December 30, 2019

PharmacologyOnLine

Archives • 2019 • vol.3 • 203-210

PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT, THROMBOLYTIC, ASTRINGENT, PRO-COAGULANT PROPERTIES INVESTIGATION OF *TAGETES LUCIDA*

Md. Sakhawat Hossain[1], Nisarat Nijhum[2], Shadhan Kumar Mondal[2], Zia Uddin Masum[3], Md. Shafiqul Islam Sovon[3], Amran Khan[4], Aditi Bhowmick[5] and Tushar Saha[3*]

[1] Department of Pharmacy, Daffodil International University, Dhaka-1207, Bangladesh.

[2] Department of Pharmacy, World University of Bangladesh, Dhaka, Bangladesh.

[3]Department of Pharmacy, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh.

[4] Department of Pharmacy, Noakhali Science and Technology University, Bangladesh.

[5] Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh.

*tushar.saha21@yahoo.com

Abstract

The aim of the present investigation was to evaluate the phytochemical and biological properties (antioxidant, thrombolytic, astringent and pro-coagulant) of *Tagetes lucida*. Freshly prepared crude extracts of *Tagetes lucida* were qualitatively tested for the presence of various chemical constituents including carbohydrates, alkaloids, flavonoids, glycosides, gums, steroids and saponins. These were identified by observing characteristic color changes using standard procedures. In phytochemical investigations, the presence of carbohydrates, alkaloids and flavonoids were identified but glycosides, gums, steroids and saponins were absent. The crude methanolic extract of *Tageres lucida* showed significant thrombolytic activity. The plant leaves extracts had anti-oxidant activity but not in a significant manner. Medical astringents are considered to stop or slow bleeding and to help wounds heal. The leaves of *Tagetes lucida* showed astringent properties. Procoagulant platelets are predominantly localized at thrombus surface. The leaves of *Tagetes lucida* showed pro-coagulant properties.

Keywords: *Tagetes lucida, astringent, thrombolytic, astringent*

Introduction

Plant sources are considered as one of the major sources of medicines [1]. Phytochemical constituents like alkaloids, glycosides, tannins etc. which are bioactive nature help to show this medicinal activity [2]. Oxidation reactions initiated by excess free radicals have been shown to lead to the formation of tumors, damage of DNA, mRNA, proteins, enzymes; cause cancer, cardiovascular diseases, nervous disorders, premature ageing, Parkinson's and Alzheimer's diseases, rheumatic and pulmonary disorders [3]. Therefore, the need for systematic screening of medicinal plants for antioxidant activity cannot be overemphasized. here is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases. Plants are the potential source of natural antioxidants. Natural antioxidants or phytochemical antioxidants are known as the secondary metabolites of plants. Carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols, tocotrienols etc., are some of the antioxidants produced by the plant for their sustenance. Beta-carotene, ascorbic acid and alpha tocopherol are the most used antioxidants. Because of the complex nature of phytochemicals, the antioxidant activities of plant extracts must be evaluated by combining two or more different *In-vitro* assays. Cerebral venous sinus thrombosis (CVST) is a common disorder which accompanied by significant morbidity and mortality [4]. Heparin, an anticoagulating agent, is the first line of treatment for CVST, because of its efficacy, safety and feasibility [5]. Thrombolytic agents such as tissue plasminogen activator (t-PA), urokinase, streptokinase etc. play a crucial role in the management of patients with CVST [6]. In medicine, astringents cause constriction or contraction of mucous membranes and exposed tissues and are often used internally to reduce discharge of blood serum and mucous secretions. This can happen with a sore throat, haemorrhages, diarrhoea, and peptic ulcers. Externally applied astringents, which may be the reason of mild coagulation of skin proteins, dry, harden, and protect the skin. People with acne are often treated with astringents if they have oily skin. Mild astringents relieve such minor skin irritations and disorders as those resulting from superficial cuts; allergies; insect bites; anal haemorrhoids; and fungal infections such as athlete's foot. Pro-coagulants mean tending to promote coagulation or a precursor of a natural substance necessary to coagulate the blood. Coagulation which is also known as clotting, is the process by which blood converts from a liquid to a gel, forming a blood clot. It helps in hemostasis, the cessation of blood loss from a damaged vessel, followed by repair [7].

Procoagulant and anticoagulant reactions play an important role in the regulation of thrombin formation during secondary hemostasis. *Tagetes lucida* is a perennial plant which is generally native to Mexico and Central America. It has medicinal property and used as a culinary herb. The plant is supposed to be rich in phytochemical constituents and thus have medicinal properties. The aim of this study is to find out the phytochemical constituents and its biological activity.

Methods

Collection and preparation of the plant material: The whole plant of *Tagetes lucida* was collected from Savar, Botanical garden and identified by taxonomist of National Herbarium, Bangladesh situated at Mirpur in Dhaka. The sample is preserved in the Phytochemical Laboratory of World University of Bangladesh for as further reference (Accession No. 48262). The leaves were air dried for several days and then oven dried for 24 hours at considerably low temperature (not more than 40°C) for better grinding. The dried leaves were then ground to a coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, World University of Bangladesh.

Extraction of the plant material: The powdered material (250gm) was taken in a cleaned, amber colored reagent bottle (5 liters) and soaked in 2.0 L of methanol. The container with its content was sealed by bottle cap and kept for a period of 10 days accompanying occasional shaking and stirring. The whole mixtures were then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. The volume of the filtrate was then all owed

to evaporate at ambient temperature until approximately 70% solvent was evaporated.

Phytochemical screening: Preliminary phytochemical analysis for alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, resins, saponins, steroids, tannins, terpenoids and triterpenoids and quantitative phytochemical analysis for alkaloids, total phenolic content, total flavonoids, tannins, saponins and ascorbic acid were done by following standard procedures.

Test for carbohydrates: Two drops of molisch's reagents were added to about 5 mg of the extract in 5 ml aqueous solution in a test tube. 1 ml of conc. H2SO4 was allowed to flow down the side of the inclined test tube so that the acid formed a layer beneath the aqueous solution without mixing with in. a red ring was formed at the common surface of the two liquids which indicated the presence of carbohydrate. On standing or shaking, a dark-purple color solution was obtained. Then the mixture was shaken and diluted with 5 ml of water. Dull violet precipitate was formed immediately. 2 ml of aqueous extract of the plant material was added to 1 ml of equal volume of Fehling's solution A and B. Then boiled for few minutes. A red or brick red color precipitate was formed in the presence of reducing sugar.

Test for glycosides: A small amount of extract was dissolved in water and alcohol then boiled with Fehling's solution. Any brick-red precipitation was noted. Another part of extract was dissolved in water and alcohol and then boiled with a few drops of dilute H_2SO_4. The acid was neutralized with NaOH solution and boiled with Fehling's solution. A brick-red precipitation was produced in this experiment which showed the presence of glycosides in the extract.

Tests for alkaloids: 300 mg extract was treated with 2M hydrochloric acid. This acidic filtrate was mixed with amyl alcohol at room temperature and the alcoholic layer was examined for the appearance of pink color which indicates the presence of alkaloids. The respective color and precipitate formation was observed by mayer's reagent, hager's reagent, wagner's reagent and dragendroff's reagent. In case of Mayer's reagent, formation of white and cream color precipitate indicated the presence of alkaloids. By applying Hager's reagent, Formation of yellow crystalline precipitate indicated the presence of alkaloids. For, Wagner's reagent, Formation of brownish-black ppt indicated the presence of alkaloids. In case of Dragendroff's reagent, formation of orange or orange-red precipitate indicated the presence of alkaloids.

Test for saponins: 300 mg of extract was taken and boiled with 5ml of water for two minutes. The mixture was cooled and mixed vigorously and left for three minutes. The formation of frothing indicates the prescence of saponins. It was taken as preliminary evidence for the presence of saponins.

Test for flavonoids: A dilute ethanolic solution of the test sample (0.5ml) was added to 0.5ml of aqueous NaOH solution (5% NaOH) in a test tube. The development of yellow to orange color indicates the presence of flavonoids. Concentrated H_2SO_4 test- A dilute ethanolic solution of test sample was taken in a test tube. Again, 4-5 drop of concentrated sulfuric acid was added. Yellow to orange color indicates the presence of flavonoids. Beside this, the presence of flavonoids was determined using 1% aluminium chloride solution in ethanol, concentrated hydrochloric acid, and magnesium chloride solution. Immediate development of a red color indicated the presence of flavanoid.

Test for steroids: About 2mg of the extract was taken in a test tube and 2ml of chloroform was added. The test tube was shaken slowly to dissolve the extract. Concentrated H_2SO_4 was poured slowly by the side of the test tube so that it formed a separate layer at the bottom. Forming a purple colored ring at the junction of the two liquids indicates the presence of steroids.

Test for alcohol: A small amount of test sample was dissolved in 0.5ml of dioxane. The solution was added to 0.5ml of citric nitrate reagent diluted to 1ml with dioxane and shaken well. A yellow –red color indicates the presence of alcohol.

Test for phenol: 2 drops of neutral ferric chloride solution was added to 1ml of diluted aqueous solution of the test sample. A greenish purple color indicates the presence of phenolic compounds.

Test for substituted amide (-NHCOR): The compound was boiled with 6N HCl for 7-8minutes and cooled under tap water and then 10% $NaNO_2$ solution was added. This solution was transferred to a test tube containing alkaline β-naphthol solution. Orange red color indicates the presence of substituted amide (-NHCOR).

Antioxidant activity investigation: The free radical scavenging activities (antioxidant capacity) of the plant extracts on thestable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method Brand Williams*et al.* [8]. 2.0 ml of a methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20µg/ml). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract and compared to that of tert-butyl-1-hydroxytoluene (BHT) and ascorbic acid (ASA) by using UV spectrophotometer. Ascorbic acid (ASA) was considered as positive control. Calculated amount of ASA was dissolved in methanol so as to obtain a mother solution having a concentration 1000 µg/ml. Serial dilution was made using the mother solution to get different concentration ranging from 500.0 to 0.977 µg/ml. Calculated amount of different extractives were measured and dissolved in methanol to get the mother solution (Conc. 1000 µg/ml). Serial dilution of the mother solution gave different concentration ranging from 500.0 to 0.977µg/ml which was kept in the marked flasks. 2.0 ml of a methanol solution of the sample (extractives/ control) at different concentration (500µg/ml to 0.977µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20µg/ml). After 30 minutes of reaction period at room temperature and in dark place the absorbance was measured at 517 nm against methanol as blank by UV spetrophotometer. Inhibition of free radical DPPH in percent (*I%*) was calculated as follows:

$(I\%) = (1 - A_{sample}/A_{blank}) \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material).

Extract concentration having 50% inhibition (IC_{50}) was calculated from the graph which was plotted inhibition percentage against extract concentration.

Thrombolytic activity investigation: The in-vitro thrombolytic activity of the crude methanolic extract *Tagetes lucida* was determined according to the method reported earlier by Prasad., *et al*. Whole blood drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy was incubated for 45 minutes at 37°C to form clot. After clot formation, the serum was completely removed without disturbing the clot and the weight of the formed clot was taken. Addition of crude extracts having thrombolytic potential result in the lysis of some of the clot. For clot lysis, the eppendorf were incubated for 90 minutes at 37°C. Thereby the weight of the clot will decrease. Weight loss of clot after application of crude extract solution was considered as the functional indication of thrombolytic activity. Weight was taken before and after clot lysis which was expressed as percentage (%) of clot lysis as shown below:

% clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100

The thrombolytic potential of the crude extract is compared with streptokinase (standard thrombolytic agent).

Clot + Extract (thrombolytic potential)
→Lysis of clot →Weight of clot decreases

Astringent activity investigation: Two Eppendorf tubes were taken and in 1st tube 5% plant extract was added in 1ml methanol. In second tube, 5% extract was added in 1ml distilled water. 100µL of milk was added in each tube and homogenized. After 3 minutes it was centrifuged for 1 minute at 3000 rpm. Presence or absence of pellets was noted.

Pro-coagulant activity investigation: Four test tubes were taken and then 10% plant extract is added with 1ml methanol. Then those test tubes were place in water bath for 1-10 min.200µL of plasma & 200 µL (0.025M) $CaCl_2$ were added in each test tube and shake quickly. They were left inclined at an angle of 45° and time of caking formation was measured using stopwatch.

Results and Discussion

Phytochemical constituents screening: Several bioactive compounds were found in the methalonic extracts of *Tagetes lucida* when it was treated with

specific reagents.
Table 1 represent the constituents which are present and absent in the methalonic extract of *Tagetes lucida.*
So the plant may be the source of these phytochemical constituents. Such constituents may have medicinal values as well as importance and can be used as folk medicines.
Antioxidant properties: Methanolic extract of leaves of *Tagetes lcida* subjected to free radical scavenging activity by the method of Brand Williams*et al*. [8]. Here, ascorbic acid (AA) was used as reference standard which value is observed 2.48µg/ml in case of IC_{50} values in DPPH method of sample were 5.80 µg/ml. Table 2 and Figure 1 represents the overall test results.

Antioxidants present in plant help in radical mediated disorder and provide health promoting ingredients. Synthetic antioxidant like tert-butyl-1-hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) etc. are used as food additives but have known toxic as well as carcinogenic effect. This why, the plant derived antioxidants are getting preferences day by day.
Thrombolytic Activity: The percentage of weight (Wt) loss of clot after the application of crude methanolic *Tagetes lucida* extract solution was taken as the functional indication of thrombolytic activity. The study was implemented on human volunteer with 5ml blood sample and value of weight loss (in %) was calculated to examine with the following formula:
% clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100
$= [(W2 – W3) / (W2 – W1)] \times 100.$
Here, W1, W2, W3 are the Weight of empty Eppendorf, Wt of Eppendorf with clot, and Wt of Eppendorf after clot lysis respectively. The percentage clot lysis for volunteer is represented by in Figure 2
The overall result of thrombolytic activity of crude plant material in compared with standard and blank is given in the Table 3.
Formation of blood clot (thrombus) may be one of the major causes of blood circulation problem can lodge in a blood vessel and hamper the flow of blood in that location depriving tissues of typical blood flow and oxygen. This may bring about harm, devastation (dead tissue), or even demise of the confined tissues (necrosis) in that area. Thrombus is formed from fibrinogen by thrombin and is lysed by plasmin, which is enacted from plasminogen by tissue plasminogen activator (tPA). All thrombolytic agents activate the enzyme plasminogen that clears the cross linked fibrin mesh. Fibrinolytic drugs can dissolve thrombi in acutely occluded coronary arteries thereby can restore blood supply to ischemic myocardium and can limit necrosis. Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial in fraction. It reduces mortality as effectively as the non-antigenic alteplas. Tissue-type Plasminogen activator (t-PA) is generally considered as being suitable and safer than either urokinase or streptokinase like activators. Currently, available thrombolytic agents have so many harmful effects. For this, large dose is required compared to plant source thrombolytic agent. Thus the thrombolytic activity of the study will be helpful.
Astringent Property: After centrifugation the result was observed. Presence of pellets was noted from this extract of *Tagetes lucida*.
So, in this experiment we observed that, the leaves of *Tagetes lucida* showed astringent properties.
Causing the contraction of skin cells and other body tissues (as witch hazel does to the pores). Medical astringents are generally used to stop or slow bleeding and to help wounds heal. Examples of this include yarrow tincture and calamine lotion.
From this experiment, it can be concluded that the extract of *Tagetes lucida* showed astringent activity.
Pro-coagulant Property: It is now well established that platelets and clotting factors have several interactive roles in haemostasis and thrombosis. After forming cake in the test tubes the time was measured. The 1st two test tubes took 18.05 time & control testtubes took 20.42 time. Figure 3 represents the pro-coagulant activity.
Pro-coagulant platelets are predominantly localized at thrombus surface. Surface distribution of pro-coagulant platelets is a result of their contraction-driven extrusion from the inner layers of the thrombus. Such distribution of pro-coagulant platelets results in surface-enhanced generation of fibrin. Contraction of arterial thrombus is responsible for the mechanical extrusion of pro-coagulant platelets to its periphery, leading to

heterogeneous structure of thrombus exterior. From this experiment, we concluded that the leaves of *Tagetes lucida* shown pro-coagulant properties.

CONCLUSION

From the study, it was found that, *Tageres lucida* contains carbohydrates, alkaloids and flavonoids. On the other hand, it has antioxidant, thrombolytic, astringent and pro-coagulant properties. So, considering the potential bioactivity, this plant can be further studied and evaluated extensively to explore its unexplored efficacy and to rationalize its medicinal use.

References

1. Thomson GE. Further consideration of Asian Medicinal plants in treating common chronic disease in West. Journal of Medicinal Plants Research 2010; 4(2): 125. 2.
2. Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology 2005; 4: 685–688.
3. Baker W, van den Broek A, Camon E, Hingamp P, Sterk P, Stösser G, Tuli MA. The EMBL Nucleotide Sequence Database *Nucl Acid Res, 2000;* 28: 19-23.
4. Watson RD, Chin BS, Lip GY. Antithrombotic therapy in acute coronary syndromes. *B. Med. J.* 2002; 325: 1348-1351
5. Biousse and Newman NJ. Venous disease of the central nervous system. *Seminars in Cardiovascular Diseases Stroke* 2004; 4: 2-17
6. Baruah DB, Dash RN, Chaudhari MR, Kadam, S.S. Plasminogen activators: A due comparison. *Vascular Pharmacol.* 2006; 44: 1-9
7. Rosing J, van Rijn JLML, Bevers EM, van Dieijen G, Comfurius P, Zwaal RF. The role of activated human platelets in prothrombin and factor X activation. *Blood* 1985; 65: 319-32
8. Brand Williams. Handbook of stable isotope analytical techniques. 1995; 2: 217

Table 1. Phytochemical constituents in *Tagetes lucida.*

Constituents present	carbohydrate, alkaloids, flavonoids.
Constituents absent	saponin, gums, steroids, glycoside.

Table 2. IC_{50} values of the standard and sample of leaves of *Tagetes lucida*

Plant part	Test Sample	IC_{50} (µg /ml)
Leaves of *Tagetes lucida*	Blank	5.82
	Sample	5.80
	Ascorbic acid (Std.)	2.48

Table 3. Thrombolytic Activity (in terms of % of clot lysis) of the extractives of *Tagetes lucida*

Test tube	Weight of empty vial W_1 gm	Weight of vial with clot W_2 gm	Weight of clot W_3= W_2-W_1 gm	Weight of vial after clot lysis W_4 gm	Weight of lysis clot W_5 = W_2-W_4 gm	%of clot lysis= 100 × W_5/W_3
Sample	3.75	4.95	1.20	4.44	0.51	42.50
Blank	3.75	4.66	0.91	4.61	0.05	5.49
SK	3.85	4.75	0.90	4.18	0.57	63.33

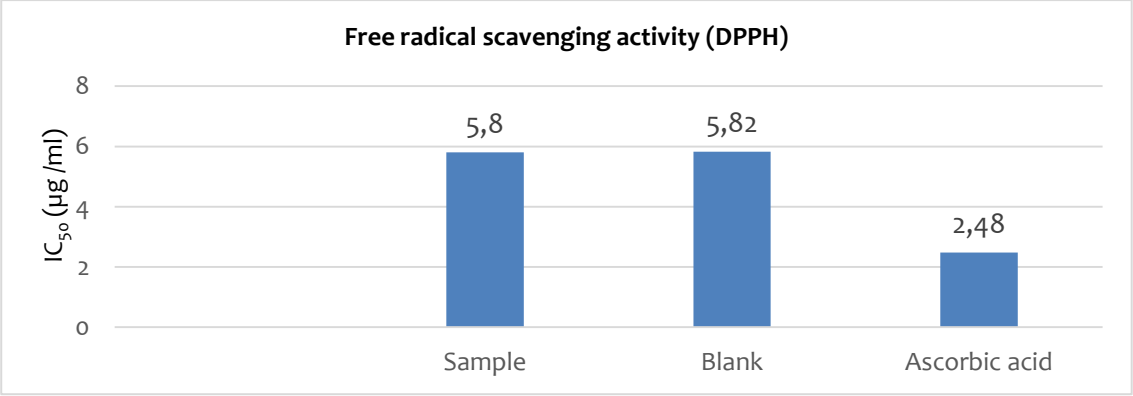

Figure 1. IC_{50} values of the standard and leaves of *Tagetes lucida*

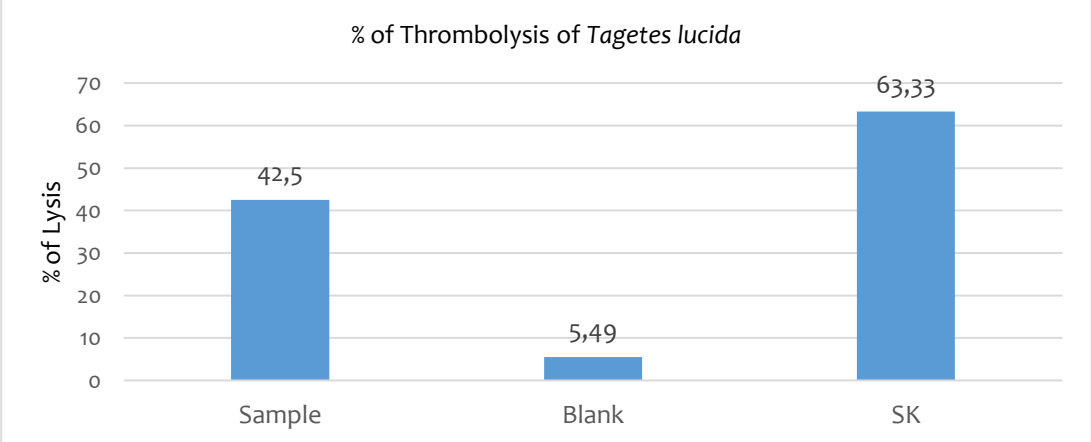

Figure 2. Thrombolytic activity of *Tagetes lucida*

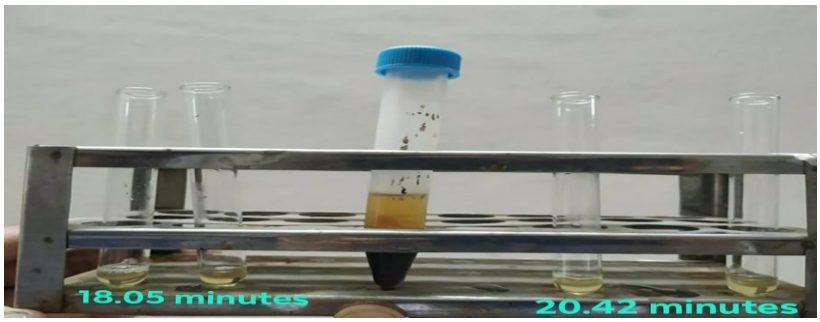

Figure 3. Pro-coagulant properties test