

COMPARATIVE STUDY OF THE FRACTIONS OF A RELATIVELY UNEXPLORED PLANT - *BYTTNERIA HERBACEA* ON HISTAMINERGIC INFLAMMATION

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ABSTRACT

Objective: In this study we have compared various fractions of *Byttneria herbacea* root extract against capillary permeability, an important feature of inflammation mediated by histamine and histamine receptor type I. Despite being widely used for the treatment of filariasis (a common disease of tropics) by tribals in India, significant scientific reports on the pharmacology and phytoconstituents are not yet available.

Methods: Quantitative estimation of alkaloid and saponin was done for *n*-butanol, ethyl-acetate and aqueous fractions. Simultaneous comparison of the three fractions was performed by *in-vivo* experiments like acetic acid (dye leakage) and histamine induced capillary permeability followed by development of HPLC chromatogram for the fractions.

Results: Maximum content of alkaloid was found in *n*-butanol fraction followed by aqueous and ethyl-acetate fraction. Similarly, saponin was found to be highest in aqueous fraction followed by ethyl-acetate fraction while it was negligible in *n*-butanol fraction. Acetic acid induced capillary permeability revealed significant maximum inhibition ($*p < 0.05$) of 78.74% and 61.21% by the *n*-butanol and aqueous fraction respectively at the dose of 200 mg/kg. Similarly, in histamine induced capillary permeability maximum significant inhibition was 76.74% and 57.42% by the *n*-butanol and aqueous fraction respectively at the dose of 200 mg/kg. HPLC chromatograms revealed few major common peaks in *n*-butanol fraction and aqueous fraction with the crude extract.

Conclusion: The results indicate *n*-butanol fraction with maximum amount of alkaloid exhibiting highest significant inhibition at the dose 200 mg/kg followed by aqueous and ethyl-acetate fraction.

Keywords: Capillary permeability, *n*-butanol fraction, Histamine and Alkaloids.

INTRODUCTION

Inflammation although a necessary function of immune system, can become hazardous and potentially fatal, when acute inflammation tends to become chronic. Such response often leads to the release of a wide range of pro-inflammatory mediators that include serotonin, histamine, bradykinin, cytokines and prostaglandins. Inflammation is also characterized by increased permeability, neutrophil migration and angiogenesis in which histamine is believed to play a key role as a potent vasodilator.

Conventional therapy of inflammation is often obscured by the adverse drug reaction which is a well known and an accepted clinical reality. Accordingly, the search for indigenous drugs with prospects of reduced adverse effects and safer management of inflammatory disorders is of much scientific interest. Several traditional medicinal plants like *Acanthus ilicifolius* Linn. (Acanthaceae)[1], *Camellia sinensis* (L.) O. Kuntze. (Theaceae)[2] and *Byttneria herbacea* Roxb. (Sterculiaceae)[3] have been studied in our laboratory for their anti-inflammatory activity. *B. herbacea* as the literature survey reveals is widely used by the traditional folklores of Kols, Birhories, Maria gonds and several other tribes from West Bengal, Chattisgarh, Bihar, Orissa, Andhra Pradesh and Jharkhand [4,5,6]. The rootstocks are used for reducing the swelling of limbs caused due to filariasis and treatment of asthma.

Reports related to the phytoconstituents and pharmacological activities of *B. herbacea*, still appears to be insufficient. Our previous study is probably the first to report, the anti-inflammatory activity of *B. herbacea* root extract via histaminic pathway probably involving histamine receptors Type-I[3]. With this background, the present study was aimed to compare the inhibition of capillary permeability in mice (a hallmark feature of inflammatory disorder like filariasis, which is mainly mediated by histamine receptor type I [7,8]) of the three fractions of *B. herbacea* roots i.e., *n*-butanol, ethyl-acetate and aqueous fractions.

MATERIALS AND METHODS

Collection of plant material and extraction

The roots of *Byttneria herbacea* were collected from Ayodhya hills of Purulia, West Bengal, in the month of July, 2009. These specimens were then taxonomically identified by Dr. Arti Garg, Senior Taxonomist, Botanical Survey of India, Howrah (specimen no. CNH/1-1/(3)/2009/Tech.II/41 dated 09/07/2009) and is kept in our laboratory for future reference. The roots were cold macerated in methanol for 48 h and filtered. The filtrate was then concentrated in rotary evaporator (Eyela, Japan). The concentrated extract obtained was then freeze dried and stored at 4°C for further use.

Animals used

Adult male Swiss albino mice of body weight 20±2 g were used for the study. The care and use of the laboratory animals were strictly in accordance to the guidelines of the Institutional Ethical Committee (constituted under the guidelines Committee for the purpose of Control and Supervision of Experiments and Animals, Reg. No. 367).

Chemicals and reagents used

Methanol (E.Merck, Mumbai, India), ethyl-acetate (E.Merck, Mumbai, India), *n*-butanol (E.Merck, Mumbai, India), histamine dihydrochloride (Himedia, Mumbai, India), pontamine sky blue, pheniramine and mizolastine (DRL, Hyderabad, India). All other chemicals and reagents were of analytical grade (SRL, Mumbai, India).

Fractionation of the methanolic extract

The methanolic extract (100 g) was partitioned in a separating funnel with ethyl-acetate (100 ml) for 4 times. The combined ethyl-acetate fraction was concentrated in rotary evaporator (Eyela, Japan) and then freeze-dried. The filtrate left after partitioning with ethyl-acetate was fractionated with water saturated *n*-butanol (100 ml) for 4 times. The combined *n*-butanol fractions were then concentrated and freeze-dried in the same manner. The fraction left after partitioning with ethyl-acetate and *n*-butanol was taken as

aqueous fraction, which was again concentrated, freeze-dried and stored at 4°C for further use.

Phytochemical investigation (Qualitative)

The three fractions were tested for the presence of alkaloids (Dragendorff's test, Mayer test and Wagner test) and for saponins (lead acetate test) [9, 10].

Phytoconstituent investigation (Quantitative)

The total alkaloid content and the total saponin content of all the three fractions were determined according to the methods of Harborne (1973) [11, 12] and Obadoni and Ochuko (2001)[13], respectively.

Thin layer chromatography (TLC) of the fractions

TLC was performed for the methanolic extract and the three fractions in the solvent system containing *n*-butanol:acetic acid:water :: 13:3:5. Afterwards the TLC plate was sprayed with Dragendorff's reagent and allowed to dry.

Acetic acid induced capillary permeability (by dye leakage)

This study was carried out following the protocol of Filderman and Kovacs (1969)[14]. Briefly, in this study, male mice were divided into groups, each containing ten animals. Control vehicle (normal saline), pheniramine maleate (10 mg/kg), *n*-butanol fraction (50, 100 and 200 mg/kg), ethyl-acetate fraction (50, 100 and 200 mg/kg) and aqueous fraction (50, 100 and 200 mg/kg) were given orally 30 min prior to the intra-venous (*i.v.*) administration of 4% of pontamine sky blue solution in normal saline. This was immediately followed by intra-peritoneal (*i.p.*) administration of 0.6% acetic acid. After 1 h, animals were sacrificed by cervical dislocation and the peritoneal cavity was opened and drained. The peritoneal exudate was then centrifuged at 1500 g for 10 min and supernatant was taken. The dye concentration of the exudates was measured at 625 nm in spectrophotometer (Thermo Scientific), taking normal saline as blank.

Histamine induced capillary permeability

This study was carried out using male mice (n = 10). Control vehicle (normal saline), mizolastine (10 mg/kg) and *n*-butanol fraction (50, 100 and 200 mg/kg), ethyl-acetate fraction (50, 100 and 200 mg/kg) and aqueous fraction (50, 100 and 200 mg/kg) were given orally 30 min prior to the *i.p.* administration of histamine (10 µg in each animal) [15]. The animals were sacrificed by cervical dislocation, viscera was then washed with normal saline containing 0.1 mM disodium ethylenediamine tetra acetic acid (EDTA). The volume of each washing was made up to 6 ml with normal saline and centrifuged at 1500 g [16]. The total amount of protein in the supernatant was estimated with Bradford reagent.

HPLC analysis

Methanolic extract and the three fractions (*n*-butanol, ethyl-acetate and aqueous fractions) were analysed in HPLC using gradient solvent system. The solvent system consisted of acetonitrile and 0.05% formic acid in Milli-Q water. From 0.01 min to 10.00 min acetonitrile was 45% which was constant from 10.00 min to 15.00 min. Again from 15.00 min to 20.00 min acetonitrile concentration gradually increased to 90%, which remained same from 20.00 min to 25.00 min. From 25.00 min to 25.01 min acetonitrile concentration decreased to 0% (formic acid concentration increasing) this continued till 30.00 min.

Statistical analysis

The results were expressed as Mean ± S.E.M. Statistical analysis were performed with one-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's tests. Here, **p*-value < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

B. herbacea belonging to Sterculiaceae family is widely used by tribal people of West Bengal, Bihar, Jharkhand, Chattisgarh and Orissa [4,5,17] for the treatment of filariasis, cholera, diarrhea and asthma. Hence considering the traditional use of *B. herbacea* roots by the folklore for the management of filariasis (the role of histamine in angiogenesis and capillary permeability in filariasis [7,8]) we have

investigated the effect of various fractions of the roots of this plant against edema grossly induced by capillary permeability. In our previous report [3], while studying the anti-edemogenic activity of *B. herbacea* root extract, it was observed that the hydroalcoholic root extract probably involves histamine receptor type-I (H₁). Accordingly, in the present study an attempt has been made to compare the fractions, which may be responsible for the anti-edemogenic activity via inhibition of capillary permeability.

The fractionation of methanolic root extract led to 25.04%, 30.1% and 6.75% (w/w) yield in *n*-butanol, aqueous and ethyl-acetate fraction respectively. Phytochemical investigation and TLC (Fig. 1) revealed the presence of alkaloids in all the three fractions i.e., *n*-butanol, ethyl-acetate and aqueous fraction. Further, a quantitative estimation of alkaloids in these three fractions exhibited presence of alkaloid to the extent of 15% in *n*-butanol fraction, 4.6% in aqueous and 3% in ethyl-acetate fraction as compared to 6.67% of alkaloid in the crude extract as reported earlier [3]. Similarly quantitative estimation of saponin revealed it's maximum content in the aqueous fraction (8.25%), as compared to ethyl-acetate fraction which was about 1%, whereas in the *n*-butanol fraction saponin test was found to be negative.

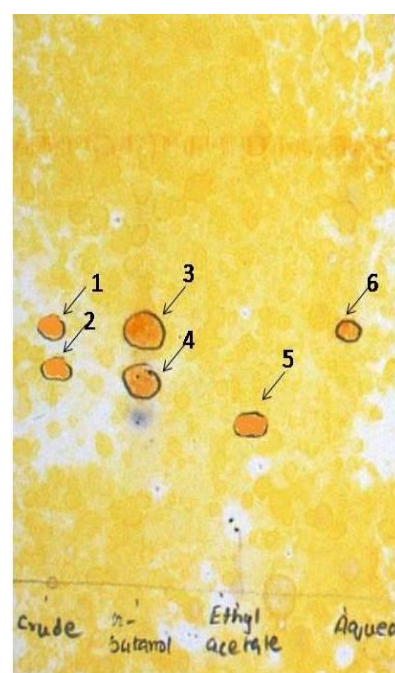


Fig. 1: TLC plate indicating alkaloid spots when sprayed with Dragendorff's reagent with the arrows indicating orange alkaloid spots (R_f of spot 1=0.58; spot 2= 0.46; spot 3= 0.58; spot 4= 0.46; spot 5= 0.4 and spot 6= 0.58).

In a report by, Spector and Willoughby (1963) [18] and Whittle (1964) [19] it has been indicated that, acetic acid induced writhing in mice is characterized by increased capillary permeability, which is grossly mediated by histamine, 5-hydroxytryptamine (5-HT) and bradykinin[20]. Histamine has also been established as an important factor in capillary permeability in yet another report by Gaddum, (1948)[21]. Since then several investigators have established the role of histamine in vasodilation and during increased capillary permeability, which is also a pivotal characteristic of acute inflammatory responses [22, 23, 24]. According to a report by Tomisawa and Sato (1973)[16], the protein content or the leaked dye of the peritoneal exudate corresponds well with the intensity of acute inflammation. Owen et al., (1980)[7] and Bhargava et al., (1977)[15], justified that the capillary permeability is solely mediated by H₁ receptors. Comparing these reports and our findings with regards to the anti-edemogenic activity of *B. herbacea*, involving histamine receptor Type-I, in the present study we have considered modulation of histamine receptor as an important factor behind the traditional use of *Byttneria herbacea* in the management of inflamed limbs during filariasis. Accordingly histamine induced

capillary permeability was selected for the comparison of the fractions of *B. herbacea* methanolic root extract.

The experimental data of acetic acid induced capillary permeability (by dye leakage method) in mice, suggests that amongst the three different fractions, in the dose range of 200 and 100 mg/kg, *n*-butanol exhibited maximum significant inhibition of 78.74% and

35.43% (Fig 2.), respectively, followed by aqueous fraction (61.21% and 31.46%, respectively) as shown in Fig 3. Ethyl-acetate fraction did not exhibit any significant inhibition at the above mentioned doses (Fig 4). It will be important to mention that pheniramine when used as a standard histamine antagonist could induce 60.89% significant inhibition even at the dose of 10 mg/kg.

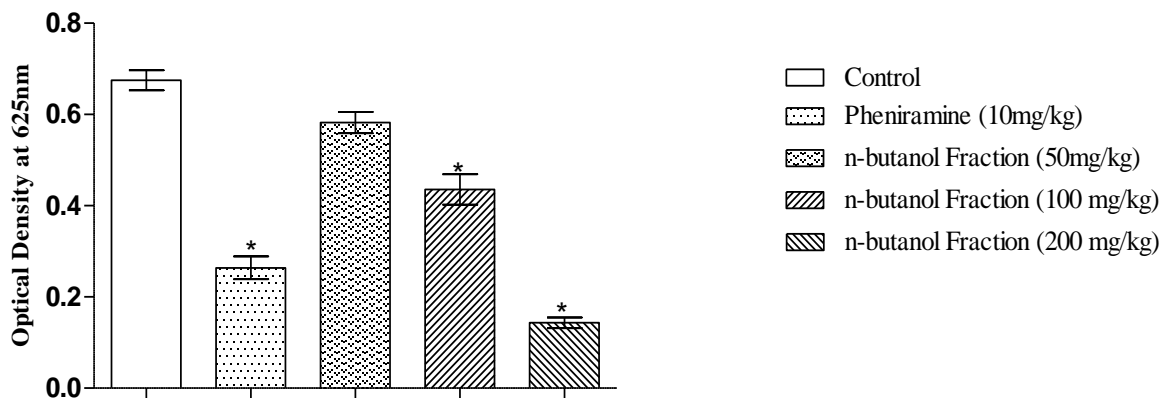


Fig. 2: Effect of *n*-butanol fraction on acetic acid induced capillary permeability (by dye leakage). Values expressed Mean±SEM; n=10; *p<0.05.

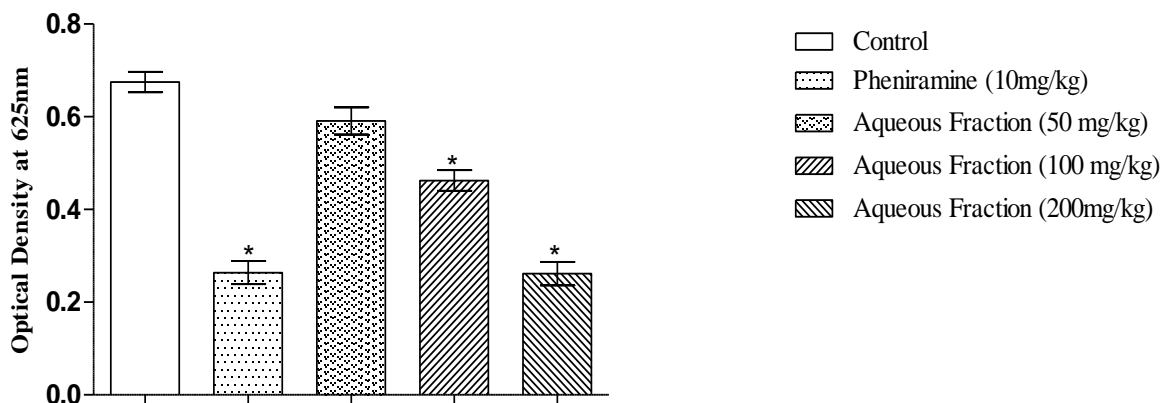


Fig. 3: Effect of aqueous fraction on acetic acid induced capillary permeability (by dye leakage). Values expressed Mean±SEM; n=10; *p<0.05.

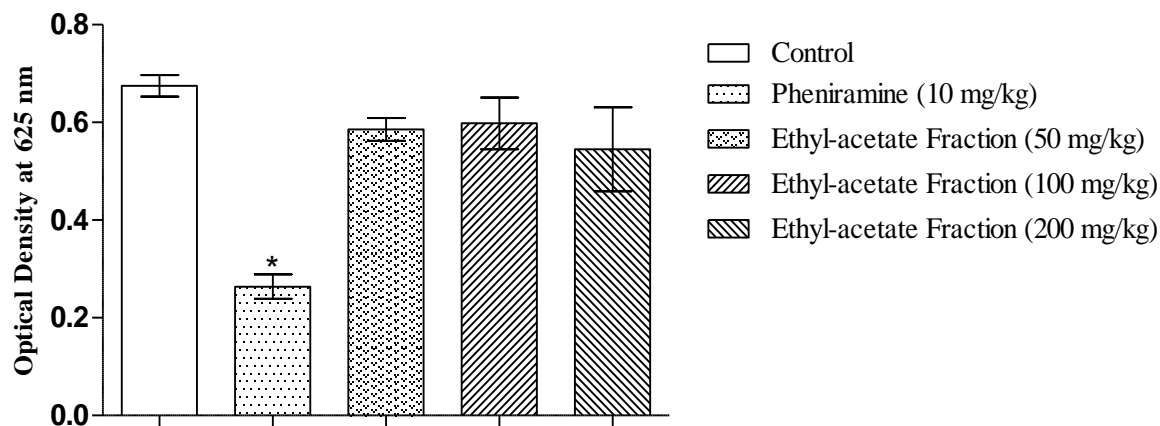


Fig. 4: Effect of ethyl-acetate fraction on acetic acid induced capillary permeability (by dye leakage). Values expressed Mean±SEM; n=10; *p<0.05.

Similarly when histamine was used to induce capillary permeability in mice, *n*-butanol fraction exhibited maximum inhibition (76.74% and 55.66%, respectively), followed by aqueous fraction (57.42% and 46.55%, respectively) in the same dose range of 200 and 100 mg/kg, in a dose-dependent manner.

Ethyl-acetate fraction did not show any significant inhibition. In this experiment to identify the specific involvement of histamine, comparatively selective histamine type- I antagonist, mizolastine was used, which also revealed 45.86% inhibition at the dose of 10 mg/kg (Table 1).

Table 1: Histamine induced capillary permeability of the three fractions of *Byttneria herbacea*.

Test Drug	Concentration (mg/kg)	Protein Content (mg/ml)	Percentage Inhibition
Control	-	0.944±0.053	-
Mizolastine	10 mg/kg	0.511±0.032	45.86%*
<i>n</i> -butanol Fraction	50 mg/kg	0.793±0.03	15.92% ^{ns}
	100 mg/kg	0.418±0.02*	55.66%*
	200 mg/kg	0.219±0.02*	76.74%*
	200 mg/kg	0.801±0.03	15.17% ^{ns}
Aqueous Fraction	50 mg/kg	0.801±0.03	15.17% ^{ns}
	100 mg/kg	0.504±0.01*	46.55%*
	200 mg/kg	0.402±0.04*	57.42%*
Ethyl-acetate Fraction	50 mg/kg	0.828±0.04	12.26% ^{ns}
	100 mg/kg	0.797±0.02	15.54% ^{ns}
	200 mg/kg	0.789±0.05	16.34% ^{ns}

Values expressed Mean±SEM; n=10; *p<0.05

The HPLC chromatograms on comparison indicates that *n*-butanol and aqueous fractions shared two major peaks with that of the crude methanol extract, while the ethyl-acetate fraction was observed to be devoid of such peaks (Fig. 5.).

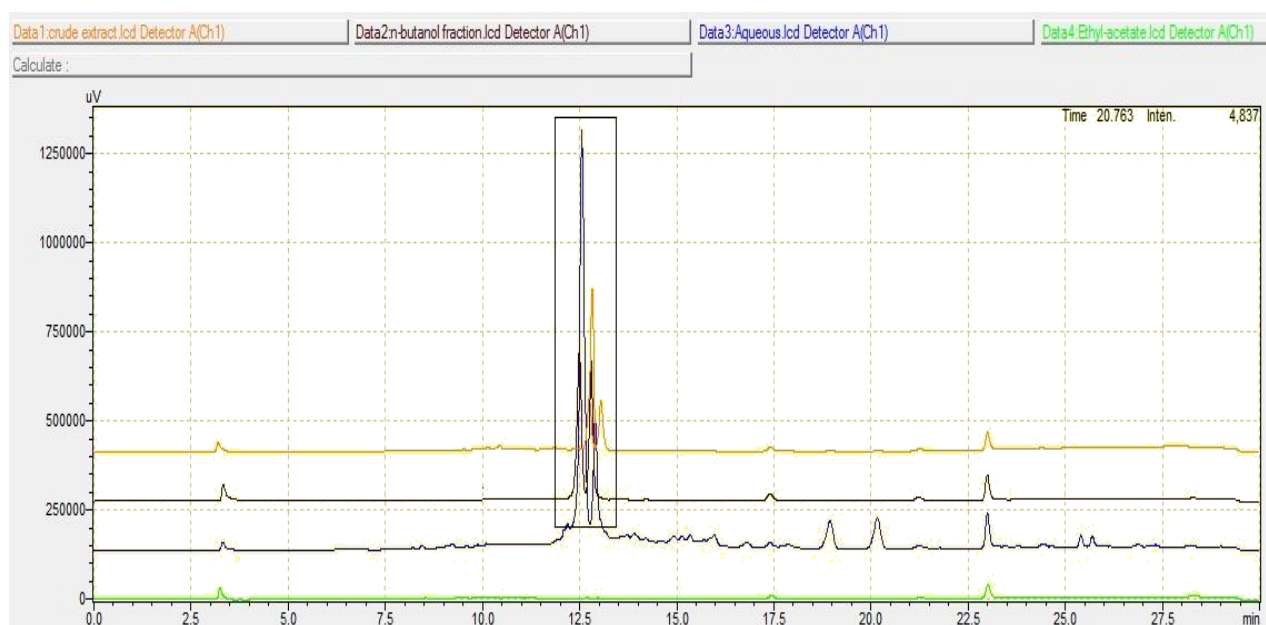


Fig. 5: HPLC chromatogram overlay of crude methanol extract, *n*-butanol fraction and ethyl-acetate fraction of *Byttneria herbacea* roots.

The results so far indicate that amongst the three fractions, *n*-butanol appears to be more potent in inhibiting capillary permeability, followed by aqueous fraction. The HPLC chromatogram is preliminary where isolation is yet to be done but the method involved for the development of the same might be helpful for further study on the plant *B. herbacea*. This is particularly important since no significant phytochemical findings have been reported, at least as per our literature survey.

It will also be important to mention, that the involvement of histamine (and/or histamine receptor), in pharmacological activity of the fractions could be indicated, considering the response of the standard histamine antagonist and our previous report [3] indicating H1 receptor specific mRNA suppression by *Byttneria herbacea*.

Further, considering the compositional study of the fraction mentioned earlier in this section it may be possible that the anti-oedemogenic activity of the *n*-butanol fraction might be attributed to the increased alkaloid content grossly involving histaminergic response. Presently, we are engaged in isolation of active compounds responsible for the proposed activity.

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