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Isolation of Chitosan from *Bridelia retusa* for Analgesic and Anti-Inflammatory Activities

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ABSTRACT

The present study was designed to isolate the flavonoid Chitosan from the ethanol extract of leaves of *Bridelia retusa.* phytochemical screening was carried out according to standard procedures from the leaves of *Bridelia retusa.* The isolated flavonoid was characterized by spectral studies and screened for anti-inflammatory and analgesic activity in experimental animal models. The anti inflammatory activity was determined by formalin induced paw edema and carrageenan induced paw-edema method, and the analgesic activity was determined by tail flick method and hot plate latency method by using external standard indomethacin and Chitosan isolated from ethanol extract of *Bridelia retusa*. Chitosan significantly ($P<0.05$) showed the anti-inflammatory and analgesic activity in experimental animals. Therefore the present study justifies that the isolated flavonoid exhibits significant analgesic and anti-inflammatory activity at a dose of 250 mg/kg.

Keywords: Bridelia retusa, chitosan, indomethacin, anti-inflammatory activity, analgesic activity.

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INRODUCTION

Bridelia retusa is a shrubs to trees, usually monoecious. Leaves distichously, simple, symmetric, basally attached, margin entire to somewhat crenate, not variegated, without glands. Different parts of About 50 species in the Old World tropics: Tropical Africa, Madagascar, Yemen and in Asia ranging from India and China throughout SE. Asia and Malaise to N. Australia, the Solomon's, and Vanuatu; 10 species in Thailand. The bark, which contains tannin (16-40 %), is having pharmaceutical use because of its antiviral, hypoglycemic, hypersensitive properties [1]. The leaves are used as fodder and are said to free intestinal worms of cattle. The claimed traditional medicinal uses have been proved on scientific basis using *in-vitro* and *in-vivo* experiments. The plant have been screened for antihistaminic activity, CNS activities (viz. hypnotic, tranquillizing, local anesthetics), analgesic, anti-inflammatory, antipyretic, antiulcer, amoebicidal, anthelmintic, antitrypanosomal to antidepressant, antiviral and immunomodulatory [2, 3]. So the present study is aimed at isolation of Chitosan from the ethanol extracts of the plant *Bridelia retusa* and the screening of the analgesic, anti-inflammatory activity.

MATERIALS AND METHODS

Collections of plant materials

The plant *Bridelia retusa* was collected in the month of November 2008 from the Chitoor district Andhra Pradesh. The plant material was taxonomically identified by the field botanist, Department of Botany, Sri Venkateswara University, and a specimen was deposited in their herbarium bearing the Voucher No.2045.

Preparation of Crude Extract and phytochemical screening

The dried leaves of *Bridelia retusa* was powdered and extracted with 70% ethanol by hot percolation method. The obtained crude residue was dried and total yield was 4.2% w/v. The fractionated residue used for isolation of flavonoid.

Isolation of Flavonoid

The ethanol extract of *Bridelia retusa* was fractionated with benzene and ethyl acetate. The ethylacetate fraction was distilled and concentrated to formed product-A. Further, the product-A was washed repeatedly with water and dried by freeze-drying. Chitosan and chitin-glucan complex were extracted from product-A wastes of citric acid production with KOH and HCl using a method developed by combination and modification of methods by Muzzarelli et al. [4]. This complex was treated with 60% aqueous KOH solution at 130^{o}C for 2 - 3 h to remove proteins, lipids and alkali-soluble polysaccharides. The insoluble material was washed with distilled water to obtain neutral pH and concentrated ethanol and freeze-dried. On applying this procedure, an insoluble fraction of cell walls consisting of chitosan and chitin - glucan

complex is left as a white powder. For chitosan isolation, this freeze-dried alkali insoluble fraction was treated with diluted 2 % HCl solution (50 ml per 1 g of dry matter) for 1-10 h at 25° or 95°C. The pH value of the acidic supernatant was increased to 9.5 with 2M NaOH. The alkali-insoluble precipitate (presumable Chitosan) obtained was repeatedly centrifuged and washed with distilled water, freeze dried and weighed.

Phytochemical test for isolated flavonoid

a) Shinoda's test

The extracts were dissolved in alcohol, to which few magnesium turnings were added followed by concentrated HCl drop wise and heated. Appearance of magenta color shows the presence of flavonoid.

b) Zn-HCl reduction test

To the test solution a mixture of zinc dust was added, few drops of concentrated HCl was added drop wise along the side of the tube, which showed red color indicating flvanoids present.

c) Extracts treated with aqueous sodium hydroxide solution, the results shown the blue to violet color (Anthocyanins), yellow color (flavones), yellow to orange (flavones), orange to crimson (flavonones).

Characterization of flavonoid Compound

Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It has a number of commercial and possible biomedical uses.

Spectral data

Structures of isolated compound were established based on IR and mass spectral studies.

IR spectrum of chitosan

IR spectra of chitosan from ethanolic extract of *Bridelia retusa* is similar to those reported in the literature of Muzzarelli et al. [4]. FTIR spectrum mentioned the absorption band occurs at 1653.00 cm^{-1} and 1321.24 cm^{-1} for C-NH2 groups. The absorption band occurs at 1737.86 cm^{-1} for c=o and c=c peak occurs at 2922.16 cm^{-1} at 3425.58 cm^{-1} for hydroxyl group.

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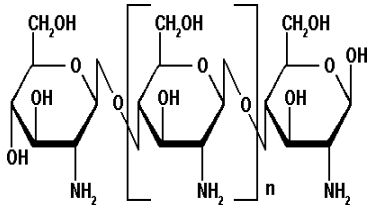
Mass spectrum of chitosan

Conclusive evidence was obtained from the Mass spectral data showed molecular ion peak value at m/z. The base peak occurs at 141, which was tallest on y-axis. Another fragmentation and characterization peak occurs at 304, 277, 248, 214, 204,167, 121, 106, 90 and 75.

Based on these results the structure of the compound was confirmed as **Chitosan**



Chitosan

EXPERIMENTAL PROTOCOL

Animals

Male Wistar albino rats (150-200g) were housed in a spacious cage for 10 days after getting approval from the "Institutional animal ethical committee". The animals were deprived of food for 15 hr before the experiment.

Grouping of animals

Animals were divided into three groups of 3 male and 3 female rats in each group and administrated with respective drugs.

Evaluation of anti-inflammatory activity

Formalin-induced paw edema

Wistar rats were administrated with the normal saline (5ml/kg, p.o), Chitosan dissolved in DMSO (250 mg/kg, p.o) and indomethacin (10 mg/kg, p.o) 60 minutes before subcutaneous injection of 50 micro liter of 1% formalin into the dorsal surface of the right hind paw was applied. The procedure was similar to that described previously Hunskaar and Hole et al. [5]. Animals were kept in the chambers and lick response was observed. A mirror was placed behind the chamber to enable the observation of the injected paw.

Carrageenan-induced paw edema

The carrageenan induced edema assay was done as described by Winter et al. [6]. The animals were administrated with the normal saline (5ml/kg, p.o), Chitosan dissolved in DMSO

(250 mg/kg, p.o) and indomethacin (10 mg/kg, p.o) 60 minutes before the injection of carrageenan (1%, w/v, in saline solution) into the subplantar region of the right hind paw. The contralateral paw was injected with an equal volume of saline. The paw volumes were determined hourly by plethysmometer (Ugo Basile, Italy) for 4 h. The edema was reported as the difference between the volumes of the right and left paws. The difference between the left and the right paw volumes (indicating the degree of inflammation) was determined and the percent inhibition of edema was calculated in comparison to the control animals.

Evaluation of analgesic activity

Tail flick test [7]

The apparatus used in the tail flick test consisted of a circulating immersion water heater. The thermostat was adjusted so that a constant temperature of 54±1 °C was maintained in the water bath. Before treatment, the terminal 3 cm of each rat's tail was immersed in the water bath and the time in seconds taken to flick the tail was recorded. Only rat showing a pretreatment reaction time less or equal to 3 sec were selected for the study. Immediately after basal latency assessment, the Chitosan (250 mg/kg), pethidine (50 mg/kg) and normal saline (5ml/kg) were administered by the oral route to different groups and the reaction time was again measured 1 and 2 h after the injections. Cut-off time was 15 sec for tail flick measurements in order to minimize tissue injury.

Hot plate latency assay in rats

The hot plate latency assay was based on the method of Eddy et al. [8]. The animals were administrated with the normal saline (5ml/kg, p.o), Chitosan dissolved in DMSO (250 mg/kg, p.o) and indomethacin (10 mg/kg, p.o), and after 30 minutes the reaction time for the animal such as lick the paw or jump from the hot plate was taken as the latency (s). The same procedure was repeated at 60^{th} and 90^{th} minute. The whole procedure was repeated for all the rats in each group. The temperature of the hot plate was maintained at 55 ± 2 °C.

Statistical analysis

The collected data were subjected to appropriate statistical tests like one-way ANOVA (Analysis of Variance) followed by Dunnet's test. P values of less than 0.05 were considered significant. The analysis was carried using Graphpad Instat software of version 3.

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RESULTS

Formalin-induced paw edema

Table: 1 Effects of isolated Chitosan in the formalin induced paw edema

Sr. No	Treatment	Lick response (sec)	
		60th minute	120th minute
1	Vehicle Control(saline)	37.38±0.17	43.73±0.02
2	Indomethacin	24.23±0.20***	22.50±0.08***
3	Chitosan	31.73±0.07***	28.78±0.12***

The values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with vehicle control group; One-way ANOVA followed by Dunnet's test.

Intraplantar injection of 2.5% formalin evoked a characteristic biphasic licking response. When compared with vehicle control group, Indomethacin and Chitosan showed a significant (P<0.001) reduction in lick response at 60th and 120th minute (table-1).

Carrageenan-induced paw edema

Table: 2 Effects of isolated Chitosan on the swelling induced by Carrageenan of rat hind paws

Sr. No	Treatment	% Edema inhibition relative to control		
		90th minute	180th minute	270th minute
1	Vehicle Control(saline)			
2	Indomethacin	74.47	78.18	83.25
3	Chitosan	65.32	69.51	71.42

The oral treatment with isolated Chitosan inhibited significantly carrageenan-induced paw edema in rats (table-2) and the percentage inhibition was found to be 65.32%, 69.51%, 71.42% at 90th, 180th, 270th minute respectively when compared with vehicle control. The Indomethacin showed percentage inhibition 74.47%, 78.18%, 83.25% at 90th, 180th, 270th minute respectively when compared with vehicle control.

Tail flick test

Table: 3 Effect of isolated Chitosan on the tail flick reaction time of rat

Sr. No	Treatment	Reaction time (minute)		
		Pre treatment	1st h	2nd h
1	Vehicle Control(saline)	2.41±0.14	2.46±0.17	2.78±0.15
2	Pethidine	2.43±0.33***	3.13±0.30***	3.91±0.25***
3	Chitosan	2.23±0.26***	3.68±0.36***	4.23±0.41***

The values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with vehicle control group; One-way ANOVA followed by Dunnet's test.

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The data concerning the antinociceptive effects of the isolated Chitosan on the tail flick test in rat are summarized in Table-3. Pretreatment of rat with Chitosan and Pethidine showed a significant (P<0.001) increase in the reaction time to thermal stimuli at the two times of measurement (1 and 2 h after drug administration).

Hot plate latency assay in rats

Table: 4 Effects of the isolated Chitosan on hot plate test in rats

Sr. No	Treatment	Reaction time(Sec)		
		30th sec	60th sec	90th sec
1	Vehicle Control(saline)	6.12 ± 0.21	5.41 ± 0.15	6.62 ± 0.22
2	Indomethacin	11.20 ± 0.92***	12.83 ± 0.18***	13.6 ± 0.12***
3	Chitosan	9.13 ± 0.16***	10.41 ± 0.15***	11.23 ± 0.19***

The values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with vehicle control group; One-way ANOVA followed by Dunnet's test.

The results showed, oral doses of Chitosan significantly (P<0.001) increased the reaction time from 6.12 ± 0.21 sec to 9.13 ± 0.16 sec when compared with vehicle control(Table 4). Similarly, at 60th and 90th minute the reaction times were significantly (P<0.001) increased. However, compared with vehicle control group Indomethacin treated group also showed a significant (P<0.001) increase in reaction time.

DISCUSSIONS

Formalin-induced paw edema

Intraplantar injection of 2.5% formalin evoked a characteristic biphasic licking response, an early phase corresponding to acute neurogenic pain, sensitive to drugs that interact with the opiod system and a late phase corresponding to inflammatory pain responses inhibited by analgesic-antiinflammatory drugs [5]. The Chitosan showed significant inhibitory activity in carrageenan-induced paw inflammation. This indicates action against release of histamine, serotonin and kinins in early phase, while later phases are suspected to be arachidonate metabolites producing an edema dependent on mobilisation of neutrophils [9]. In the present investigation, Chitosan produced a marked reduction of the duration of the licking in the late phase, corresponding to the inflammatory reaction. Inhibition of only the second phase of the formalin test is a typical characteristic of cyclooxygenase inhibitors, suggesting a anti-inflammatory activity of Chitosan.

Carrageenan-induced paw edema

The development of enema in the paw of the rat after the injection of carrageenan is due to release of histamine, serotonin and prostaglandin like substances [10]. Significantly high anti-inflammatory activity of isolated Chitosan (250 mg/kg) may be due to inhibition of the

mediators of inflammation such as histamine, serotonin and prostaglandin. The present result indicates the efficacy of Chitosan as an efficient therapeutic agent in acute anti-inflammatory conditions.

Tail flick test

The thermal model of the tail flick test is considered to be a spinal reflex, but could also involve higher neural structures and this method identifies mainly central analgesic. Drugs that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs inhibit the late phase [11]. The Chitosan at a dose 250 mg/kg showed significantly high antinociceptive activity may be due to inhibition of both the central and peripherally mediated activities.

Hot plate latency assay in rats

The analgesic property was studied using sensitive models that could provide different grades of noxious stimuli (in thermal stimulus and chemically induced tissue damage). Antinociceptive activity of opioid agonist, opioid partial agonist, and non-steroidal anti-inflammatory agents can be determined by writhing test [12]. In the present study the thermal test was selected because of several advantages including the sensitivity to strong analgesics and limited tissue damage. The results of study indicated that the isolated Chitosan at a dose o 250 mg/kg exhibited central and peripheral antinociceptive activities.

CONCLUSION

The extracts and isolated compounds (leaves) of *Bridelia retusa* has Anti-inflammatory and analgesic effect of supporting the Ethno pharmacological uses. The results of this study might be valuable in finding pure natural products having analgesic and anti-inflammatory activity. This effect may be explored in the use of the leaves in the management of CNS.

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