

Full Length Research Paper

***In vitro* anti-arthritic and thrombolytic activities of methanolic extract of *Protium serratum* leaves**

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The present study was engineered to find out the anti-arthritic and thrombolytic activity of methanolic extract of *Protium serratum* by using *in vitro* method. Methanolic extract of *P. serratum* was assessed with the inhibition of protein denaturation model which was used to evaluate anti-arthritic potential and this extract assessed with human blood to evaluate thrombolytic effect. The extract showed remarkable anti-arthritic activity which was evaluated by the percentages of inhibition of protein denaturation in different concentration of plant extract compared to diclofenac sodium, a reference drug. The maximum percentage inhibition of protein denaturation was observed as 83.94% at 1000 µg/ml. It has significant thrombolytic effect compared to streptokinase. During assay for thrombolytic activity, the methanolic extract of *P. serratum* revealed $59.653 \pm 8.626\%$ lysis of clot, while standard streptokinase (SK) and water used as positive and negative controls, demonstrated 72.835 ± 5.702 and $2.725 \pm 0.983\%$ lysis of clot, respectively. These findings demonstrate that the leaves extract of *P. serratum* have excellent anti-arthritic and thrombolytic effect.

Key words: *Protium serratum*, anti-arthritic, thrombolytic, % lysis of clot, protein denaturation.

INTRODUCTION

Arthritis is an auto immune disorder characterized by pain, swelling and stiffness. Its prevalence depends upon age. It occurs more frequently in women than in men. It is an inflammation of synovial joint due to immune mediated response. All anti inflammatory drugs are not anti arthritic because it does not suppress T-cell and B-cell mediated response. Rheumatoid arthritis (RA) is an autoimmune disorder characterized by synovial proliferation, inflammation, subsequent destruction like deformity of joints or

destruction of cartilage and bone (Firestein, 2003). RA is the most common inflammatory joint disease in humans and has long been classified among the autoimmune diseases in which skeletal complications start with focal erosion of cartilage followed by marginal and subchondral bone loss. Extended joint destruction with ankylosis and generalized bone loss are characteristic for late complications (Feldmann et al., 1996). These long-term skeletal complications have serious consequences as they can lead

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not only to painful joint deformities but also to progressive functional disability and increased mortality rates (Pincus et al., 1993).

The production of auto antigens in certain arthritic diseases may be due to *in vivo* denaturation of proteins (Brown et al., 1968). The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Grant et al., 1970). So, by controlling the production of auto antigen and inhibiting denaturation of protein and membrane lysis in rheumatic disease leads to anti-arthritic activity. Hence, inhibition of protein denaturation and membrane lysis were taken as a measure of the *in vitro* anti-arthritic activity (Volluri et al., 2011).

Formation of blood clots is one of the vital reasons of blood circulation problem. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area (Thrombus, 2011). A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin which is activated from plasminogen by tissue plasminogen activator (tPA). Fibrinolytic drugs has been used to dissolve thrombi in acutely occluded coronary arteries there by restoring blood supply to ischemic myocardium to limit necrosis and to improve prognosis (Laurence, 1992).

Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic alteplase in most infarct patients while having the advantages of being much less expensive. Tissue-type plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs (Nicolini et al., 1992; Adams et al., 1991; Lijnen et al., 1991; Marder, 1993; Wu et al., 2001).

The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and protective/disease preventive properties (phytochemicals). Nearly 50% of drugs used in medicine are of plant origin and only a small fraction of plants with medicinal activity has been assayed. Therefore much current research have been devoted to the phytochemical investigation of higher plants which have ethno-botanical information associated with them. The phytochemicals isolated are then screened for different types of biological activity like thrombolytic potentials (Harborne, 1998). Herbal preparations are used potential source of medicine since ancient times to maintain health and regain healthy state of mind. Herbs showing thrombolytic activity have been studied and some significant observations

have been reported (Basta et al., 2004).

Protium serratum (Syn. *Bursera serrata*) belonging to the family Burseraceae is an evergreen resinous small sized tree, distributed mostly in hilly areas, deciduous and semi-evergreen forests in Bangladesh, Native India and the Philippines. It yields aromatic oil and edible fruits (Huq et al., 1987). *Protium* species showed anti-inflammatory (Carretero et al., 2008), anti-tumor (McDoniel et al., 1972) and agglutinating and immobilizing activities (Huacuja et al., 1990). Terpenoids and coumarin were analyzed by Ara et al. (2009). Since there is no scientific report for anti-arthritic and thrombolytic potential of *P. serratum* leaf extract, the present study was an attempt to evaluate the anti-arthritic and thrombolytic effect by *in vitro* analysis.

MATERIALS AND METHODS

Plant

The leaves of *P. serratum* were collected from Bandarban district of Chittagong hill tracts region of Bangladesh in 2013 and authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

Extracts preparation

The collected plant was washed thoroughly with water and air dried for a week at 35 to 40°C and pulverized in electric grinder. The obtained powder was successively extracted in methanol (55 to 60°C). The extracts were made to dry by using rotary evaporator under reduced pressure.

In vitro test analysis

In vitro anti-arthritic activity

For the evaluation *in vitro* anti-arthritic activity of *P. serratum*, the method used was "inhibition of protein denaturation" (Mishra et al., 2011; Lavanya et al., 2010; Singh et al., 2011; Chippada et al., 2011) using diclofenac sodium a standard. The test solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of test solution (methanolic extract of *P. serratum*). The test control solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control (0.5 ml) consists of 0.45 ml of distilled water and 0.05 ml of test solution. Standard solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of diclofenac sodium. Various concentrations (62.5, 125, 250, 500, 1000 µg/ml) of methanolic extract of *P. serratum* (MEPS) and diclofenac sodium (standard) were taken, respectively. All the solutions were adjusted to pH 6.3 using 1 N HCl. The samples were incubated at 37°C for 20 min and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, 2.5 ml of phosphate buffer was add to the previous solutions. The absorbance was measured using UV-Visible spectrophotometer at 416 nm. The control represents 100% protein denaturation. The results were compared with diclofenac sodium. The percentage inhibition of protein denaturation of different concentrations is tabulated in Table 1. The percentage inhibition of protein denaturation can be calculated as:

Table 1. Percentage inhibition of protein denaturation.

| Concentration ($\mu\text{g/ml}$) | Percentage inhibition in protein denaturation | |
|------------------------------------|---|-------------------|
| | MEPS (Test solution) | Diclofenac sodium |
| 62.5 | 45.21 | 68.05 |
| 125 | 54.29 | 72.65 |
| 250 | 62.24 | 79.21 |
| 500 | 71.09 | 85.04 |
| 1000 | 83.05 | 92.94 |

Table 2. Effect of herbal extracts on *in vitro* clot lysis.

| Extracts/Drugs | Mean \pm SD (% of clot lysis) |
|---|---------------------------------|
| Water (Negative Control) | 2.725 \pm 0.983% |
| Streptokinase (Positive Control) | 72.835 \pm 5.702%* |
| Methanolic extract of <i>P. serratum</i> (L.) | 59.653 \pm 8.626%* |

Statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) done by paired t-test analysis; % clot lysis is represented as mean \pm S.D. and * $p < 0.0001$, significant compared to control.

$$\% \text{ of Inhibition} = \left[\frac{100 - (\text{OD of test solution} - \text{OD of product control})}{\text{OD of product control}} \right] \times 100$$

Where OD = optical density

The control represents 100% protein denaturation. The results were compared with diclofenac sodium.

***In-vitro* thrombolytic test**

The thrombolytic activity of this extractive was evaluated by the *in vitro* thrombolytic test (Prasad et al., 2006) using streptokinase as standard. The dry crude extract (10 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 min. 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 micro-centrifuge 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 min 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. % of clot lysis = (weight of released clot / clot weight) \times 100

Statistical analysis

The significance between % clot lysis by herbal extract by means of weight difference was tested by the paired t-test analysis. Data are expressed as mean \pm standard deviation.

RESULTS

Different concentrations of methanolic extract of *P. serratum* and diclofenac sodium were tested for anti-arthritic activity and found significant percentage inhibition in protein denaturation (Table 1). The methanolic extract of *P. serratum* (MEPS) also showed significant thrombolytic activity with 59.653 \pm 8.626% (Table 2) lysis of clot. The positive control (streptokinase) showed 72.835 \pm 5.702% and negative non thrombolytic control (distilled water) showed 2.725 \pm 0.983% lysis of clot.

DISCUSSION

In *in vitro* protein denaturation test, the MEPS have shown significant activity at various concentrations and its effect was compared with the standard drug diclofenac sodium. The maximum percentage inhibition of protein denaturation was observed as 83.94% at 1000 $\mu\text{g/ml}$ which was close to the percentage of inhibition of diclofenac sodium (92.94%) as shown in Table 1. In *in vitro* thrombolytic test, *P. serratum* possesses good thrombolytic activity in comparison with streptokinase. So that, we may assume that these extracts can be considered as a potential source of natural anti-arthritic as well as thrombolytic agent. In context of the discussion, it would be interesting to investigate the causative components/mechanism for clot lysis by these plant extracts and for protein denaturation activity. This is only a preliminary study and to make final comment, the extract should thoroughly be investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

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Conflict of Interest

The authors have declared that there no conflict of competing interest.

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