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Anticoagulant activity of partially purified coumarin(s) extracts of *Sonchus oleraceus*

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ABSTRACT

The present investigation was aimed to isolate and characterize coumarin from *Sonchus oleraceus*. By all the three standard tests presence of couamrin in the plants was confirmed. The same extract was used for anticoagulant assays. aPPT assay and PT assay were performed on citrated plasma of healthy volunteer donors with different concentration of coumarin extract. The coumarin extract prolonged/delayed the time taken for blood clotting in all the tested methods. The activity was increased with the concentration of extracts used. The coumarin extract can be used as anticoagulant and further in detail work is needed for the characterization of the active coumarin molecule.

Keywords: *Sonchus oleraceus,* coumarin, anticoagulant, aPPT, PT.

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INTRODUCTION

Haemostasis is the process that retains the blood within the vascular system during periods of injury. The coagulation mechanism may be thought of as a complex series of cascading reactions involving development of enzymes from their precursor (zymogens, procoagulants proenzymes). Most of the substances which are necessary for coagulation are present in an inert form and must be converted to an activated state. As one enzyme is formed it then becomes available to convert the next zymogen to its activated enzyme (serine protease). This process continues until a fibrin meshwork clot has formed. In addition to the zymogens, protein cofactors and membrane phospholipids surfaces, calcium ions play an active role in the final development of the fibrin clot (Dey and Bhaktha, 2012). Most adult cardiovascular disorders involving hypertension, cerebral hemorrhage, coronary thrombosis, arteriosclerosis and congestive heart failure are caused by problems in the blood circulatory system as blood clotting disorders which constitute a serious medical problem.

A number of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) such as aspirin and indomethacin have been used as antithrombotic agents. These drugs *in vitro* and *in vivo* cause inhibition of platelet aggregation and thromboxane formation (Dey and Bhaktha, 2012).

Anticoagulant drugs include; heparin and derivative substances e.g. low molecular weight heparin and coumarins (vitamin K antagonists) such as Warfarin. Warfarin acts via inhibition an enzyme vitamin K epoxide reductase, which recycles oxidized vitamin K to its reduced form after it has participated in the carboxylation of several blood coagulation proteins, mainly prothrombin and factor VII. Reduced vitamin K must be regenerated from the epoxide for sustained carboxylation and synthesis of biologically competent proteins (Thumber et al., 2011).

Determination of Activated Partial Thromboplastin Time (APTT) helps in estimating abnormality in most of the clotting factors of the intrinsic pathway including congenital deficiency of factor VIII, IX, XI and XII. The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of a series of plasma proteins in a highly ordered and

complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues. Activated Partial Thromboplastin Time is prolonged by a deficiency of coagulation factors of the intrinsic pathway of the human coagulation mechanism such as factor XII, XI, IX, VIII, X, V, II and fibrinogen.

The prothrombin time test (also known as the pro test or PT test) is a useful screening procedure for the extrinsic coagulation mechanism including the common pathway. It detects deficiencies in factor II, V, VII and X. The prothrombin time test is frequently used to follow oral anticoagulant therapy that inhibit factors II, VII, IX and X. Thromboplastin activates the extrinsic coagulation system in plasma in the presence of calcium ions. The subsequent clotting time is dependent on the concentration of factors II, V, VII and X. Thus prolongation indicates a deficiency in one or more of these factors (Quick, 1966, 1970).

The normal prothrombin time is 11 to 15 s. Each prothrombin time within this range indicates that the person has normal amounts of clotting factors VII and X while prolongation in prothrombin time is considered abnormal (Dey and Bhaktha, 2012).

The cost of developing a novel anticoagulant is also given due consideration, whereby a cheaper yet effective alternative would be of immense welcome (Spyropoulos, 2008). In addition, there is a pressing need for an orally available anticoagulant agent to replace warfarin (Franchini and Mannucci, 2009). At present, lavish focus is being given to potent anticoagulant sources hailing from natural origin. There is compelling scientific evidences demonstrating that the consumption of dietary anticoagulants or phytochemicals with anti-coagulant properties can ultimately reduce or eliminate the risks of thromboembolic diseases (Matsubara et al., 2001). New discoveries in medical science are reaffirming much of the old herbal lores in order to extend the horizons of botanical medicine. Hence, anticoagulants of natural origin furnish a good source for the aforementioned disorders (Wang and Ng, 1999). We found in this preliminary study that one such potential candidate for anticoagulant agent is *S. oleraceus* (common name sowthistle) that is quite common in India. This plant is a winter to spring annual adaptable and is found in full or partial sun, moist to slightly dry sites and fertile soil, so it is a weed. The seeds are used for medicine and young leaves are edible. In this work, we employed plant *S. oleraceus* for identification of coumrin and its series of functional clot-based *in vitro* screening and confirmatory assays to validate its anticoagulant activity.

MATERIALS AND METHODS

Preparation of crude extracts

Plant, *S. oleraceus* was collected locally from Tumkur district (Karnataka), India in April 2014. The shade dried plant was ground into fine powder and the total mass was subjected to extraction by microwave assisted extraction method (Martino et al., 2006) with water, ethanol and methanol in a closed-vessel system (GMS 17M 07 WHGX SOLO Microwave). Two grams of powdered samples were subjected to extraction in a vessel with of solvent up to the volume of 2 ml. Microwave assisted extraction method is followed for the extraction process using three solvents at different temperature for 5 min followed by one or two cycle extraction method at different temperature. The resulting extracts were kept in refrigeration for further studies.

Phytochemical analysis

Phytochemical analysis for major phytoconstituents of the plant extract was undertaken using standard qualitative methods as described by various authors. The plants extracts were screened for the presence of biologically active compounds like as steroids, terpenoids, alkaloids, flavonoids, coumarins, saponins, tannins, phenols, catechin, anthraquinone and quinine (Obdoni and Ochuko, 2001). The following qualitative tests were carried out as follows:

Identification of coumarin in extracts

Test 1: To the concentrated alcoholic extract of drugs few drops of alcoholic ferric chloride ($FeCl_3$) solution was added. Formation of deep green color, which turned yellow on addition of conc. nitric acid (HNO_3), indicates presence of coumarins.

Test 2: The alcoholic extract of plant was mixed with 1N sodium hydroxide (NaOH) solution (one ml each). Development of blue-green fluorescence indicates presence of coumarins.

Test 3: 3 ml of methanol extract was evaporated to dryness in a vessel and the residue was dissolved in hot distilled water. It was then cooled and divided into two test portions, one was reference, second was the test. To second test tube, 0.5 ml of 10% ammonium hydroxide (NH_4OH) was added. The occurrence of intense/fluorescence under UV light is a positive test for the presence of coumarins and derivatives. The experiment was carried out for all tests in three replicates (Jagessar and Cox, 2010).

Anti coagulant assay

Preparation of plasma

Blood samples were collected from healthy volunteers, using a disposable polypropylene syringe and then anti-coagulated using 3.8% tri-sodium citrate in a polypropylene container (9 parts of blood to 1 part of tri-sodium citrate solution). It was immediately centrifuged at 4000 × g for 15 min and plasma was separated and pooled. The freshly prepared plasma was stored at 4°C until its use (Premanathan et al., 1999).

APTT assay (effect of coumarin on intrinsic pathway)

In a test tube 0.1 ml test plasma and 0.1 ml Liquiceilin-E (a phospholipid preparation derived from rabbit brain with ellagic acid as an activator; Tulip Diagnostics Pvt Ltd., India) were added and shaken briefly to mix the reagent and plasma. The tube was placed at 37°C for 20 min for incubation. After the incubation, 0.1 ml pre-warmed calcium chloride solution was forcibly added into the mixture of plasma and reagent. To this, one ml of purified coumarin extract was added separately in different concentrations and kept at 37°C. A stopwatch was started to record the coagulation time in seconds. The tube was shaken to mix the contents and it was

Table 1. Phytochemical analysis of three solvent extracts of *Sonchus oleraceus.*

Samples	Phytochemical analysis					
	Steroids	Flavonoids	Anthraquinone	catechin	Alkaloids	Phenol
Ethanol extracts						
1E	+	+	-	-	+	+
2E	+	+	-	-	+	+
3E	+	+	-	-	+	+
Methanol extracts						
1M	+	+	-	-	+	+
2M	+	+	-	-	+	+
3M	+	+	-	-	+	+
Water extracts						
1W	+	-	-	-	+	+
2W	+	-	-	-	+	-
3W	+	-	-	-	+	-

Repeated each experiment thrice, 1: one cycle at 50°C, 2: two cycle at 50°C, 3: one cycle at 70°C, +: present, - : absent, M: Methanol, E: Ethanol, W: Water.

started to record the coagulation time in seconds. The tube was shaken to mix the contents and it was tilted gently back and forth and the stopwatch was stopped as soon as the clot formation began (Shanmugam and Mody, 2000). The activity is expressed in term of clotting time ratio in relation to control. The steps were repeated three times for each sample and average of the test value was noted. Normal saline was used in place of the extracts for the negative control and 10 mg/ml of EDTA for the positive control.

PT assay (effect of coumarin on extrinsic pathway)

In a test tube 0.1 ml of plasma was added and the test tube was placed in a water bath for 3 to 5 min at 37°C. Then, 0.2 ml of Liquiplastin reagent (Tulip Diagnostic Pvt Ltd, India) pre-warmed at 37°C was added. To this mixture, one ml of purified coumarin was added separately in different concentrations and kept at 37°C. A stopwatch was started to record the coagulation time in seconds. The tube was shaken to mix the contents and it was started to record the coagulation time in seconds. The tube was shaken to mix the contents and it was tilted gently back and forth and the stopwatch was stopped as soon as the clot formation began. The activity is expressed in term of clotting time ratio in relation to control. The steps were repeated three times for each sample and average of the test value was noted. Normal saline was used in place of the extracts for the negative control and 10 mg/ml of EDTA for the positive control.

Table 2. Identification of coumarin compound in solvent extract of *S. oleraceus.*

Samples	Identification of coumarin compound		
	Test 1	Test 2	Test 3
Ethanol extract			
1E	-	-	-
2E	-	-	-
3E	-	-	-
Methanol extract			
1M	-	-	-
2M	+	+	+
3M	+	+	+
Water extract			
1W	-	-	-
2W	-	-	-
3W	-	-	-

Repeated each experiment thrice, 1: one cycle at 50°C, 2: two cycle at 50°C, 3: one cycle at 70°C, +: present, - : absent, M: Methanol, E: Ethanol, W: Water.

RESULTS

Extraction was performed by the microwave assisted extraction method using three solvents (ethanol, methanol, water) followed by one or two cycle extraction at different temperatures.

Table 1 shows the phytochemical analysis of the methanol, ethanolic and aqueous extracts. It was performed in order to know the presence of different phytoconstituents such as alkaloids, flavonoids, saponins, tannins, terponoids, phenol, quinine etc. Results obtained showed the presence of steroid and alkaloids in all six extracts and flavonoids in methanolic and ethanolic extracts and it was absent in aqueous extracts. Anthraquinone and catechin were not found in all six extracts. Phenolic compound was present in the ethanolic and methanolic extracts and it was absent in aqueous extracts.

Table 2 shows the presence of the coumarin in

Table 3. APTT assay for M2 extracts (effect of coumarin on intrinsic pathway).

Samples	Concentration (µg ml^{-1})	Clotting time in sec
M2	10	90
	20	120
	30	150
	40	220
+ve control (EDTA)	10	3000
-ve control (without any sample/ EDTA)	-	45

Repeated the each experiment thrice, 2: two cycles at 50°C, M – Methanol.

Table 4. APTT assay for M3 extracts (effect of coumarin on intrinsic pathway).

Samples	Concentration (µg ml^{-1})	Clotting time in sec
M3	10	60
	20	64
	30	71
	40	90
+ve control (EDTA)	10	3000
-ve control (without any sample/ EDTA)	-	45

Repeated the each experiment thrice, 3: one cycle at 70°C, M: Methanol.

Table 5. PT assay (effect of coumarin on extrinsic pathway).

Samples	Concentration (mg ml^{-1})	Clotting time in sec
M2	10	40
	20	105
	30	120
	40	180
+ve control (EDTA)	10	2400
-ve control (without any sample/ EDTA)	-	15

Repeated the each experiment thrice, 2: two cycles at 50°C, M: Methanol .

methanolic extracts in all three tests. Presence of coumarin is confirmed in two cycled methanolic extracts.

Anticoagulant activity at four different concentrations of methanolic extracts was tested in blood plasma using two assay system and the results were tabulated.

In APTT assay, the activity was maximal in the M2 extracts. M2 extracts exhibited greater potency with prolonged clotting time 90, 120, 150 and 220 s at 10µg ml^{-1}, 20 µg ml^{-1}, 30 µg ml^{-1} and 40 µg ml^{-1} concentrations respectively. A similar trend of effect was observed with M3 extracts, which exhibited 60, 64, 71 and 90 s at 10 µg ml^{-1}, 20 µg ml^{-1}, 30 µg ml^{-1} and 40 µg ml^{-1} concentrations respectively (Table 3 and 4).

In PT assay, M2 extracts showed the clotting time of 40, 105, 120 and 180 sec at 10 µg ml^{-1}, 20 µg ml^{-1}, 30 µg ml^{-1}and 40 µg ml^{-1}. And for M3 extracts showed the clotting time of 30, 50, 60 and 80 s respectively in 10 µg ml^{-1}, 20 µg ml^{-1}, 30 µg ml^{-1} and 40 µg ml^{-1} (Table 5 and 6).

These assays prove the anticoagulant property of coumarin extracted from the *S. oleraceus.* M2 extracts showed higher blood clotting time compare to the M3 extracts but slightly lower than the EDTA.

DISCUSSION

Different solvents yielded different phytochemicals. The steroids, alkaloids were yielded in all the solvent extracts. Anthraquinone and catechin were absent in all the solvent extracts.

The fractions (2M and 3M) methanol extracts exhibited

Table 6. PT assay (effect of coumarin on extrinsic pathway).

Samples	Concentration (µg ml^{-1})	Clotting time in sec
M3	10	30
	20	50
	30	60
	40	80
+ve control	10	2400
-ve control (without any sample/ EDTA)	-	15

Repeated the each experiment thrice, 3: one cycle at 70°C, M: Methanol .

the presence of coumarin in all the 3 tested methods. M2 fractions of methanol extract showed highest blood clotting time compared to other fractions and also other solvent extracts in APPT and PT assay. The intrinsic and extrinsic anticoagulant activity may be due to presence of coumarin, steroids and alkaloids. The coumarins are strong anticoagulant molecules reported by Rosselli et al. (2007) and Gazard et al. (2005). Steroids (Derbyshire et al., 2006; Dey and Bhakfs, 2012) and alkaloids (Mahajan and More, 2012; Kashani, 2012) also possessing anticoagulant properties. Further, the present research will be helpful to identify anticoagulant potent molecules and then biological characterization to produce new anticoagulant drug.

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