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Evaluation of Membrane Stabilizing Activity, Total Phenolic Content, Brine Shrimp Lethality Bioassay, Thrombolytic and Antimicrobial Activities of *Tagetes patula* L.

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Abstract: The methanol extract of leaf of *Tagetes patula* L. as well as its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates were subjected to screening for total phenolic content, brine shrimp lethality, membrane stabilizing, thrombolytic and antimicrobial activity. The membrane stabilizing activity was assessed by hypotonic solution-and heat-induced methods and was compared with acetyl salicylic acid. In the present studies, the *n*-hexane soluble fraction demonstrated strong membrane stabilizing activity in both hypotonic solution-and heat-induced methods with 44.48% and 42.68% inhibition of haemolysis, respectively. The total phenolic content was also determined and expressed in gallic acid equivalent. In brine shrimp bioassay, the crude methanol extract of leaf showed strong cytotoxic activity with LC_{50} value of 8.58 µg/ml compared to that of 0.451 µg/ml exhibited by standard vincristine sulphate. During assay for thrombolytic activity, the *n*-hexane soluble fraction revealed 43.7% lysis of clot while standard streptokinase and water, used as positive and negative controls, demonstrated 65.8% and 3.62% lysis of clot, respectively. In antimicrobial assay by disc diffusion method, all the samples exhibited moderate to significant antimicrobial activity (zone of inhibition = 9.0-22.0 mm) against all the test organisms. Among all the samples, the carbon tetrachloride soluble fraction displayed strong antimicrobial activity against *Escherichia coli* (22.0 mm).

Keyword: *Tagetes patula* L, Membrane Stabilizing, Total Phenolic Content, Brine Shrimp Lethality, Thrombolytic, Antimicrobial.

1. Introduction

Tagetes patula L. (Family: Asteraceae or Compositae,) commonly known as Genda, is native to South America but introduced and naturalized in most parts of the world and currently is a cosmopolitan ornamental plant. It is a common garden plant[1]. The plant and its preparations have been used as astringent, diuretic, sedative, diarrhea, vomiting, fever, skin diseases and hepatic disorders[2]. Furthermore, divergent biological activities such as antibacterial, antifungal, insecticidal, nematicidal and larvicidal[3-7] have been devoted to the active principles of this plant. Essential oils and acetylenic thiophenes derived from roots are the most important secondary metabolites of French marigold[7]. Monoterpenes, sesquiterpenes and α-terthienyl have been reported as the main

ingredients of the herb and roots of *T. patula*, respectively[8,9]. The insecticidal and nematicidal activities of this plant are due to the presence of high levels of sesquiterpenes and thiophene compounds in the essential oil and organic solvent extracts of roots[3].

As part of our ongoing research with medicinal plant of Bangladesh,[10-12] the present study has been undertaken to evaluate the membrane stabilization, total phenolic content, brine shrimp lethality, thrombolytic and antimicrobial activities of *T. patula* as well as to find out the logical evidence for its folk uses.

2. Materials and Methods

2.1 Plant materials

The leaves of *T. patula* were collected from Mirpur in August 2010. A voucher specimen (Accession no. 35455) for this plant has been maintained in Bangladesh National Herbarium, Dhaka, Bangladesh for future reference.

2.2 Reagents and chemicals

All chemicals i.e. methanol, *n*-hexane, carbon tetrachloride, chloroform and other reagents used in these experiments were of the highest analytical grade.

2.3 Extraction and fractionation

The samples were sun dried for several days and then oven dried for 24 hours below 40 °C to facilitate grinding. The powdered materials (500 gm) were macerated in 2.0 L of methanol for 7 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator at low temperature (40-45 ºC) and reduced pressure. The concentrated methanol extract was partitioned by the modified Kupchan method[13] and the resultant partitionates i.e., *n*-hexane (1.2 gm), carbon tetrachloride (1.0 gm), chloroform (800 mg), and aqueous soluble (1.6 gm) fractions were used for the experiment.

2.4 Membrane stabilizing activity: The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane[14]. The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced mice erythrocyte haemolysis[15]. To prepare the erythrocyte suspension, whole blood was obtained using syringes (containing anticoagulant EDTA) from mice through cardiac puncture. The blood was centrifuged and blood cells were washed three times with 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 gm.

2.5 Hypotonic solution-induced haemolysis: The test sample comprised of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1. 0 mg/ml) or acetyl salicylic acid, (ASA, 0.1 mg/ml). The control sample consisted of 0.5 ml of RBCs mixed with hypotonic buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 gm and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation-

$$\% \text{ Inhibition of haemolysis} = 100 \times (OD_1 - OD_2/OD_1)$$

where, OD_1 = optical density of hypotonic-buffered saline solution alone (control) and OD_2 = optical density of test sample in hypotonic solution.

2.6 Heat-induced haemolysis: Isotonic buffer containing aliquots (5 ml) of different extractives were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µl) was added to each tube and mixed by gentle inversion. A pair of the tubes was incubated at 54 ºC for 20 min in a water bath. The other pair was maintained at 0-5 ºC in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 gm and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of hemolysis in tests was calculated according to the equation:

% Inhibition of hemolysis = 100 x [1- (OD_2-OD_1/ OD_3-OD_1)]

where, OD_1 = optical density of unheated test sample, OD_2 = optical density of heated test sample and OD_3 = optical density of heated control sample

Table 1: Effect of extractives of leaf of *T. patula* on hypotonic solution-and heat-induced haemolysis of erythrocyte membrane.

Sample	Concentration (mg/mL)	Hemolysis inhibition (%)	
		Hypotonic solution induced	Heat induced
Hypotonic medium	50 mM	- -	- -
CME	2.0 mg/ml	40.10	36.36
HSF	2.0 mg/ml	44.88	42.86
CTSF	2.0 mg/ml	37.20	33.33
CSF	2.0 mg/ml	43.17	41.67
AQSF	2.0 mg/ml	31.74	30.00
Acetyl salicylic acid	0.1 mg/ml	71.9	42.12

Here**,** CME = Crude methanolic extract; HSF = Hexane soluble fraction; CTSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = aqueous soluble fraction of the methanolic extract of *T. patula***.**

2.7 Total phenolics analysis: The total phenolic content of *T. patula* was measured by employing the method [16] involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as standard. To 0.5 ml of extract solution (2.0 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5% w/v) solution were added. After 20 minutes of incubation at room temperature, the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolics were quantified with the help of calibration curve obtained from gallic acid (0-100 µg/ml). The phenolics content of the sample was expressed as mg of GAE (gallic acid equivalent)/gm of the dried extract.

2.8 Cytotoxicity screening: Dimethyl sulfoxide **(**DMSO) solutions of the extractives were applied against *Artemia salina* in a one-day *in vitro* assay [17]. For the experiment, 4 mg of each of the Kupchan fractions was dissolved in DMSO and solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.78125 µg/ml were obtained by serial dilution technique. Vincristine sulphate and DMSO were used as the positive and negative control, respectively.

2.9 Thrombolytic activity: The thrombolytic activity of all extractives was evaluated by the method [18] using streptokinase as standard. The dry crude extract (100 mg) was suspended in 10 ml of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered. Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in five different pre weighed sterile micro centrifuge tube (1 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone).

To each microcentrifuge tube containing pre-weighed clot, 100 µl aqueous solutions of different partitionates along with the crude extract was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non-thrombolytic control, 100 µl of distilled water were separately added to the control tubes. All

the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The differences in weights taken before and after clot lysis were expressed as percentage of clot lysis as shown below:

% of clot lysis = (wt of clot after lysis /clot wt) × 100

2.10 Antimicrobial activity: The preliminary antimicrobial activity of the extractives was determined at 400 µg/disc by the disc diffusion method[19] against a number of Gram positive and Gram negative bacteria and fungi (Table-3). The bacterial and fungal strains used in this experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. Here, standard Kanamycin (30 µg) disc was used as reference.

Table 2: Total phenolic content, cytotoxic activity (LC_{50} µg/ml) and thrombolytic activity (% Clot lysis) of different Kupchan fractions of *T. patula.*

Sample	Total Phenolic Content (mg of GAE/gm of dried extract)	Cytotoxic activity (LC_{50} µg/ml)	Thrombolytic activity (% Clot lysis)
VS	ND	0.451	ND
SK	ND	ND	65.8±1.04
CME	53.92±1.17	8.58±0.96	18.3±1.41
HSF	64.54±1.26	37.19±1.34	43.7±0.82
CTSF	42.5±0.85	34.91±0.67	30.24±1.23
CSF	67.44±1.21	43.84±0.57	15.0±0.67
AQSF	14.85±0.63	58.79±1.13	8.2±0.35

Here, VS= Vincristine sulphate, SK = Streptokinase, ND = Not determined

3. Results and Discussion:

The present study was undertaken to evaluate the membrane stabilization, total phenolic content, brine shrimp lethality bioassay, thrombolytic and antimicrobial activities of the organic soluble materials of a methanol extract of *T. patula* and the results have been summarized in Table 1-3.

The extractives of *T. patula,* at concentration 2.0 mg/ml, significantly protected the lysis of mice erythrocyte membrane induced by hypotonic solution and heat, as compared to the standard acetyl salicylic acid (0.10 mg/ml) (Table 1). In hypotonic solution-induced haemolysis, the *n*-hexane and chloroform soluble fraction inhibited 44.88% and 43.17% haemolysis of RBC, respectively as compared to 71.9% produced by acetyl salicylic acid. The methanolic crude extract also revealed significant inhibition of haemolysis of RBCs. On the other hand, in heat induced haemolysis, both the hexane soluble fraction (HSF) and chloroform soluble fraction inhibited 42.86% and 41.67% haemolysis of RBC, respectively as compared to 42.1% produced by acetyl salicylic acid.

The total phenolic content (TPC) varied for different Kupchan fractions of methanolic extract ranging from 14.85 to 67.44 mg of GAE/gm of dried extract (Table-2). The highest total phenolic was found in chloroform soluble fraction (67.44 mg of GAE/gm of extractives) demonstrating the significant antioxidant potentials. The hexane soluble Kupchan fraction and crude methanol extract revealed moderate antioxidant activity. The lowest phenolic was seen in aqueous soluble fraction (14.85 mg of GAE/gm of extractives).

In brine shrimp lethality bioassay, the crude methanol extract showed strong cytotoxic activity with LC_{50} value of 8.58 µg/ml while the carbon tetrachloride, *n*-hexane, chloroform and aqueous soluble fraction were also moderately cytotoxic with LC_{50} values of 34.91, 37.19, 43.84 and 58.79 µg/ml, respectively compared to 0.451 µg/ml produced by standard vincristine sulphate (Table 2).

Table 3: Antimicrobial activity of *T. patula* extractives at 400 µg/disc.

Test microorganisms	Diameter of zone of inhibition (mm)					
	CME	HSF	CTSF	CSF	AQSF	Ciprofloxacin
Bacillus megaterium	13.0	11.0	14.0	11.0	9.0	40.0
B. cereus	9.0	14.0	17.0	12.0	11.0	40.0
B. subtilis	12.0	13.0	16.0	10.0	10.0	42.0
Sarcina lutea	15.0	13.0	20.0	12.0	10.0	41.0
Staphylococcus aureus	9.0	12.0	15.0	12.0	9.0	41.0
Escherichia coli	13.0	11.0	22.0	12.0	16.0	40.0
Pseudomonas aeruginosa	10.0	9.0	12.0	11.0	9.0	42.0
Salmonella paratyphi	9.0	10.0	10.0	9.0	12.0	40.0
Salmonella typhi	9.0	9.0	11.0	10.0	12.0	38.0
Shigella boydii	12.0	11.0	16.0	10.0	13.0	41.0
Sh. dysenteriae	10.0	10.0	16.0	13.0	9.0	42.0
Vibrio mimicus	9.0	11.0	15.0	12.0	12.0	40.0
V. parahaemolyticus	10.0	10.0	18.0	12.0	9.0	41.0
Aspergillus niger	9.0	9.0	12.0	10.0	10.0	40.0
Candida albicans	9.0	9.0	12.0	11.0	9.0	40.0
Saccharomyces cerevisiae	9.0	11.0	14.0	12.0	9.0	40.0

In order to identify the drugs with the ability to promote lysis of blood clot from natural sources, the extractives of *T. patula* were assessed for thrombolytic activity. Addition of 100 µl streptokinase (SK), a positive control (30,000 I.U.) to the clots of human blood and subsequent incubation for 90 minutes at 37 °C, showed 65.8% lysis of clot. On the other hand, distilled water when treated as negative control, showed negligible lysis of clot (3.62%). The mean difference in percentage of clot lysis between positive and negative control was found to be statistically significant. In this study, all the samples showed mild to moderate activity where the hexane and carbon tetrachloride soluble fractions exhibited 43.7 ± 0.82 and 30.24 ± 1.23 clot lysis, respectively (Table-2).

During screening for antimicrobial activity, all the samples exhibited moderate antimicrobial activity (zone of inhibition = 9.0-22.0 mm) against the test organisms. The crude methanolic extract exhibited moderate antimicrobial activity against *Sarcina lutea* (15.0 mm), *Bacillus megaterium* (13.0 mm) and *Escherichia coli* (13.0 mm) while the *n*-hexane soluble fraction showed maximum activity against *Bacillus cereus* (14.0 mm). Among all the samples, the carbon

tetrachloride soluble fraction displayed strong antimicrobial activity against *Escherichia coli* (22.0 mm), *Sarcina lutea* (20.0 mm), *Vibrio parahaemolyticus* (18.0 mm). The aqueous soluble fraction was found to be active against *Escherichia coli* (16.0 mm) while the chloroform soluble fraction revealed little activity against the test organisms.

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5. References

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