Research Article

A STUDY OF THE ANTIINFLAMMATORY ACTIVITY OF STEM BARK OF *RHAMNUS PURPUREUS*

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ABSTRACT

The present study was facilitated to screen the anti-inflammatory effect of bark of the *Rhamnus purpureus. Rhamnus purpureus* commonly known as Gaunta a deciduous shrub found in the Himalayan range, Himachal Pradesh and Bhutan. In present study the stem bark of *Rhamnus purpureus* was chopped and extracted in ethanol butenol, chloroform and evaluated for carregenum, cotton pallet induced granulloma in Wister Albino Rats. The results revealed that crued ethonolic extract showed maximum inhibition in caragenum induced rat paw edema followed by ethanol, butenol, and chloroform extract. The ethonolic extract of infective dose 200mg/kg body weight showed significant anti inflammatory activity with inhibition of 41.05%, 49.35%, 54.30%, and 54.77%, and butanolic extract showed significant results viz. 46.01%, 49.80%, 49.17%, 50.28%. and the standard drugs indomethacin also compared (10mg/ kg) showed inhibition of 50.69%, 51.63%, 50.46%, 51.78% after 1,2,3,and 4 hours of treatment respectively. The result indicates that the ethonolic extract as well as butonilic extract revealed strong inflammatory activity while chloroform showed less effective in comparision with indomethicin. The order of inhibition of carregenum induced rat paw edema was found to be - indomethecine > ethanol > butonol>chloroform>. In conclusion to above study it has been seen that the *Rhamnus purpureus* was found to have great anti-inflammatory response and can be substitute or alternative for synthetic or chemically derived drugs.

Key words: *Rhamnus. Antiinflammatory,Carregenum*

INTRODUCTION

The genus *Rhamnus* belongs to the family Rhamnaceae is a large genus of small trees and shrubs, commonly known as 'buckthorns', distributed chiefly in the temperate and warmer regions of the world. About ten species of *Rhamnus* occur in India [1]. *Rhamnus purpueus* Edgew locally known as *'Gaunta'* is a deciduous shrub or small tree found in submontane to montane Himalaya, Himanchal Pradesh to Bhutan up to 1000-2400 m [2]. Its stem are dark purple-brown, 3-6m high; leaves aleternate, ovate-lanceolate, 7-14x2-2.4cm, serrulate, acuminate, membranous; flowers pale-white, ca 4mm across, 5-merous, in-axillary branches or simple racemes; peduncle 6-10cm long. Calyx cup-shaped; segment acute. Petals globose or obovate [2].*Rhamnus* species have been reported to possess anti-inflammatory, anti-oxidant, anti-spamodic, cardio-stimulating, anti-ulcer and hypotensive activity [3-5}. Preliminary phytochemical screening of different parts (leaves, stem bark and roots) of *R. purpureus* showed that all these parts have similar chemical constituents but the higher concentration of the chemical constituents (monitored by TLC) was found in stem bark, therefore, pharmacological study of aqueous ethanol extract was carried out. The aqueous ethanol extract showed significant anti-inflammatory and anti-oxidant activity, therefore, the ethanol extract was fractionated with $CHCl_3$ and n-butanol and anti-inflammatory and anti-oxidant activity of aqueous ethanol, chloroform and butanol extract was carried out.

Anti-inflammatory Activity of *R. purpureus*

INFLAMMATION

The definition of inflammation is the body's response to tissue injury [8]. Inflammation is defense reaction of the organism and its tissue to injurious stimuli that lead to the local accumulation of plasmatic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be included, maintained or aggravated by many diseases such as vasomotor rhinnorrhoea, rheumatoid arthritis, and atherosclerosis. [9-11]. Inflammation is caused due direct damage (cuts, sprains), chemical such as acid, ischemia and cell necrosis, allergic reaction, physical agents such as injuries or burns. Inflammation usually involves a sequence of events which can be categorized under three phases viz. acute transient phase, delayed sub acute phase and chronic proliferate phase. In the first phase, inflammatory exudates develop due to enhanced vascular permeability and leads to local edema. It is followed by the migration of leukocytes and phagocytes from blood to vascular tissue which is the second phase, in the third phase; tissue degradation is followed by fibrosis. Inflammation

results in the liberation of endogenous mediators like histamine, serotonin, bradykinin, prostaglandins etc. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation. These mediators even in small quantities can elicit pain response [12]. Inflammation can be classified based on duration of inflammation as acute and chronic inflammation.Acute inflammation begins within seconds to minutes following the injury of tissues. The damage may be purely physical, or it may involve the activation of an immune response [13]. The cardinal signs of acute inflammation are heat, redness, swelling, pain and loss of function. Acute inflammation is initiated by the cells. At the onset of an infection, burn or injuries, the cells undergo activation and release inflammatory mediators responsible for the clinical signs of inflammation. Vasodilation and its resulting increased blood flow cause the redness and increased heat. Increased permeability of the blood vessels results in an exudation of plasma proteins and fluids into the tissue (edema), which manifests itself as swelling. Some of the released mediators such as bradykinin increase the sensitivity of pain. The mediator molecules also alter the blood vessels to permit the migration of leukocytes, mainly neutrophils, outside of the blood vessels into the tissue.The acute inflammation response requires constant stimulation to be sustained. Inflammatory mediators have short half lives and are quickly degraded in the tissue. Chemical mediators of inflammation include histamine, prostaglandins, leukotriene B4, and nitric oxide. Histamine, the main pre-formed mediator of inflammation, released from mast cells, basophils, and platelets, causes transient dilation of arterioles, increases permeability in venules and is the primary cause of increased vascular permeability in the first hour of injury. Prostaglandins released from mast cells are responsible for vasodilation, fever and pain. Leukotriene B4 is able to mediate leukocyte adhesion and activation, allowing them to bind to the endothelium and migrate across it. Nitric oxide which is locally synthesized by endothelium and macrophages through the activity of the enzyme is responsible for the skin inflammation [14].Chronic inflammation has slow onset and persist for weeks or more. The symptoms are not as severe as with acute inflammation, but the condition is insidious and persistent. An acute inflammation will become chronic if the immune system is unable to get rid the foreign agents from the body. In the case of persistent infections, such as tuberculosis, and autoimmune diseases, chronic fatigue will arise directly. The main cells involved in chronic infection are macrophages and lymphocytes [14]. In order to comprehend the inflammatory process, antagonists of mediators are generally employed in both Ayurveda and Allopathy treatment. Most of the anti-inflammatory drugs now available are potential inhibitors of cyclooxygenase (COX) pathway of arachidonic acid metabolism which produces prostaglandins. Prostaglandins are hyperanalgesic, potent vasodilators and also contribute to edema and pain. Hence for treating inflammatory diseases analgesic and anti-inflammatory agents are required. Now a day's herbal drug are routinely used for curing diseases rather than chemically derived drugs having side effects Inflammation has become the focus of global scientific research because of its implication in virtually all human and animal diseases. Inflammatory diseases include different types of rheumatic disorders such as rheumatic fever, rheumatoid arthritis, ankylosing spondylitis, polyarthritis nodosa, systemic lupus erythematosus and osteoarthritis. Therefore, new anti-inflammatory and analgesic drugs lacking these side effects are being researched as alternatives to NSAID and opiates [15, 17]. Attention is being focused on the investigation of the efficacy of plant-based drugs used in the traditional medicine because they are cheap, have little side effects and according to WHO, about 80% of the world population still rely mainly on herbal remedies [15, 17].

Matrial and method

PREPRATION OF EXTRACT

The stem bark of *R. purpureus* Edgew locally known as *'Gaunta'* was collected from Kamad region district Uttarkashi, Uttrakhand, in the month April. The plant was identified by Dr. Sumer Chand, Systematic Botany Divison, FRI, Dehradun, Uttrakhand, India. A voucher specimen (No. 70) was deposited in the herbarium of Department of Botany, Govt P.G. College Uttarkashi, Uttrakhand. The stem bark are chopped in to small pieces and dried under shade, and powdered in a grinder. The powdered plant material (3.0 kg) was defatted by petroleum ether (60-80ºC) for 72 h. The defatted plant material was dried in room temperatures and extracted with Ethanol (95%) for 72 h. The Ethanol extract was concentrated under reduced pressure at a low temperature (40-50°C) yields crude ethanol extract (71.3g). A suspension of ethanol extract (60.0g) was made with water, which was successively partitioned with chloroform and n-butanol. The chloroform and butanol layers were separated out and concentrated under reduced pressure afforded chloroform (21.5 g) and n-butanol extract (27.3g), respectively.

Animals Used

Albino Wistar rats of either sex weighing between 150-200 g were, purchased from CCS Haryana Agriculture University, Hisar, Haryana, India, used in present study and were The animals had free access to food and

water and were maintained under controlled temperature (27±2^{0}C) and 12 h: 12 h light and dark cycle. Initial body weight of each animal was recorded. Institutional Animal Ethics Committee (IAEC), Department of Pharmacy, GGD University, Bilaspur, had approved the experimental protocol and care of animals was taken as per the guidelines of CPCSEA, Department of Animal Welfare, Government of India.

For determination of acute-inflammation the most commonly used method is Carrageenan-induced paw edema in rats. This model is based on the principle of release of various inflammatory mediators by carrageenan [20]. Edema formation due to carrageenan in the rat paw is biphasic event. The initial phase is attributed to the release of histamine and serotonin. The second phase of edema is due to the release of prostaglandins, protease and lysosomes and **carrageenan induced granuloma pouch model (Sub** acute model**) and carrageenan induced air pouch model (chronic** model**).**

Table 2: Effect of Ethanol, butanol, and chloroform extracts of stem bark of *R. purpureus* on cotton pellet granuloma in rats.

Treatment	Dose	Weight of dry cotton pellet (mg)	% Inhibition
Control	Vehicle	85.95	--
Indomethacin	10mg/kg	39.47[a]	54.08
Crude Ethanolic Extract(CEE)	100mg/kg	65.27[ab]	24.06
	200mg/kg	54.77[ab]	36.28
	400mg/kg	39.00[a]	54.62
Chloroform Fraction(CFM)	100mg/kg	57.92[ab]	32.62
	200mg/kg	42.07[a]	51.06
	400mg/kg	39.07[a]	54.55
Butanol Fraction (BFM)	100mg/kg	47.38[a]	44.88
	200mg/kg	40.62[a]	52.74
	400mg/kg	39.07[a]	54.55

Values are given as mean ±SEM (n=6)

[a]P<0.05 as compared to control; [b]P<0.05 as compared to Indomethacin treated group.

Acute toxicity studies

Acute toxicity study was performed as per OECD-423 guidelines. The test substance was administered in a single dose by an intubation canula. Three animals were used for each step. The test substance was administered orally at a doses ranging from 5, 50, 300 and 2000 mg/kg body weight. Animals were observed individually after dosing once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia, mortality were observed. All test animals were subjected to gross necropsy. No signs of toxicity were observed. The doses selected were 50, 100 and 200 mg/kg [35].

Carrageenan–Induced Rat Paw Edema Method

The animals were divided into eight groups of six animals each. The first group (control) was treated with 5% acacia i.e. vehicle only 10ml/kg and second group (standard) was treated with indomethacin (10mg/kg). The third, sixth and ninth group received ethanol, chloroform and n-butanol extract at 100mg/kg, body weight; fourth. Seventh and tenth group received ethanol, chloroform and n-butanol extract 200mg/kg, respectively and fifth, eighth and eleventh group received ethanol, chloroform and n-butanol 400mg/kg, body weight respectively. All the drugs were given orally half an hour before the administration of carrageenan suspension with the help of an oral catheter. One percent suspension of carrageenan in 5% acacia was prepared and 0.1 ml of it was injected in sub plantar region of right hind paw of each rat. The paw volume was measured immediately after injection (i.e. 0 hrs) and after 1, 2, 3 and 4 hrs with the help of Plethysmometer. The average paw swelling in rats treated with plant extracts was compared with control and standard group [20].

The percent change in edema was calculated by the formula:

% edema inhibition = [(Vc – Vt) / Vc] X 100

Where, Vt = Volume of treated group; Vc = Volume of control group

Cotton Pellet-Induced Granuloma

The rats were divided into eight groups (n = 6) as discussed under acute model. The rats were anesthetized and an incision was made on the lumbar region by blunted forceps, a subcutaneous tunnel was made and a sterilized cotton pellet (100 ± 1 mg) was inserted in the groin area. From the day of cotton pellet insertion all the animals received either vehicle (1% CMC) or indomethacin or plant extracts orally depending upon their respective grouping for seven consecutive days [20]. On 8th day, animals were anesthetized and cotton pellets were removed and dried to constant mass.

Table 3: Effect of Ethanol, butanol, and chloroform extracts extracts of stem bark of *R. purpureus* on excudate volume, neutrophil and monocyte count in carrageenan induced air-pouch inflammation.

Treatment	Dose	Exudate volume	Neutrophils (X 10 cells)	Monocytes (X 10 cells)
Control	Vehicle	3.17±0.021	213.33±6.67	88.67±4.25
Indomethacin	10mg/kg	0.65±0.034[a]	68.00±2.38[a]	38.50±2.81[a]
Crude Ethanolic Extract(CEE)	50mg/kg	2.95±0.148[b]	185.83±3.75[a]	70.33±3.86[ab]
	100mg/kg	2.62±0.040[a]	166.17±6.26[ab]	61.00±3.82[ab]
	200mg/kg	1.17±0.056[ab]	113.17±4.30[ab]	56.33±2.46[ab]
Chloroform Fraction(CFM)	50mg/kg	2.57±0.033[ab]	137.67±2.51[ab]	59.17±2.48[ab]
	100mg/kg	1.58±0.048[ab]	130.17±1.28[ab]	55.67±2.06[ab]
	200mg/kg	1.48±0.060[ab]	122.83±1.17[ab]	51.00±1.59[ab]
Butanol Fraction (BFM)	50mg/kg	1.5±0.045[ab]	114.17±1.40[ab]	55.00±2.49[ab]
	100mg/kg	1.1±0.037[ab]	98.33±1.82[ab]	48.17±2.34[a]
	200mg/kg	0.95±0.022[ab]	95.50±1.38[ab]	45.00±3.51[a]

Values are given as mean ±SEM (n=6)
[a]P<0.05 as compared to control; [b]P<0.05 as compared to Indomethacin treated group.

Cronic toxicity studies

Carrageenan induced air-pouch model

The rats were divided into different groups (n = 6) as discussed under acute model. Air-pouch was produced according to the method described by Salvemini et al. 1996 [36]. Briefly, rats were anesthetized and air cavities were produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back (that is, 0 day). An additional 10 ml of air was injected into the cavity every 3rd day (3rd and 6th day) to keep the space open. On the 7th day, 2 ml of 1% solution of carrageenan dissolved in saline was injected directly into the pouch to induce an inflammatory response. The rats were orally pre-treated with either vehicle or CEE/CFM/BFM or indomethacin 2 h prior to the injection of carrageenan. The second dose of treatment was repeated after 24 h of the first treatment. 48 h after carrageenan injection, the rats were anesthetized with ether and the pouch was carefully opened by a small incision. The volume of exudates was collected and measured. An aliquot of the exudate was used for differential cell count (neutrophils and monocytes) using a manual cell counter after staining with Wright's stain.

Statistical analysis

The results were expressed as Mean ± SEM. Statistical analysis was carried out by using one way ANOVA followed by Dunnett,s test.

Result

Effect on Carrageenan Induced Rat Paw Edema

The effect results crude ethanol, chloroform extract and btanol extract are shown in table 3.1. The results showed that the crude ethanolic extract showed maximum inhibition in carrageenan induced rat paw edema followed by butanol and chloroform extracts. The crude ethanolic extract showed significant ($P < 0.05$) anti-inflammatory activity with inhibition of

41.05%, 49.39%, 54.50% and 54.77% after 1hr, 2hr, 3hr and 4hr of treatment, respectively at the dose of 200mg/kg body weight whereas the n-butanol extract showed 46.01%, 49.80%, 49.17 and 50.28% inhibition after 1hr, 2hr, 3hr and 4hr of treatment at 200mg/kg dose, respectively. The standard drug indomethacin (10mg/kg) showed inhibition of 50.69, 51.63%, 50.46%, and 51.78% after 1hr, 2hr, 3hr and 4hr of treatment, respectively. The effects of crude ethanol extract at the dose of 100 and n-butanol extract at the dose of 200 mg/kg body weight at 4 hr after treatment were found comparable to that of indomethacin whereas the ethanol extract at the dose 200 mg/kg body weight at 3hr, and 4hr were found greater than that of standard drug indomethacin. Furthermore, it is evident from the table 3.1 that the anti-inflammatory activity of these extracts was found dose and time dependent. These results indicate that the ethanol extract as well butanol extract showed strong anti-inflammatory activity where as chloroform extract showed weak activity. The order of inhibition of carrageenan induced rat paw edema was found to be indomethacin>ethanol>butanol>chloroform.

Effect on Cotton Pellet Granuloma in Rats

The effect of ethanol, chloroform and butanol extract on granuloma formation in cotton pellet method was presented in table 3.2. The ethanol extract showed maximum inhibition of 51.06 (at 200 mg/kg) and (54.62% (at 200 mg/kg) in granuloma formation followed by butanol extract with inhibition of 52.74% and 54.55% at 100 mg/kg and 200 mg/kg dose, whereas the chloroform extract showed inhibition of 54.08%. The standard indomethacin showed 54.55% inhibition in granuloma formation (Table 3.2). The order of inhibition was found to be ethanol>indomethacin=n-butanol>chloroform extract.

Effect on exudate volume, neutrophil and monocyte count in carrageenan induced air pouch inflammation.

The effect of crude ethanol, chloroform and butanol extracts on exudates volume, neutrophil, and monocyte count in carrageenan induced air pouch in showed that these extracts elicited significant ($P<0.05$) dose dependent reduction in exudates volume and infiltration of netrophil and monocytes into the air pouch compared to control group (table 3). Indomethacin at a dose of 10 mg/kg body weight also showed significant ($P < 0.05$) result. The butanol extract showed significant effect on observed parameters than ethanol and chloroform extracts when compared with standard group. The activity was found to be in following order: Indomethacin>butanol>ethanol>chloroform extracts.

Discussion

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. Although it is an defense mechanism, the complex events and mediators involved in the inflammatory reaction can be included, maintained or aggravated by many diseases [9]. The aim of inflammation is to repair the damage or at least to limit it and also to remove the cause. Causes of inflammation include direct damage, chemical such as acids, ischemia and cell necrosis of infarction and infections [8]. Carragenan-induced rat paw edema is an acute inflammatory model involved several mediators released in sequence [37, 38]. An initial phase during the first 1.5 hr is caused by the release of histamine and serotonin. The second phase mediated by bradykinin from 1.5 to 2.5 hr and te third phase, in which the mediator is likely to be prostaglandin, occurs from 2.5 to 5 hrs after carragenan injection [39]. The carragenan-induced paw edema in rats is known to be sensitive to cyclooxygenase inhibitors, but not to lipoxygenase inhibitors. This test has been used to evaluate the effect of NSAIDs which primarily inhibit the cylclooxygenase involved in prostaglandins synthesis [39]. In the present study, the crude ethanolic and butanolic extracts at the doses of 100 and 200 mg/kg significantly decreased the rat paw edema induced by carragenan in all phases, suggesting that the possible mechanism of action of these extracts may involve inhibition of these inflammatory mediators release in all phases.The cotton pellet-induced granuloma formation in rats is a model involved with chronic inflammation. The inflammatory responses have been divided into in to three phases; transudative, exudative and proliferative phases. The fluid absorbed by the pellet greatly influenced the wet weight of the granuloma, and the dry weight correlated well with amount of granulomatous tissue formed [40, 41]. Chronic inflammation occurs by means of the development of proliferate cells. These cells can be either spread or in granuloma form. Non steroidal anti-inflammatory drugs decrease the size of granuloma which results from cellular reaction by inhibiting granulocyte infiltration, preventing generation of collagen fibers and suppressing mucopolysaccharides [42, 43]. In the present study, the crude ethanolic, butanolic and chloroform extract at all tested doses significantly reduced cotton pellet-induced granuloma formation in rats, indicating that the transudative, exudative and proliferative phases of inflammation were partially inhibited. In order to assess the efficacy of the extract against proliferative phase of inflammation, we selected carrageenan induced air pouch model in which tissue degradation and fibrosis occurs. During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small blood vessels, which are the basic sources of forming a highly vascularised reddish

mass, termed granulation tissue [44, 45]. In this model the crude ethanol, chloroform and n-butanol extracts significantly reduced infiltration of neutrophils and monocytes. These results indicate that the tested extracts may alter the action of endogenous factors that are involved in the migration of these substances to the site of inflammation. There is increasing evidence that lysosomal enzymes play an important role in the development of acute and chronic inflammation [46-49 process . It is thus apparent that the ant-inflammatory effect produced by these extracts might be due to either inhibiting the lysosomal enzymes, stabilizing the membrane or by altering the action of endogenous factors that are involved in the migration of these substances to the site of inflammation.

Conclusion

In the present study the crude ethanol and its n-butanol fraction showed strong anti-inflammatory activity than that of chloroform extract, which indicate that the active constituents that produced anti-inflammatory effect are present in higher concentration in the ethanol and n-butanol, extracts than that of chloroform extracts. It is thus apparent that the anti-inflammatory effect produced by ethanol, chloroform and n-butanol extracts of *R. purpureus* might be due to either inhibiting the lysosomal enzymes, stabilizing the membrane or by altering the action of endogenous factors that are involved in the migration of these substances to the site of inflammation.

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