ISSN: APPLIED
2018, Volume – 1; Issue – 1;

IN VITRO MILK CLOTTING AND ANTICOAGULANT PROPERTIES OF THE LATEX OF *JATROPHA GOSSYPIFOLIA L*.

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ABSTRACT

The present study was carried out aiming to evaluate the milk clotting and anticoagulant activities of the latex of *J. gossypiifolia*. The 500 mg concentration of the *Jatropha* latex strongly coagulated skimmed milk solution within four minutes (which corresponds to a specific clotting activity of 42.6 U/mg, protein content of 3.8 mg), forming a white and firm curd. Among the three concentrations of enzyme used, 250 µg concentrations showed maximum anti-coagulant activity of (20:36mins) in 250 µg enzyme concentrations. Hence, the presented results showed that *J. gossypiifolia* latex, have significant beneficial effects as an anticoagulant agent for treating deep vein thrombosis, pulmonary embolism, myocardial infarction and stroke.

Key words: *Jatropha gossypifolia*, Protease, milk clotting and anticoagulant activity.

INTRODUCTION

The use of plants with medicinal purposes for the prevention and/or treatment of diseases are one of the most ancient forms of primary health care (Calixto, 2000; Newman and Cragg, 2012). Plants produce several secondary metabolites that present many important biological activities. Anticoagulant and antioxidant activities could be highlighted amongst these.

Anticoagulants are chemical agents that inhibit blood clotting.1 They are categorized broadly as; agents that inhibit clotting factors in the intrinsic pathway through enhancing the effect of antithrombin III (AT III) (e.g., unfractionated heparin and low molecular weight heparin), agents that exert their anticoagulation effect by inhibiting Vitamin K reductase which invariably prevents the gamma-carboxylation of glutamic acid residue of

factors II, VII, IX, and X (e.g., warfarin), agents that inhibit specific clotting factors (e.g., rivaroxaban - factor Xa inhibitor, or dabigatran - thrombin inhibitor), and agents that prevent platelet activation and aggregation thereby preventing primary hemostasis (e.g., aspirin, ticlopidine, tirofiban, and clopidogrel).2 Anticoagulants have found use clinically in the management of cardiovascular disorders.

While, the currently established anticoagulants such as, warfarin, dabigatran, unfractionated heparin (UFH), enoxaparin, fondaparinux, bivalirudin (thrombin inhibitors), low molecular weight heparin and antiplatelet agents such as, aspirin, thienopyridines, dipyridamole, dlopidogrel, dpoprostenol, abciximab, eptifibatide and tirofiban (glycoprotein IIb/IIIa inhibitors) are having numerous limitations with several side effects, including lack of reversibility, a sheer dose response, food and multiple drug-drug interactions, narrow therapeutic index, internal bleeding, birth defects and miscarriage (Rosendaal et al., 1993; Hurlen et al., 2002; Antman and Van de Werf. 2004) Therefore, identifying the novel anticoagulant and ant platelet agents from the natural sources with least side effects is the challenging task to the researchers.

Jatropha gossypiifolia L. is a medicinal plant belonging to Euphorbiaceae popularly known in Brazil as “pinhão-roxo” or worldwide as “bellyache-bush”. Several human and veterinary uses in traditional medicine are described for different parts (leaves, stems, roots, seeds and latex) and preparations (infusion, decoction, maceration, among others) based on this plant, by oral or topical use. The most frequent reports regards to its antihypertensive, anti-inflammatory, antiophidian, analgesic, antipyretic, antimicrobial, healing, haemostatic, anti-anemic, antidiabetic, anti-hemorrhagic, among many other examples (Mariz et al., 2010; Albuquerque et al., 2007; Sabandar et al., 2013). Regarding its phytochemical constitution, alkaloids, coumarins, flavonoids, lignoids, phenols, saponnins, steroids, tannins and terpenoids were already detected in different extracts from different parts of this plant (Zhang et al., 2009). The present study, therefore, examined the anticoagulant and antiplatelet properties of the latex of *C. papaya* by *in vitro* experimental protocols.

MATERIALS AND METHODS

The stem of growing *Jatropha gossypifolia* plants was cut and latex was collected into glass tubes containing 1 ml of 10 % sodium metabisulphite by incision of the bark of the trunk and branches of the plant. The crude latex was strained through cotton wool to remove suspended coarse inert materials and then centrifuged at 7000 rpm and 4°C for 30 mins. The supernatant was collected and used as the crude enzyme extract.

The crude enzyme solution was precipitated by ammonium sulphate (40 - 60% w/v). The solution was kept overnight in cold condition (2 – 8 °C) and then centrifuged (7000 rpm, 30 min, 4 ºC). The precipitate was dialysed in 0.02M phosphate buffer (pH 7.0).

Milk clotting activity of the crude enzyme from all plant latex was performed by the method of Arima *et al.*,1970. The substrate(10% w/v of skim milk in 0.01M calcium chloride) at pH 6 was pre incubated at 37ºC for 5 minutes. To 1000 µg of substrate added 250, 500 and 1000 µg of the crude enzyme, individually and curd formation was observed by manually rotating the test tubes time to time. The time taken for the visible discrete particles to form was noted**.** One clot unit was defined as the amount of enzyme that clots 10ml of the substrate within 40 minutes.

MCA (U/ml) = (2400/clotting time in sec) ×dilution factor. Specific milk clotting activity was calculated as milk clotting activity per milligram of protein.

Protein concentration in the enzyme extract was determined using Folin Ciocalteu reagent as per the procedure of Lowry *et al*., 1951, Crystalline Bovine Serum Albumin used as standard protein for preparation of standard curve. The absorption of the blue color developed was measured at 660 nm using spectrophotometer.

The blood samples were obtained from normal individuals by using sterile syringes, withdrawn from vein of right arm of each individual and placed separately in containers containing tri-sodium citrate to prevent the clotting process. Centrifugation (15 minutes at rate 3000 rpm) was carried out to separate the blood cells from plasma in order to obtain pure platelet plasma (ppp) for prothrombin time test. The obtained plasma sample of each individual were

poured separately in plane containers using automatic pipette and stored at room temperature (Biggs and McFarlane, 1962; Hull et al., 1982).

0.2 ml plasma, 0.1 ml of crude extract of different concentration (125, 250 and 500 µg) and $CaCl_2$ (25 *m*M) were added together in a clean fusion tube and incubated at 37^0C in water bath. For control experiment extract solution was replaced by same volume of 0.9% saline water. The clotting time was recorded with stopwatch by tilting the test tubes every 5 seconds. This time is called the prothrombin time (BuLoeliger et al., 1985; Colman et al., 1994).

RESULT AND DISCUSSION

The results of protease purification from Latex of *Jatropha gossypiifolia* L. are summarized in following Table 1. After fixed fermentation time period, the fermented broth was centrifuged at 7000 rpm and 4°C for 30 mins to obtain a cell free extract containing crude enzyme. The initial activity of culture supernatant was 209. Among all the used concentration of ammonium sulphate the 60% w/v final concentration showed a good result for fractionation of enzyme. The optimum ammonium sulphate precipitation at 60% w/v final concentration showed 2.1 fold increases in specific activity (3.5 U/mg) compared to the crude enzyme supernatants and the 0.7 fold increases its specific activity when compared with 60% ammonium sulphate fractionation. According to Sadia *et al*., 2009 the fractionation of protease by ammonium sulphate was carried out at 60% recovered 3.6 fold purification of protease.

Table 1: Purification of protease from Latex of *Jatropha gossypiifolia* L

Purification	Activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude enzyme	2302	209	1.7	100	1
Ammonium Sulphate	748	75	3.5	76	3.8
Dialysis	1400	120	4.7	81.4	3.7

The 500 mg concentration of the *Jatropha* latex strongly coagulated skimmed milk solution within four minutes (which corresponds to a specific clotting activity of 42.6 U/mg, protein content of 3.8 mg), forming a white and firm curd (Table 2). A closely related species to *C. gigantea*, *C. procera* has been extensively explored for its milk-clotting efficiency. Several studies on vegetable milk coagulants have reported high MCA associated with latex of this *Calotropis* species (Aworh, 1986; Oseni and Ekperigin, 2013).

Sample (Conc)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
250 µg	88	1.9	38.8
500 µg	162	3.8	42.6
100 µg	148	2.1	40.1

Table 2: Milk clotting activity of latex of *Jatropha gossypiifolia* L.

A growing fascination for natural anticoagulants discoveries stemming from the overwhelming consumer response seeking remedies devoid of unfavorable side effects has prompted the execution of this study (de Medeiros et al., 2000; Trento et al., 2001; Low, 2008).

Among the three concentrations of enzyme used, 250 µg concentrations showed maximum anti-coagulant activity of (20:36mins) in 250 µg enzyme concentrations and a minimum of (15:36mins) in 125 µg (Table 3).

Table 3: Anticoagulant activity of Latex of *Jatropha gossypiifolia* L.

Sample (Conc)	Amount of plasma	Amount of extract	$CaCl_2$ Solution	Time of coagulation
Control	0.2 ml	0.1 ml	0.3 ml	75 sec
125 µg	0.2 ml	0.1 ml	0.3 ml	15:36 mins
250 µg	0.2 ml	0.1 ml	0.3 ml	20:43 mins
500 µg	0.2 ml	0.1 ml	0.3 ml	16:25 mins

Many researchers study the anticoagulant activity of some plant such as Manicam *et al*., 2010 studied the anticoagulant activity of *Melastoma malabathricum*, their study revealed the prolonged the coagulation time. Several plant extracts were found to exhibit antithrombotic and/or anticoagulant activity *Sutherlandia frutescens* leaf extract, *Gloriosa superba* and *Zantedeschia aethiopica* leaf extracts and *Leonotis leonurus* root extract displayed anticoagulant properties (Kee *et al*., 2008)

In conclusion, the presented results showed that *J. gossypiifolia* latex, have significant beneficial effects as an anticoagulant agent. Anticoagulants reduce blood clotting and also prevent deep vein thrombosis, pulmonary embolism, myocardial infarction and stroke. These extracts showed significant anti-coagulant activity based on the concentrations dependent manner. This information will be helpful to create interest towards the plant and may be useful in developing new formulations, which are more effective and having more therapeutic value.

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