ISSN 1996-3351

Asian Journal of Biological Sciences

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OPEN ACCESS Asian Journal of Biological Sciences

ISSN 1996-3351
DOI: 10.3923/ajbs.2019.36.41

Research Article
Therapeutic Potentiality of *Diospyros blancoi* Linn. Seeds Against Pain, Thrombus and Inflammation: An *in vivo* and *in vitro* Study

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Abstract

Background and Objective: Although some investigations have previously been reported with this plant, the seed of the plant was not studied yet to date. This present study aimed to investigate *in vivo* and *in vitro* bioactivities of *Diospyros blancoi*. Cold methanolic extract of *D. blancoi* has been considered for evaluation of therapeutic activities such as analgesic, thrombolytic activity and anti-inflammatory. **Materials and Methods:** Analgesic activity was tested by formalin induced licking and biting in mice, thrombolytic activity was carried out by using human blood sample and the anti-inflammatory effect was tested by carrageenan induced paw edema method. The seeds of *D. blancoi* was extracted with methanol. **Results:** The extracts were used for the observation of analgesic activity, thrombolytic activity and anti-inflammatory activity. The *D. blancoi* had showed dose dependent significant analgesic activity in swiss albino mice in formalin induced method. They produced a protection of 55.67 and 73.47% from extract 100, 200 mg kg^{-1}, respectively. In thrombolytic activity the extract shown 38.17% of clot lysis from 10 mg mL^{-1}, in anti-inflammatory test they produced an inhibition of 24.81 and 27.23% at last hour. from extract (200 and 400 mg kg^{-1}), respectively. **Conclusion:** The results demonstrated that the methanol extract of *D. blancoi* seed had the moderate analgesic, anti- inflammatory and thrombolytic properties.

Key words: *D. blancoi*, analgesic, thrombolytic, anti-Inflammatory, dose-dependent, swiss albino mice

Citation: Md. Ibrahim, Md. Saddam Hussain, Farhana Sultana, Nazmul Abhi, Tanvir Ahmed, S.M. Naim Uddin, Atikur Rahaman, Maria Azmerin, Tutun Das Aka, Sakib Mosharraf and A.H.M. Mazbah Uddin, 2019. Therapeutic potentiality of *Diospyros blancoi* Linn. seeds against pain, thrombus and inflammation: An *in vivo* and *in vitro* study. Asian J. Biol. Sci., 12: 36-41.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

At present most of the people depends on the herbal medicine due to its fewer side effects, easily accessible for all kind of people and like other established medicine it does not produce any drug resistance[1]. Pain produces a discomfort feeling. Variety of physical, psychological, biological states produces painful appreciation[2]. On the other hand inflammation is a cellular process which acts against foreign substances result into the dilatation of blood vessels, swelling of a part of the body[3,4]. Including prostaglandin other inflammatory mediators like cytokines, histamine, serotonin, leukotrienes and activation of nociceptors also responsible for cellular damage and ultimate result is pain[5]. A wide number of analgesic drugs agents are used either alone or incorporation with other drug to reduce pain and inflammation. Though this drugs are successfully used to reduce pain but their use are limited because of some undesirable side effects[6,7]. Several studies supported that a huge number of medicinal plants provide excellent result against several disorders and it also provide safer outcome than any other established drug[8]. Besides analgesic and anti-inflammatory properties medicinal plants also contain thrombolytic properties. Clotting blood in the cardiovascular system refers to the thrombosis is the main reasons of acute coronary disorder[9-10]. Treatment of this pathophysiological condition requires depletion of platelet coagulation which can be achieved by the right selection of thrombolytic agents[11]. At present, new researcher found some medicinal plant which contains notable thrombolytic property. One of them is *D. blancoi* which containing large number of pharmacologically active chemical constituent and provide positive impact on analgesic, inflammation, microbial growth, diarrhea, dysentery, fever, cough exhibiting[12-16]. The *D. blancoi* is a popular plant and several studies were done previously with its various part. Although some investigations have previously been reported with this plant, the seed of the plant was not studied yet to date. This present study was aimed to investigate some *in vivo* and *in vitro* bioactivities of *D. blancoi*. During this study cold methanolic extract of *D. blancoi* have been considered to screen their biological properties such as analgesic, anti-inflammatory and thrombolytic activity.

MATERIALS AND METHODS

Collection, identification and extraction of sample: For this present investigation, the medicinal plants was collected from Barisal and was identified by Bangladesh National Herbarium, Mirpur, Dhaka. The every collected plant components (seeds) were separated from undesirable materials or other plants or plant components. They were preserved for one week. The plant components were ground into a rough powder with the assistance of an appropriate grinder. The powder was held on an airtight condition in a cool, dark and dry place till analysis. About 400 g of high-powered material of plant was taken in an exceedingly clean, flat bell-bottomed glass and soaked in 1600 mL of 80% methanol. The glass was sealed and waited with shaking and stirring in regular intervals for 12 days. The entire mixture then underwent a rough filtration by a chunk of unpolluted, white cotton material. Then it had been filtered through Whatman paper (Bibby RE200, Sterilin Ltd., UK). Experimental research had been approved by ethical committee of Noakhali Science and Technology University (NSTU-E-1623).

Experimental animals: Swiss albino mice used for this study were collected from Jahangir Nagar University, Savar, Dhaka, Bangladesh and were kept in polypropylene cages exposing them to alternate cycle of 12 h dark and light at temperature 25±20°C and relative humidity 55±10%. Mice were fed with standard laboratory pellet diet and water at libitum and were allowed to acclimatize for 7 days to the laboratory conditions before the experiment. Mice were give adequate human care throughout the experimental period.

For determining analgesic and anti-inflammatory activities 20 experimental healthy mice were randomly selected for each method and divided into four groups with five mice in each group.

Following groups had been considered during evaluation of Analgesic activity

- **Group I:** Control group, where all of the mice were fed with normal food and water
- **Group II:** Diclofenac sodium at dose of 10 mg kg^{-1} b.wt., i.p., had been administered
- **Group III:** Mice were treated with methanolic extract of 200 mg kg^{-1} b.wt.,
- **Group IV:** Mice were treated with methanolic extract of 400 mg kg^{-1} b.wt.,

Again, for the determination anti-inflammatory activity following groups have been constructed:

- **Group I:** Control group, where all of the mice were fed with normal food and water
- **Group II:** Ibuprofen at dose of 10 mg kg^{-1} b.wt., i.p., has been administered

- **Group III:** Mice were treated with methanolic extract of 200 mg kg^{-1} b.wt.,
- **Group IV:** Mice were treated with methanolic extract of 400 mg kg^{-1} b.wt.,

Collection of blood samples: Three milliliter of blood was collected from each healthy Bangladeshi male human volunteer (n = 3) under standard condition. The collected blood was kept into a test tube containing ethylenediaminetetra acetic acid (EDTA) to prevent clotting and stored until analysis.

***In vivo* analgesic activity test:** The analgesic activity for methanolic extract of *D. blancoi* was inferred in mice with the modification by Hussain *et al.*[17]. Aliking and biting test was conducted in mice. During this study 12 mice were randomly divided into four groups where each group contained 3 mice. Control group and two study groups with different *D. blancoi* doses.

Thrombolytic activity: *In vitro* clot lysis activity of *D. blancoi*was carried out according to the method of Prasad *et al.*[18], with minor modifications. About 7 mL of venous blood was drawn from healthy volunteers (n = 3) and transferred to different pre weighed sterilized micro-centrifuge tube (1 mL/tube). The micro-centrifuged tubes were exposed to incubation at 37°C for 45 min. After the formation of clot, serum was completely discarded from the tubes (carried out without disturbing the clot formed) and each tube having clot was again weighed to determine the weight of the clot (clot weight = weight of clot containing tube-weight of tube alone). Each micro-centrifuge tube containing clot was appropriately labeled and 100 µL of the plant extract with various concentrations (2, 4, 6, 8 and 10 mg mL^{-1}, respectively) was added to the tubes accordingly. As a positive control, 100 µL of streptokinase and as a negative non-thrombolytic control, 100 µL of sterilized distilled water were distinctly added to the control tubes numbered. After that the tubes were incubated again at 37°C for 90 min and observed for clot lysis. The following incubation, the obtained fluid was discarded from the tubes and they were again weighed to observe the difference in weight after clot disruption. Finally, difference obtained in weight was calculated and the result was expressed as percentage of clot lysis following the under beneath equation:

$$\text{Clot lysis (\%)} = \frac{\text{Weight of released clot}}{\text{Clot weight}} \times 100\%$$

Carrageenan-induced paw edema method: The mice were divided into four groups each containing five mice. Acute inflammation was induced by injecting 0.1 mL of 1% carrageenan into the plantar surface of the rat hind paw[19]. The extract (200 and 400 mg kg^{-1}),normal saline (1 mL kg^{-1}) and Ibuprofen (10 mg kg^{-1}, i.p.) as the referral agents were administered 30 min before carrageenan injection. The paw volume was measured at 0, 1, 2, h using a vernier caliper to determine the diameter of edema. The difference between the readings at time 1 h and different time interval was taken as the thickness of edema.

Statistical analysis: All the results were expressed as Mean±SEM. p-value was calculated by one-way ANOVA using SPSS software, version 22.0 (IBM Corporation, New York, NY, U.S.A.). Where, *p<0.05, **p<0.01, ***p<0.001 stands for significant, more significant and most significant respectively.

RESULTS

Investigation of analgesic test: Table 1 demonstrated the results of analgesic effect measured by formalin induced licking and biting method. The significant (p<0.05) inhibition was found at 400 mg kg^{-1} b.wt., of *D. blancoi* at both early and late phase. On the other hand, a dose of 200 mg kg^{-1} b.wt. of *D. blancoi* also provided a significant (p<0.05) inhibition with a rate of 43.12% at early phase.

Investigation of thrombolytic activity: Table 2 showed the results of the clot lysis of the plant extracts. Both positive (100 µL) and negative control provided a significant lysis of clot, whereas water (as negative control) was only provided

Table 1: Effects of *D. blancoi* extract on acetic acid induced writhing in mice

Groups	Dose (mg kg^{-1})	Early phase	Protection (%)	Late phase	Protection (%)
Group I	Vehicle	40.00±3.29	-	25.50±2.41	
Group II	10 (standard)	11.25±0.98	71.88	8.25±0.98	67.65
Group III	100	17.20±1.12*	43.12	9.32±1.08	55.67
Group IV	200	8.10±1.82*	63.77	7.03±2.33*	73.47

Values are expressed at mean (n = 5)±SEM (standard error mean) *p<0.05 compared with vehicle control (one way ANOVA followed by Dunnett' test)

Table 2: Comparative thrombolytic activity of methanolic extract of *D. blancoi*

Treatment groups	Dose (mg mL^{-1})	Clot lysis (%) (Mean±SEM)
ME	2	15.68±1.56
ME	4	23.03±1.08
ME	6	27.82±2.56*
ME	8	34.79±1.18*
ME	10	38.17±2.45*
Water		3.87±0.45*
Streptokinase		65.17±2.15*

Values are expressed at mean (n = 5)±SEM (standard error mean) *p<0.05 compared with vehicle control (one way ANOVA followed by Dunnett' test)

Table 3: Effect of methanolic extract of the *D. blancoi* on carrageenan-induced paw edema in mice

		Edema diameter (mm)			Inhibition (%)		
Groups	Dose (mg kg^{-1})	0 min	1 h	2 h	0 min	1 h	2 h
Group I	Vehicle	3.85±0.24	3.88±0.41	3.86±0.45		-	-
Group II	10	2.75±0.45*	1.88±0.46*	1.63±0.46*	32.2	51.4	58.12
Group III	200	2.93±0.1	2.82±0.53*	2.79±0.19	13.1	22.5	24.81
Group IV	400	2.91±1.4	2.87±2.9*	2.72±1.03*	18.01	23.16	27.23

Values are expressed at mean (n = 5) ±SEM (standard error mean) *p<0.05 compared with vehicle control (one way ANOVA followed by Dunnett' test)

3.87% clot lysis. In study groups, crude methyl alcohol extract (ME) showed their activity significantly at 6, 8 and 10 mg mL^{-1} dose, where as other level of dose showed non-significant results.

Investigation of anti-inflammatory test: Table 3 showed the results of Carrageenan-induced paw edema method of *D. blancoi*. Standard Ibuprofen showed significant inhibition of inflammation at all three phases (0, 1 and 2 h), whereas at 1 h *D. blancoi* showed significant outcome for 200 mg kg^{-1} dose. On the other hand 400 mg kg^{-1} dose showed statistically significant result at 1 and 2 h period.

DISCUSSION

Discovery of new drug with less adverse effect and high potency is always a matter of great interest for all kind of people. For that reasons study on plants always find major attention[7]. Phytochemicals obtained from phytochemical screening present on plant is responsible for therapeutic activity[19]. Tannins, glycosides, flavonoids, alkaloids, saponins, cardiac glycosides and steroids are the constituents of plant. Among them flavonoids, a polyphenol compound is major constituent found in various plant acts as anti-inflmmatory, anticancer, antimicrobial, antithrombolytic, analgesic etc. Chalcones, flavan-3-ols, flavanones, flavones and flavonols, iso-flavones and biflavonoids are the types of flavonoids and also 4000 derivatives found from nature[20]. Many studies showed that flavonoids is very effective against inflammation especially chronic inflammation and also it possess analgesic activity[20]. Anti-inflammatory and analgesic effects of flavonoids can be achieved by several mechanisms. One of them is increasing cellular movement of mast cells, macrophages, lymphocytes and neutrophils which are known as inflammation-related cells[21,22]. Through GABA receptors, opioids receptors and alpha-adrenergic receptors flavonoids can pass the blood-brain barrier and activate different parts of the nervous system such as ventral medulla oblongata (medulla Rostral ventrolateral), the alpha-adrenergic, GABA receptors, as a result repression of the responsible enzymes of pain and inflammation can be obtained[23]. Flavonoids reduce arachidonic acid (AA) metabolizing enzymes such as phospholipase A2 (PLA2), cyclooxygenase (COX) and lipoxygenase (LOX) and the nitric oxide (NO) producing enzyme, nitric oxide synthase (NOS) results the suppression of preeminent mediators of inflammation and pain[24-26]. *D. blancoi* contains flavonoid, sterols, saponins, terpenes, gum, sugar, tannins, alkaloids and phenolic acids. Among all constituents our plant may show analgesic and anti-inflammatory activity due to flavonoids, so thus by using one of the above mechanisms the constituents present in our plant *D. blancoi* may show analgesic and anti-inflammatory activity and our present finding have been endorsed by some previous studies[24-26].

In our present study thrombolytic activity was also examined along analgesic, anti-inflammatory activity. Formation of thrombus requires initiation and accumulation of several tissue factors, platelets, fibrin to the injurious part of the endothelial cell which starts immediately after wound[27]. Development of thrombus hampers the smooth movement of blood through the vessel, consequence of several diseases like atherothrombotic such as myocardial or cerebral infarction.

When thrombus converted into embolism death may occur. To split clot, plasmin can be used which is a thrombolytic agents activated from plasminogen may break two main component of clot called fibrinogen and fibrin[28,29]. Scientist always wants to discover high potency with less side effects thrombolytic drugs and by the help of recombinant technology many modified drugs are prepared[30,31]. That's why present study was undertaken to examine thrombolytic effect of *D. blancoi* because medicinal plant does not produce any noticeable side effects. Our plant contains saponins, tannins, alkaloids which are the well-known thrombolytic agents, thus may be those constituents are responsible for the thrombolytic activity. The findings of present study showed a moderate analgesic, anti-inflammatory and thrombolytic property. The exact mechanism of this effect yet not studied. So, advanced research work give us better understanding about plant constituents, their way of action like interaction with the binding site and produce effects against various diseases.

CONCLUSION

From the overall discussion, it is clear that *D. blancoi* is very effective against pain, inflammation and thrombosis. So this present study can use successfully as it is very safe and do not produce any unacceptable adverse effects. Though present study showed moderate effects but more study will help us to comprehend this plant pharmacological movement and increasing its use for the betterment of undesirable conditions.

SIGNIFICANCE STATEMENT

This study has demonstrated that treatment with methanolic extract of *D. blancoi* seeds to swiss albino mice. This study will provide baseline data to help the researcher effectively probe the active principles of the plant extract responsible for the analgesic, thrombolytic and anti-inflammatory activities. The study will also give a lead to the researcher to unravel the mechanism of action.

ACKNOWLEDGMENTS

The authors are grateful to Beacon pharmaceuticals Ltd., Bangladesh for their generous supply of necessary drugs. The authors are also thankful to all the teachers and staffs of the all enlisted universities for their cordial co-operation to carry out the research work with full sensations.

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