinone	Hydroxy anthraquinone test	–	–
Resin	Test with acetone solution	+	–

*MEL = Crude methanol extract of leaves, and MES = Crude methanol extract of stem of *O. basilicum* var. *pilosum*; (+) = Present, (-) = Absent

Table 2 Evaluation of anxiolytic activity of crude methanol extract of *O. basilicum* var. *pilosum* leaves and stem by using Hole-board test.

Treatment Dose (mg/kg)	Number of Head dipping			
	30 minutes	60 minutes	90 minutes	120 minutes
Control	10.60 ± 1.21	9.43 ± 1.36	9.20 ± 1.39	11.40 ± 1.03
Diazepam (1mg/kg)	19.20 ± 1.36[a]	23.80 ± 1.77[a]	23.20 ± 1.93[a]	29.40 ± 1.81[a]
MEL (200 mg/Kg)	21.80 ± 1.96[a]	29.80 ± 2.42[a]	29.60 ± 3.67[a]	13.80 ± 1.66
MEL (400 mg/Kg)	29.60 ± 2.04[a]	28.80 ± 4.43[a]	8.40 ± 1.57	8.80 ± 1.93
MES (200 mg/Kg)	22.28 ± 1.28[a]	19.40 ± 2.58	9.01 ± 1.61	3.62 ± 0.81
MES (400 mg/Kg)	23.80 ± 1.56[a]	24.20 ± 1.69[a]	18.21 ± 2.80	13.05 ± 1.52[a]

*Each value represents the mean ± SEM. (n = 5), [a] $p < 0.001$ was statistically significant in comparison with control; MEL = Crude methanol leaves extract, and MES = Crude methanol stem extract of *O. basilicum* var. *pilosum*

indicated that Diazepam (1.0 mg/kg) significantly increased the number of head dipping from 19.20 ± 1.36 to 29.40 ± 1.81 times for 120 minutes compared to the control group. At the dose of 200 mg/kg, MEL showed an increase in the number of head dipping for 120 minutes (21.80 ± 1.96 to 29.80 ± 2.42 times), whereas MEL at the dose of 400 mg/kg increased the number of head dipping (29.60 ± 2.04 times) within 30 minutes, in comparison with that of the negative control significantly ($^{a}p<0.001$). On the other hand, MES at doses of 200 mg/kg and 400 mg/kg significantly increased the number of head dipping during 30 to 60 minutes compared to the negative control group. The obtained data showed that both MEL and MES exhibited significant potential as anxiolytic agents in a dose-dependent manner.

The reference standard Diazepam is a CNS (central nervous system) depressant that is used in modern medicine to treat anxiety disorders and has a binding site on the Gamma amino-butyric acid receptor type-A ionophore complex ($GABA_A$). GABA binding to the receptor decreases neuronal excitability by raising the action potential threshold, resulting in reduced activity, excitement moderation, and a sense of calmness[30-33]. Several studies found that some phytoconstituents, including flavonoids, saponins, and tannins, could be associated with anxiolytic action. These phytochemicals might bind to the neurotransmitter receptors like that of the neuroactive steroids such as GABA. Given that the anxiolytic effect of MEL and MES were comparable to that of Diazepam, they might have acted through the same GABA receptor complex. Thus, natural substances with GABA-mimetic action may potentially displace synthetic medications because of their less adverse effects[34,35].

Evaluation of cytotoxicity activity

The brine shrimp lethality bioassay (BSLA) against *Artemia salina* is an easy way to assess the cytotoxicity of the crude extracts as well as the isolated compounds[23]. It serves as a marker for antiviral, antiplasmodial, antifilarial, antimalarial, and anticancer as well as pesticidal properties[36,37]. The toxicity level for the brine shrimp was categorized as non-cytotoxic when the LC_{50} value was above 1000 µg/ml and cytotoxic when it was less than 1000 µg/ml[23]. The % of mortality was plotted against the concentration of the sample to determine the median lethal concentration (LC_{50}) of the test samples after 24 hours. The best-fit line was derived from the curve data using regression analysis. The control group showed no signs of brine shrimp death, indicating their non-cytotoxic profile. The reference standard vincristine sulfate was lethal with an LC_{50} value of 0.16 µg/ml, compared with the control (seawater). The LC_{50} values of MEL and MES were 244.45 µg/ml and 475.95 µg/ml, respectively. Thus, they exhibited a moderate cytotoxic effect compared to the control group, where mortality was the highest at the higher concentrations of MEL and MES and lowest at the lower concentrations. The results are represented in Figure 1, Figure 2, Figure 3, and Table 3. Exposure to various dosage levels of the test samples resulted in varying degrees of mortality for *Artemia salina*. The ovicidal and larvicidal properties of the plant extract could be attributed to the toxic compounds, which either hampered embryonic development or killed the eggs. The phytochemical analysis of the plant revealed the presence of tannins and flavonoids, which have anticancer properties[38,39]. Modern chromatographic separation techniques will allow us to identify the exact compounds causing those actions.

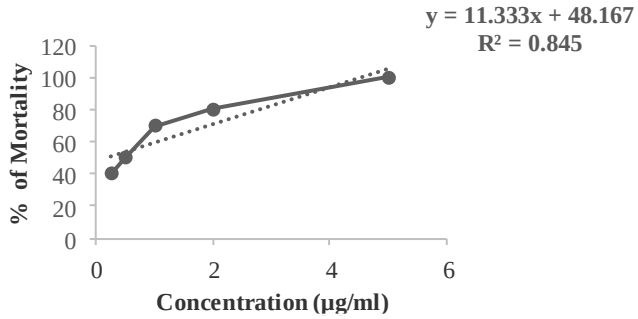

Figure 1. The cytotoxic effect of Vincristine sulfate against brine shrimp. The straight line represents % of mortality, and the dotted line represents Linear (% mortality)**.**

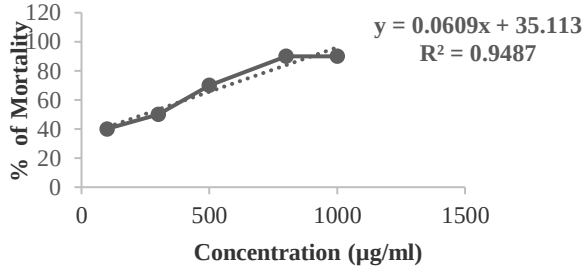

Figure 2. The cytotoxic effect of crude methanol extract of leaves (MEL) of *O. basilicum* var. *pilosum* against brine shrimp. The straight line represents % of mortality, and the dotted line represents Linear (% mortality).

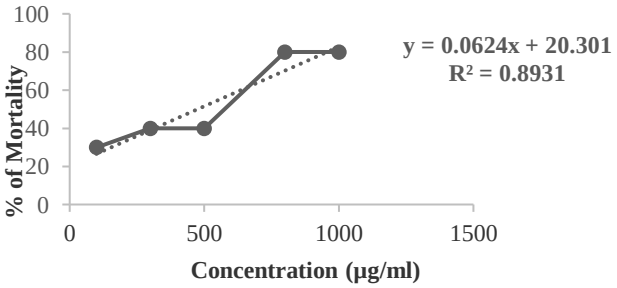

Figure 3. The cytotoxic effect of crude methanol extract of the stem (MES) of *O. basilicum* var. *pilosum* against brine shrimp. The straight line represents % of mortality, and the dotted line represents Linear (% mortality).

Table-3. A brief overview of the cytotoxic activity of crude methanol extract of *O. basilicum* var. *pilosum* leaves and stem

Samples	Concentration (µg/ml)	% Mortality	Regression Equation	R^2	LC_{50} (µg/ml)
Vincristine sulphate (Standard)	0.25	40	y = 11.33x + 48.17	$R^2 = 0.85$	0.16
	0.50	50			
	1	70			
	2	80			
	5	100			
MEL	100	40	y = 0.06x + 35.11	$R^2 = 0.95$	244.45
	300	50			
	500	70			
	800	90			
	1000	90			
MES	100	30	y = 0.06x + 20.30	$R^2 = 0.89$	475.95
	300	40			
	500	40			
	800	80			
	1000	80			

*MEL = Crude methanol extract of leaves and MES = Crude methanol extract of stem of *O. basilicum* var. *pilosum*

Conclusion

The acquired data suggested that MEL and MES of *O. basilicum* var. *pilosum* possessed anticancer as well as anxiolytic activities. These effects might be attributed to its phenols, flavonoids and terpenoids components. However, more study is required to determine the precise mechanism and effective components of *O. basilicum* var. *pilosum.*

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