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PHYTOCHEMICAL CHARACTERIZATION AND ANTI-INFLAMMATORY ACTIVITY OF BRIDELIA RETUSA BARK SPRENG. IN ACUTE AND CHRONIC INFLAMMATORY CONDITIONS: A POSSIBLE MECHANISM OF ACTION

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ABSTRACT

Several species of Bridelia have been used to relieve pain in arthritic condition (rheumatic pain) in Indian folk medicine. This study was designed to explore the preliminary phytochemical analysis of Bridelia retusa spreng. (Euphorbiaceae) and further evaluation of its analgesic, anti-inflammatory and anti-arthritic activity as well as underlying mechanism of fraction in rats. Bridelia retusa spreng bark was extracted using pet.ether,ethyl acetate and acetone. All the extracts were significantly inhibit abdominal writhings response of mice and inhibit licking time in late phase by inducing formalin. Test samples could also significantly inhibit mean paw edema of rats induced by carrageenan & histamine at dose of 200 & 400 mg/kg, ip. Test materials also showed significant dose dependent reduction in cotton pellet granuloma & acetic acid induced vascular permeability at 400 mg/kg. In FCA induced arthritic rats, physical changes, biochemical and hematological parameters observed in arthritic animals were altered significantly to near normal condition after oral administration of Bridelia retusa fractions. The maximum paw edema inhibition at day 21 was observed at 400 mg/kg. It also proved significant protection against protein denaturation & RBC membrane damage. The GC-MS analysis of EA extract revealed the presence of β-sitosterol, stigmasterol, lupeol and friedelin (Pentacyclic triterpenoid). In conclusion, the current study has demonstrated the analgesic; anti-inflammatory and anti-arthritic activities of Bridelia retusa bark fractions and suggested that the molecular membrane might be associated with inhibition of biochemical and hematological parameters. Overall bioactive profile of Bridelia retusa makes it an attractive candidate for future development as a drug or phytomedicine.

KEYWORDS: Bridelia retusa Spreng. Lupeol, Anti-inflammatory; Freund's complete adjuvant; Euphorbiaceae; Protein denaturation; Leukocyte migration.

INTRODUCTION

Bridelia retusa Spreng. Syn:Bridelia airy shawii (Family: Euphorbiaceae) is a small to moderate sized deciduous tree, spinous when young with the grey bark, found throughout India up to altitude of 1000 m except in very dry regions. In Pharmacological trials the bark of Bridelia retusa exhibited anti-viral, hypoglycemic and hypotensive properties.[1] According to Ayurveda, the bark is good for removal of urinary concretions, useful in lumbago and hemiplegia. The bark is also used as liniment with gingelly oil in rheumatism.[2] Bark is documented to be used ethno botanically to promote antifertility. The presence of triterpene ketone and 16-40% of tannins in the bark has been reported.[3] Literature survey revealed that there is no systematic approach has been made on Bridelia retusa bark therefore it was needed to investigate the phytochemical

and pharmacological property. Bridelia retusa bark has been previously investigated for the acute inflammatory effect by carrageenan-induced hind paw edema.[4] Recently reported that there has been a growing interest in plants as a significant source of new pharmaceuticals. Species of Bridelia have been widely used in folk medicine for their rheumatic property. In general, phytosterols, triterpenoid and tannins have been reported to display anti- inflammatory, anti-ulcer, anti-nociceptive and antiarthritic properties. There is lack of scientific validated data in support to traditional use of plant; hence the present study was designed to evaluate the analgesic and anti-inflammatory effects of Bridelia retusa bark and to elucidate its possible mechanisms of action.

MATERIALS AND METHODS
Plant material and preparation of extracts
The plant material of Bridelia retusa bark was collected from Toranmal region of Satpuda hills, India and it was identified by Dr. D. A. Patil, Taxonomist, SSVPS Science College, Dhule, MS, India. A voucher specimen (B-12) of plant was deposited at the RCPCOP herbarium for reference purpose.

The shade-dried bark powder (1 kg) was successively extracted with petroleum ether (60-80oC) (PE), mixture of methanol: dichloro methane (1:1) and acetone: water mixture (70:30) (ACE). Further residue obtained from methanol: dichloro methane (1:1) was again partitioned with ethyl acetate (EA; 4x500ml). The yields of PE, EA and ACE extracts were 0.7, 1.1 and 9.2% (w/w), respectively. proximate chemical analysis and pharmacological activity of all the extracts was carried out according to standard method.

Separation and Isolation of Phytoconstituents
The unsaponifiable fraction of PE (5 g) was subjected to column chromatography on a silica gel (60-120 mesh)with gradient elution using petroleum ether: ethyl acetate[(90:10,82:18 v/v) yielded compound 1 (28 mg) and compound 2 (22 mg). similarly EA fraction was chromatographed over silica gel (60-120 mesh) column, eluted with methanol and chloroform (80:20 v/v) in order of increasing polarity to gave compound 3. All the compounds were identified and characterized by melting point, UV, FT-IR, NMR, HPLC, HPTLC and GC-MS analysis. The compounds were identified by comparison with the mass fragmentation pattern of standards available in NIST library, USA.

Defatted ACE extract of Bridelia retusa bark was hydrolyzed with 2 M H_2SO_4. Hydrolyzed portion were further partitioning with solvent ether and dried. Dry residue dissolved in methanol and spotted on TLC plate using toluene: ethyl acetate: formic acid (2.5:5:0.5) as solvent system. It revealed the presence three spot on plate. Prominent blue colored spot, spraying with alcoholic ferric chloride reagent, was separated by preparative TLC technique, dried and labeled as compound 4; the same was subjected to melting point, UV, FTIR, HPTLC and LC-MS/MS study.

Liquid chromatography-Mass spectroscopy (LC-MS/MS)
LC-MS: Thermo scientific USA, Model-LCQ fleet and TSQ quantum access with surveyor Plus HPLC system. Mass range 50-2000amu, ESI & APCI source, HPLC system with auto sampler and PDA detector. LC-MS analysis for identification of chemical compound by using standard library.

HPLC analysis
The HPLC system of Agilent 1200 series system (Agilent Technologies, Waldbronn, Germany) with photodiode array detector, ODS C18 (5 μm) column was used (150mm×4.6mm) interfaced with an IBM Pentium 4 personal computer. Elution of the phyconstituents with isocratic of two solvents denoted as A and B was employed (Solvent A: acetonitrile and B: water (60:40%), flow rate: 1ml/min, injection volume: 20μl, wavelength: 286nm. The HPLC profile of isolated compounds was compared with reference compound at a specific wavelength. Identification of compounds was performed on the basis of the retention time, co-injections, and spectral matching with standards. The chemical structures of the isolated compounds are given in Fig. 2.

HPTLC
High-performance thin layer chromatography (HPTLC) was carried out by using precoated silica gel 60 F 254 HPTLC plates (4 x10 cm, Merck, Germany) on CAMAG TLC Scanner 3 (Camag, Switzerland) system. The extracts were applied over the plate with a Linomat IV applicator. The resolved peaks were scanned at different wavelengths.

Experimental Animals
Adult albino wistar rats and swiss albino mice of either sex weighing between 150-230 and 30-40 g, respectively were used for the study. The animals were housed in well ventilated colony cages in the air conditioned animal house at (12:12 hr) and fed with standard pellet diet and water ad libitum. All the experimental procedure and protocols used in the study were approved by Institutional animal Ethical committee (Protocol number 045/2005) under North Maharashtra University, Jalgaon, India in accordance with Committee for the purpose of Control and supervision on experiments on Animals (CPCSEA), guidelines, Chennai, India.

Acute oral toxicity study
Acute oral toxicity studies were performed according to OECD-423 guidelines (acute toxic class method). Swiss mice of either sex selected by random sampling technique were employed in this study. Mortality was observed in 2/3 or 3/3 animals and then the dose administered was considered as toxic dose.

Anti-Inflammatory Activity
Carrageenan and histamine induced rat paw edema
According to a modification of method Winter et al., (1962), [5] male wistar rats (150–200 g each) divided into groups of six animals each were used for acute inflammation, induced in the right hind paw of rats by subcutaneous injection of a 0.1 ml of 1% freshly prepared carrageenan in saline and sub-plantar injection of histamine (0.1ml of 0.1%).volume of the injected paws was measured every hour for 6 h using a plethysmometer (Ugo Basile, Italy). The percentage increase in paw volume or swelling was calculated based on the paw volume prior to injection.[6]

Acetic acid induced vascular permeability

The method of Whittle (1964)[7] was used to evaluate the effect of Bridelia retusa on vascular permeability in adult albino mice of both sexes. One hour after oral administration of the extract, 0.1 ml/10 g b. w. of 1% Evans blue solution was intravenously administered through the tail vein into group of mouse(n=6), immediately followed by an intra-peritoneal injection of, 0.1 ml/10 g of 0.7% acetic acid. Thirty minutes after the administration of acetic acid, the mice were killed by cervical dislocation. The peritoneal cavity washed with normal saline (10 ml) into heparinized tubes and centrifuged. The concentration of Evans blue in the peritoneal cavity was measured by absorbance at 630 nm using an ELISA Analyzer (Biotek, USA). The vascular permeability was represented in terms of the absorbance (A630) that leaked into the cavity.[8]

Granulomatous tissue induction

The effect of Bridelia retusa fractions on chronic inflammation was evaluated using cotton-pellet granuloma test in rats.[9] On day 1, adult albino rats of either sex received orally 200 & 400 mg/kg of PE, EA, ACE fractions. Control group received vehicle only (1%,w/v,carboxymethylcellulose,5ml/kg).Thirty minutes later, one autoclaved cotton pellets 15±1.0 mg were aseptically implanted under the previously depilated back of rats anaesthetized with diethyl ether. Test materials were administered once daily for the next 7 days. On day 8, animals were killed by overdose of ether. The pellets were dissected out, freed of tissue attachments and dried in the oven overnight at 60◦C. The dry pellets were weighed and the mean weight of the granuloma tissue formed around each pellet determined. The level of inhibition of granuloma tissue development was calculated.[10]

ANALGESIC ACTIVITY
Acetic acid induced Writhing method

Male wistar albino mice (n=6) weighing 30-35 g were used. The total number of writhings response was produced by intra peritoneal administration of acetic acid solution (1%, 10 mL/kg). The mice were treated with PE, EA and ACE of B. retusa (200 &400 mg/kg), thirty minute before administration of acetic acid. The number of abdominal writhings was counted during continuous observation for 20 min beginning at 5 min after the acetic acid injection. Percentage of protection was calculated by using the following ratio: (control mean treated-- mean)×100/control mean.[11,12]
Formalin induced nociception

The antinociceptive activity of the Bridelia retusa fractions were evaluated using the formalin test according to Hunskar et al.[13] One hour before testing, the rat was placed in a standard cage that served as an observation chamber. The dried extract (PE, EA, ACE) and ethyl morphine hydrochloride (10 mg/kg) were administered i.p. in a volume of 1.5 ml. control group received only vehicle (2 ml). Fifteen minutes after treatment, 50 μl 2.5% formalin was injected to the dorsal surface of the left hind paw. The rat was observed for 30 min after the injection of formalin, and duration of paw licking was measured for 0–5 min (early phase) and 15–30 min (late phase). The percentage inhibition of licking was calculated.

ANTI- ARTHRITIC ACTIVITY
Freund's complete adjuvant (FCA) induced arthritis

Freund's complete adjuvant (FCA, Sigma, USA) induced Arthritis model was used to assess the anti-arthritic activity in albino rats. Animals were randomly divided into groups of six animals each (n=6). Group I served as control received 1% tween 80, Group II received dexamethasone (5mg/kg, p.o.) served as reference standard and Group III, IV and V received the crude fractions of PE, EA and ACE of B.retusa at the dose of 200 & 400 mg/kg,bw.p.o. respectively. Arthritis was induced by injecting a 0.1ml (0.5% w/v) suspension of killed Mycobacterium tuberculosis bacteria homogenized in liquid paraffin into the sub plantar region of left hind paw.[14-15] Drug treatment was started from the initial day i.e. from the day of adjuvant injection (0 day), 30 minutes before adjuvant injection and continued till 21st day. Paw volume was measured on every three days after the induction of arthritis till day 21 using plethysmometer (Ugo Basile 7140, Italy). The mean changes in injected paw edema with respect to initial paw volume, were calculated on respective days and % inhibition of paw edema with respect to untreated group was calculated. On day 21st, at the end of the experimental period, the animals were sacrificed by cervical decapitation and blood was collected and separated plasma for assaying the hematological & biochemical parameters.[16] Arthritis was also assessed by body and spleen weight of rats.

Hematological investigation

Blood samples were collected from the retro-orbital plexus for laboratory tests. The hematological parameters like Red Blood Cell (RBC), White Blood Cell (WBC), and Erythrocyte Sedimentation Rate (ESR) were determined.[17]

RA and CRP Test

Rheumatoid arthritis (RA) testing was carried out by utilizing the agglutination reaction between immunoglobulin and rheumatoid factor. C-reactive protein (CRP) factor based on the immunologic reaction between CRP and latex particles. Both the test was measured in serum using a commercial kit.

Determination of tissue marker enzymes

The marker enzymes glutamate oxalo acetate transaminase/aspartate aminotransferase (GOT/AST) and glutamate pyruvate transaminase/alanine aminotransferase (GPT/ALT) and alkaline phosphatase (ALP) were analyzed in serum. A lysosomal enzyme such as acid phosphatase (ACP) was estimated in plasma. Spleen were dissected out, washed and

transferred to an ice-cold saline solution. The organs were weighed.

In- vitro evaluation of membrane stabilizing property Leukocyte migration assay in mice

The test was carried out using the technique of Hong-Yue Ma et al.[8] After oral administration of test samples of B.retusa, animals received an intraperitonial injection of 1% CMC-Na solution in normal saline after 1 h. Four hours later, mice were sacrificed and the peritoneal cavities were washed with 5ml of the normal saline. Twenty micro liter of peritoneal fluid was mixed with 0.38 ml of Turk's solution (0.01% crystal violet in 3% acetic acid) and the number of leukocytes was counted under a light microscope.

Heat induced haemolysis

Preparation of erythrocyte suspension: Fresh rat blood was collected and transferred to heparinized centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min, and washed three times with equal volume of normal saline. The blood volume was measured and reconstituted as a 40% v/v suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4). The inhibitory effect of test samples on rat erythrocyte haemolysis was assayed by the method described by Perez et al.[18] The percent inhibition of haemolysis was   calculated using.[19-20] Percent inhibition = A 540 Control - A 540 Sample X100 / A 540 Control.

Inhibition of protein denaturation

The reaction mixture consisted of bovine serum albumin (5% aqueous solution) and test extract. PH was adjusted at 6.3 using a small amount of 1N HCl. The samples were incubated at 370c for 20 min and then heated at 570c for 3 min, after cooling; phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm. For control tests distilled water was used instead of extracts while product control tests lacked bovine serum albumin.[21] The percentage inhibition of protein denaturation was calculated as: Percentage inhibition =   100 - (O.D of test – O.D. of product control)/ O.D. of control X100.

Statistical analysis

Data are reported as Mean±SD. and were analyzed statistically by analysis of variance (ANOVA) followed by Dunnett's test. Results with $p < 0.05$ were considered significant.

RESULTS

Phytochemical analysis of Bridelia retusa

Extracts of Bridelia retusa gave positive test for steroidal/triterpenoidal glycosides (Liberman Burchard test, Salkowski test for steroids/triterpenoids; Molisch test, Fehling's test for carbohydrates) and tannins (ferric chloride test), etc.

The gas chromatography-mass spectroscopy analysis of Bridelia retusa extract resulted for the presence of β–sitosterol, stigmasterol, friedelin & Lupeol as displayed in Fig.1.
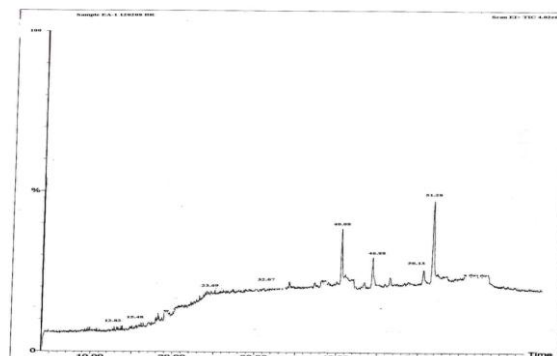

Fig. 1: GC-MS chromatograph of EA of Bridelia retusa stem bark.

Time (min) 40.08: β-sitosterol; 46.88: stigmasterol; 50.13: Lupeol; 51.28 Friedelin

The melting point of Compound 1 and 2 were 1340 and 1760C respectively and both of them showed positive Lieberman-Burchared chemical test. Mass spectrum of compound 1 & 2 showed a parent molecular ion [M+H]+ peak at m/z 414 and 412, respectively which corresponds to C29H50O and C29H48O. Further evidence in support of the structure of compound 1 & 2 was provided by its IR, 1H-NMR spectral data. The 1H NMR spectra of compound 1 & 2 was similar to those of a known compound β-sitosterol and stigmasterol respectively, thus, it was as assigned as β-sitosterol and stigmasterol.

Similarly the melting point of compound 3 was 215-217OC. molecular ion [M+H]+ peak at m/z 426, which corresponds to C30H50O. Besides the molecular ion peak at 426, EI mass spectrum also showed other fragment ion peak at m/z 411 (M-CH3), 218 (M-C14; H28), 207 (M-16; H- 27) which are characteristic for pentacyclic triterpenoid .The 1H NMR, 13C-NMR spectra of compound 3 was similar to those of a known compound Lupeol, thus the structure of compound 3 was assigned as Lupeol.

LCMS spectra and mass fragmentation pattern of compound 4 was identified with standard spectra of gallic acid (m/z ration of 175). The highest relative abundance was reported in compound 4 and in standard gallic acid with 174 mol. wt. According standard calibration curve and linearity equation 49.20 mg of compound 4 as gallic acid present in 100 mg of ACE extract. The percentage purity of compound 4 was found to be 94.26 %. The structures of chemical compound are shown in Fig.2 a, b, c, d.
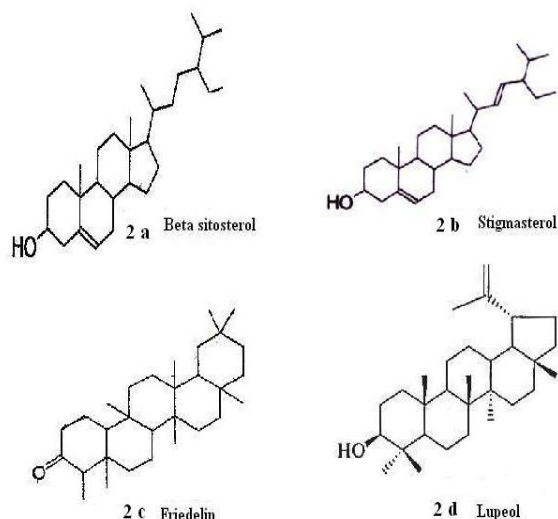
Fig. 2: Probable structure of Phytoconstituents. 2a: β-sitosterol; 2b: stigmasterol; 2c: Friedelin; 2d: lupeol.

In this paper, reversed phase HPLC chemical fingerprinting method was utilized and developed for identifying and determining the phytoconstituents of B.retusa extract. A total of 9 characteristic peaks in the chromatogram 1 and 8 peaks in chromatogram 2 were observed. β-sitosterol and stigmasterol were identified in Chromatogram 1 whereas lupeol was identified in chromatogram 2 according to corresponding standards (Fig.3). In quantitative determination, calibration plot of all reference compound showed linearity was good within the test range.
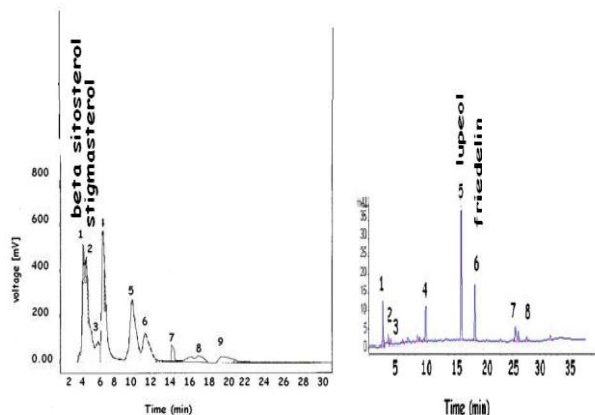
Fig. 3: HPLC chromatogram of petroleum ether (a) and ethyl acetate extract (b) of Bridelia retusa.

Anti-inflammatory activity
Carrageenan and histamine-induced rat paw edema
PE, EA and ACE extracts of Bridelia retusa (200 & 400 mg/kg, p.o.) significantly ($P<0.01$) inhibited the mean paw volume at 3 h after carrageenan injection. The % inhibition of rat paw edema was gradually increases in PE, ACE and EA at higher dose study. Anti-inflammatory activity of PE, EA and ACE (400 mg/kg,

p.o.) produced statistically significant ($P < 0.01$ and $P < 0.001$) inhibition of the edema induced by histamine at 2nd & 3rd h., when compared to the vehicle treated control groups. The observations are given in Table 1.

Acetic acid induced vascular permeability
Oral administration of Bridelia retusa (200 & 400 mg/kg) extract evoked a significant ($P < 0.001$) dose-related inhibition of vascular permeability induced by acetic acid in mice. The observations are given in Table 5.

Cotton Pellet-Induced Granuloma Formation
Fig.4.shows the effect of B.retusa bark extracts on cotton pellet-induced granuloma formation in rats. The results indicate that PE, EA, and ACE at an oral dose of 400 mg/kg significantly inhibited the transudative weight and granuloma formation. Diclofenac (mg/kg) elicited significant reduction.
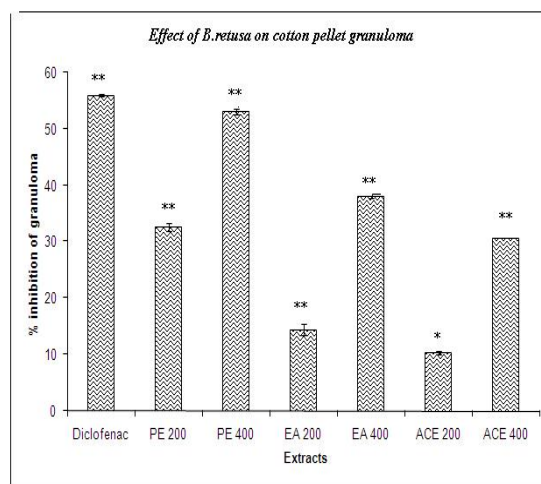

Fig. 4: Effects of B.retusa bark on cotton pellet granuloma.

$*P<0.05$, $**P < 0.01$, as compared with the control group. (One-way ANOVA followed by Dunnett's test)

Analgesic activity
Writhing test
The peripheral analgesic action of EA (400 mg/kg) was found to be comparable to that of morphine (10mg/kg). The peripheral analgesic activities of B.retusa and its extracts seem to be related to the inhibition of the synthesis of the arachidonic acid metabolite. Maximum percentage of inhibition of writhing response was observed with EA (400mg/kg) as 65.62%.PE, EA and ACE extract significantly ($P<0.01$) reduced number writhings at dose dependent manner (200 & 400 mg/kg). The observations are given in Fig. 5.
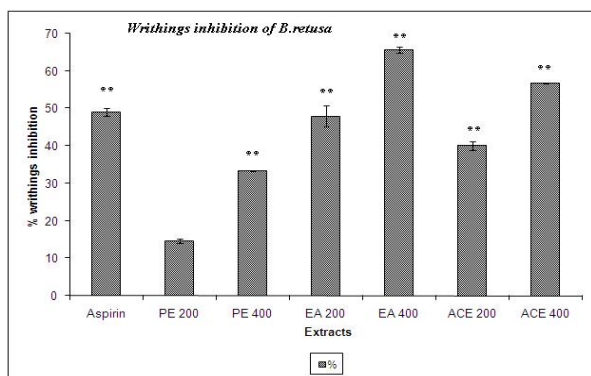
Fig. 5: Effects of PE, EA and ACE Fraction of Bridelia retusa on Acetic Acid-Induced Writhing Response in Mice.

**p<0.01, significant as compared to the control by Dunnett's t-test.

**Formalin induced nociception method in rat**
The results given in Table 2. that the time spent on licking the injured paw was significantly attenuated in the late phase (15–30 min) by the of B.retusa extracts i.e.PE, EA and ACE at 400 mg/kg dose .When tested in the formalin induced-pain as a reference, morphine showed significant inhibition (p < 0.01) of licking responses in the late phase (51-53 % inhibition).

Anti-arthritic activity
Freund's complete adjuvant-induced arthritis in rats
The inflammatory effect of B.retusa bark on a chronic arthritis model was evaluated using FCA-induced arthritis in rats. Fig.6 showed that extracts of B.retusa was capable of reducing the severity of arthritic lesions and a statistically significant (P<0.05) inhibition of the paw edema as compared to the control group. The 400 mgkg−1 of PE, EA and ACE of B.retusa showed

significantly potent inhibition from day 6-21 at 31-60%, 27-41% and 26-44 % respectively. Swelling and redness developed over a 24 h period in the foot injected with adjuvant. This inflammatory reaction subsided slightly during the next 11 to 14 day and then increased at that time when disseminated arthritis appeared. In rats treated from the day of adjuvant injection, the paw swelling was completely suppressed and no secondary sign was seen. The drug treatment for 14 d from the day of adjuvant injection suppressed the secondary increase in swelling of the injected foot that occurred with the appearance of polyarthritis.
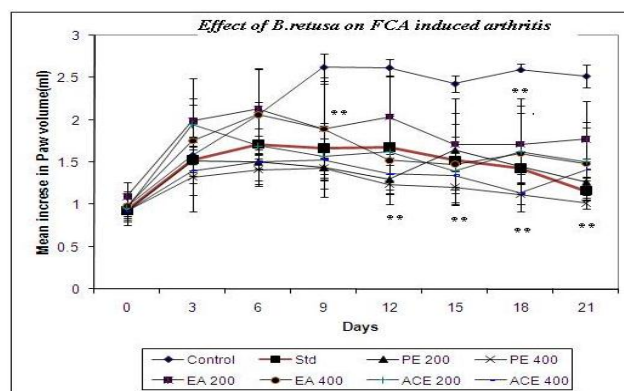
Fig 6: Effects of B.retusa bark on FCA induced arthritis rat.

0.1 ml of Freund's complete adjuvant (FCA) into the sub plantar region in the right hind paw. The adjuvant contained 10 mg heat killed Mycobacterium tuberculosis in 1ml paraffin oil.*P<0.05, **P < 0.01, ***P < 0.001 as compared with the control group. (One-way ANOVA followed by multiple Dunnett's test)

Table 1: Effect of Bridelia retusa bark on Carrageenan and histamine induced rat paw edema.

Test Group & Dose (mg/kg)	Mean(±SD) Increases in Paw Volume in ml				
	Carrageenan			histamine	
	3 h	5h	1h	2h	3h
Control	0.533±0.024	0.721±0.014	0.383±0.063	0.333±0.054	0.283±0.074
Diclofenac 13.5	0.252±0.011** (52.72)	0.282±0.016** (60.88)	0.237±0.064** (38.12)	0.225±0.038* (32.43)	0.195±0.033* (31.09)
PE 200	0.266±0.036** (50.09)	0.339±0.011** (52.98)	0.170±0.079*** (55.61)	0.208±0.073** (37.53)	0.123±0.020** (56.53)
PE 400	0.255±0.017** (52.15)	0.310±0.012** (57.00)	0.260±0.066** (32.11)	0.287±0.067 (13.81)	0.243±0.068 (14.13)
E 200	0.327±0.022** (38.64)	0.423±0.016** (41.33)	0.216±0.029*** (43.60)	0.230±0.043* (30.93)	0.131±0.049** (53.71)
EA 400	0.292±0.014** (45.21)	0.383±0.015** (46.87)	0.221±0.058** (42.29)	0.233±0.072* (30.03)	0.247±0.088 (12.72)
ACE 200	0.380±0.010** (28.70)	0.471±0.017** (34.67)	0.203±0.042*** (46.99)	0.173±0.050** (48.04)	0.137±0.035** (51.59)
ACE 400	0.282±0.007** (47.09)	0.342±0.011** (52.56)	0.146±0.048*** (61.87)	0.115±0.047** (65.46)	0.106±0.045** (62.55)

Values are expressed as Mean ± S.D. (n=6), while those in parenthesis represent percentage inhibition of paw edema. *P<0.05, **P<0.01, ***P<0.001. Compared with vehicle control (ANOVA followed by multiple Dunnet´s test).

Table 2: Effect of various fractions of Bridelia retusa stem bark on the licking time of rats in formalin test.

Treatment / Dose mg/kg		Liking time (sec)		% Inhibition	
		0-15min	15-30min	1st phase	2nd phase
Control	----	52.08±2.29	66.17±4.24	----	----
PE	200	32.97±1.73	37.01±1.80	36.69	44.06
PE	400	21.33±1.23	27.57±1.90	59.04	58.33
EA	200	31.11±1.05	36.47±1.65	40.26	44.88
EA	400	23.44±3.56	27.70±2.27	54.99	58.13
ACE	200	43.46±3.35	46.46±2.76	16.55	29.78
ACE	400	28.45±2.84	34.49±1.92	45.37	47.87
EMH	----	25.32±3.53	31.30±1.71	51.38	52.69

Values are expressed as Mean ± S.D. (n=6) compared with vehicle control (ANOVA followed by multiple Dunnet´s test). All are significant at $p<0.01$. PE=pet. ether, EA=ethyl acetate, ACE=acetone extracts; EMH= Ethyl morphine hydrochloride

Table 3: Effect of B. retusa extract on hematological parameter of rats treated with Freund's complete adjuvant.

Group	Dose (mg/kg)	WBC count (10^3 /mm3)	RBC	Platelet count (10^5 /mm3)	ESR (mm/hr)
Normal	-----	8.50 ±0.165	6.02±0.529	2.35±0.223	3.78 ±0.185
Control	----	12.21±0.288	4.25±0.232	3.46±0.180	5.54 ±0.490
PE	200	11.32±0.460	4.74±0.320	2.89±0.151	4.99 ±0.209*
	400	8.98±0.417***	5.87±0.198**	2.58±0.077	4.06 ±0.439***
EA	200	11.29±0.718	4.60±0.035	2.94±0.219	4.87 ±0.496
	400	9.25±0.563***	5.66±0.340**	2.61±0.217	4.25 ±0.323**
ACE	200	11.04 ±0.659*	4.57±0.385	2.57±0.201	5.15 ±0.447
	400	10.45±1.228***	5.22±0.057**	2.70±0.144	4.16 ±0.167***
Diclofenac	13.5	9.28±0.320***	5.63±0.415**	2.48±0.283	4.44±0.468**

Table 4: Effect of B. retusa extract on biochemical parameter of rats treated with Freund's complete adjuvant.

Group	Dose (mg/kg)	Acid phosphatase (IU/L)	Alkaline phosphatase (IU/L)	SGOT(AST) (U/L)	SGPT(ALT) (U/L)	Spleen weight (mg)
Normal	-----	10.70±1.002	35.81±2.734	147.3±27.34	51.91±6.438	433.3±32.49
Control	----	17.35±2.857	64.30±5.372	206.2±56.35	68.99±8.656	529.5±23.61
PE	200	14.50±0.834	45.47±2.357	200.3±36.32	66.29±8.937	496.3±60.76
	400	12.86±1.718**	39.15±1.024	147.7±17.20*	54.25±5.267*	441.3±13.15**
EA	200	14.50±1.901	45.25±4.873	178.2±44.79	64.26±3.451	499.5±14.82
	400	13.16±1.866*	40.43±3.591	145.0±20.79*	53.59±5.408*	454.5±31.55**
ACE	200	16.90±2.281	50.78±6.593	197.8±26.28	66.88±8.092	533.3±22.38
	400	14.68±1.041	39.98±0.627	177.0±4.848	65.31±8.710	461.5±10.15*
Diclofenac	13.5	11.68±1.322**	77.00±4.149	209.8±13.85	61.81±12.95	439.3±16.13**

The observations are given as Mean SD. n=6; *$p<0.05$, ** $P< 0.01$ *** $p<0.001$. Compared with saline treated control group (ANOVA followed by multiple Dunnet´s test)

Table 5: Effects of B. retusa on leucocytes migration induced by Na-CMC and vascular permeability induced by acetic acid.

Treatments	Dose (mg/kg)	leucocytes migration assay		Vascular permeability assay	
		Total leucocytes (x103	% inhibition of Leucocytes Migration	Absorbance	Inhibition (%)
Normal	--	3.31±0.23	--		
Control	--	5.49±0.09	--	2.019 ±0.033	-------
PE	200	4.93±0.16*	10.20	0.490 ±0.089**	75.73
	400	3.74±0.13**	31.87	0.359 ±0.023**	82.21
EA	200	4.92±0.65*	10.38	0.394 ±0.046**	80.48
	400	3.85±0.61**	29.87	0.289 ±0.010**	85.68
ACE	200	5.06±0.11*	7.83	0.541 ±0.056**	73.20
	400	4.14±0.09**	24.59	0.416 ±0.020**	79.39
Diclofenac	10	3.23±0.11**	41.16	0.325 ±0.012**	83.90

Each data represents the Mean ± SD.n=5. * $p <0.01$, ** $p <0.001$ significantly different from control group. (ANOVA followed by multiple Dunnet´s test).

Table 6: Effects of B.retusa extracts on heat induced erythrocytes haemolysis and protein denaturation.

Conc. (µg/ml)	% inhibition of heat induced							
	erythrocytes hemolysis				% inhibition of protein denaturation			
	AA	PE	EA	ACE	PE	EA	ACE	AA
200	29.19±2.89	08.07±0.89	03.10±0.05	16.77±1.80	37.77±2.32	42.22±3.364	30.00±2.23	63.33±4.52
250	37.26±2.36	15.52±1.00	12.42±1.20	20.49±1.99	46.66±3.64	51.11±6.310	40.00±2.87	80.00±5.20
300	42.85±3.36	29.81±2.10	21.73±2.25	32.29±2.90	61.11±4.52	63.33±5.21	54.44±4.52	86.66±5.98
350	54.65±3.87	36.02±2.89	30.43±2.52	37.88±3.31	72.22±5.20	75.55±5.00	68.88±5.210	88.88±5.98
400	63.97±4.52	44.72±4.20	42.23±3.96	50.31±4.50	80.00±5.11	83.33±6.28	77.77±5.21	94.44±5.69

PE=Pet. ether, EA=ethyl acetate, ACE=acetone extract.

Hematology and biochemical investigation

Table 3 represents the hematological changes associated with arthritic condition. Levels of RBC were decreased in arthritic rats with concomitant increases in WBC, platelet count and ESR. These changes were observed to near normal levels in B.retusa treated animals. ESR of test group decreased up to 4 as compared to control 5.54 mm/hr. arthritis induced rats with B.retusa test samples exhibited potent inhibitory effects on RA and CRP factors. In this AIA rat model, both ESR and CRP were found to be markedly associated with the development of the disease, and significantly elevated ESR and CRP levels were noted throughout the course of the experiment as compared to control rats.

Table.4. shows the effect of test drugs on changes of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase (ALP) in the control and experimental animals. A marked increase in AST, ALT and alkaline phosphatase was observed in serum of arthritic rat. Aminotransferase and alkaline phosphatase was significantly reduced in arthritic rat after the administration of PE and EA at 400 mg/kg test drugs when compared to the arthritic control rat. It also showed the significant effect of PE (p<0.01) and EA (p<0.05) on lysosomal enzymes in the plasma of control and experimental animals. The administration of test drugs at 400 mg/kg to arthritic rat significantly reversed the biochemical changes to a normal level. The enlargement of spleen weight was significantly suppressed in rats treated with B.retusa test samples at higher dose. A change in the body weight is a useful index to assess the course of the disease and the response to therapy of anti-inflammatory drugs under investigation. Membrane stabilizing activity
B.retusa at 400 mg/kg significantly increased % inhibition of leucocytes migration from 24-32%. The positive control, dexamethasone showed (41.16%) a remarkable inhibition. The observations are given in Table 5.B.retusa test samples inhibited heat-induced haemolysis of RBCs to varying degrees. it evoked conc. related inhibition of haemolysis. PE, EA and ACE exhibited the most potent and conc. dependent inhibition of hemolytic activity. Denaturation of protein as a one the cause of inflammation. Effect of B.retusa extracts on inhibition of protein denaturation at different concentrations (200 and 400 µg/ml) provided significant protection against denaturation of proteins. The observations are given in Table 6.

DISCUSSION

In the present study, the evaluation of anti-inflammatory and analgesic property of Bridelia retusa were evaluated by different experimental models and the results demonstrate that the phytosterol, triterpenoid and tannins rich fraction can play a significant role in the inhibition of pain and inflammatory processes. Hydrolysable tannins like gallic acid are reported first time in the bark of this plant. Carrageenan-induced paw edema model is thought to be a biphasic event; in the early phase histamine and serotonin are released, then peak at 180min to release of kinin-like substances, while in the late phase, prostaglandins, proteases and lysosymes are released.[22-23] The obtained results indicated that Bridelia retusa extracts and its fractions significantly inhibited the formation of the rat paw edema, in the early and late phases, in fact somewhat more active in the late phase. It has been reported that the second phase of edema is sensitive to most clinically effective anti-inflammatory agents.[24] Test drugs produced significant (p<0.01) dose-related inhibitory effect on peritoneal capillary permeability produced by acetic acid in mice. Chemical-induced vascular permeability causes an immediate sustained reaction that is prolonged over 24 h and its inhibition suggests that the extract may effectively suppress the exudative phase of acute inflammation.[25]

Administration of Bridelia retusa appears to be effective in inhibiting the dry weight of cotton pellet. Hence a decrease in the granuloma weight indicates a suppression of the proliferative phase being effectively inhibited by the extracts. The effect of PE (500 mg, 52.82 %) on dry weight of the cotton pellet was almost near to that of diclofenac (55.95%). This effect may be due to the cellular migration to injured sites and accumulation of collagen and mucopolysaccharide.[10]
The result of the acetic acid-induced writhing strongly suggests that the mechanism of Bridelia retusa extract may be linked partly to inhibition of lipooxygenase and/or cyclooxygenase in peripheral tissues, thereby reducing PGE2 synthesis and interfering with the mechanism of transduction in primary afferent nociceptor.[27,28]

The formalin test is considered a model for chronic pain.[29] In this experiment, the analgesic effects of Bridelia retusa extracts were significant in the both phase of formalin test suggest that the anti-nociceptive might

be due present of active analgesic principles acting both centrally and peripherally.

Rheumatoid arthritis is a disease whereby tissues are damaged by the overproduction of reactive oxygen species (ROS).[30] In the investigation of adjuvant induced arthritis rats, showed a soft tissue swelling that was noticeable around ankle joints and was believed due to edema of periarticular tissues such as ligaments and joint capsules. The initial reduction of edema and soft tissue thickening at the deposit site is probably due to the effect of the adjuvant, whereas the late occurring circulated arthritis and burn in the injected foot are presumably immunological events. Petroleum ether and EA (triterpenoidal rich fractions) significantly suppressed the swelling of the paws. In the present study, the migration of leucocytes into the inflamed area is significantly suppressed by the standard drug and petroleum ether extract as seen from the significant decrease in total WBC count.[31] The decrease in RBC levels in arthritic rats reflects the presence of anemia, is the most common extra cellular manifestation in RA and a moderate hypo chromic; normocytic anemia due to reduction in the RBC count. RA factor is observed positively in 80% of arthritis control rat and is also increased in diffuse collagen disease. CRP factor is a diagnostic index of bacterial infection, chronic rheumatoid arthritis, supprurative arthritis, gout, malignant tumor and rheumatoid fever. The acute phase proteins in ESR and C-reactive protein (CRP) share the property of showing elevations in the concentration in response to stress or inflammation like injection, injury, surgery and tissue necrosis.[32] The ESR count, which drastically increased in arthritic control group, has been remarkably counteracted by the standard, sample and back to normal thus justifying its significant role in arthritic conditions. The level of aminotransferase and ALP were significantly increased in FCAIA rats due to increased lipid per oxidation, permeability of cell membrane or altered metabolism of these enzymes, since these are good indices of liver injury.[30] The decreased enzyme levels on herbal drugs treatments emphasize the decreased bone loss and organ protective role of Bridelia retusa in AIA rats. The enhanced protective effect of sample might be due to the combined effect of phytosterol and triterpenoid along with tannins.

Reduction in the release of lysosomal enzymes into the extra cellular compartment would prove beneficial and this indirectly confirms the protective effect of the drug. Bridelia retusa administration decreases the lysosomal enzyme release in adjuvant-induced arthritic rat which indicate its anti-inflammatory effect.[35-36]

Splenomegaly occurs as a result of profound induction of extramedullary hematopoiesis in the extracts conjunction with pyogranulomatous inflammation. Ideally, an agent active in adjuvant disease should restore the spleen weights and morphology to normal as is the case with both of herbal drugs.[37] A change in the body weight is a useful index to assess the course of the disease and the response to therapy of anti-inflammatory drugs under investigation. The loss of body weight observed in FCA induced arthritic rats may be due to the reduced absorption of glucose and leucine in the rat intestine.[38] But on the treatment with anti-inflammatory drugs, the decrease in absorption was nullified and it shows that the anti-inflammatory drugs correct the decreased absorption capacity of intestine during inflammation. The increased body weight during treatment of B.retusa fraction may be due to the restoration of absorption capacity of intestine.

Recent studies have shown that polyphenols significantly reduce the incidence of collagen-induced arthritis in mice. Viana et al.(1997) demonstrated that tannin-enriched fraction represents potent analgesic and anti-inflammatory effects.[39-40]

Generally, leukocytes migrate to sites of inflammation in response to chemotactic stimulus.[41-42] Subsequent studies of the effect of test materials on leukocytes migration induced by CMC -Na revealed a significant (P< 0.05) reduction of total leukocyte comparable to those of indomethacin (84.3%). These activated leukocytes at inflammation sites release super oxide radicals during phagocytosis.[43] Thus, herbal sample containing phytosterols and tannins may reduce the incidence of super oxide radicals' release by inhibiting leukocyte migration. It has been demonstrated that herbs containing phytoconstituents were capable of stabilizing the RBC membrane, considered as model of the lysosomal membrane, may be indicative of their ability to exert anti-inflammatory activity.[25]

Membrane stabilization study was performed for the mechanism of anti-inflammatory action of Bridelia retusa, inhibited both heat- induced lysis and protein denaturation. PE, EA and ACE significantly inhibited heat-induced erythrocytes lysis. These provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. Denaturation of protein as a one the cause of inflammation. Production of auto antigens in inflammation disease may be due to in vivo denaturation of protein. Nonsteroidal anti-inflammatory (NSAIDs) drugs could also act by protecting endogenous proteins against denaturation.[44]

Phytochemical analysis showed the extract contained sterols and terpenoids, which are documented anti-inflammatory constituents of some plants. Sterols such as sitosterol isolated from several plants as well as some terpenoids have demonstrated anti-inflammatory activity.[45]

In conclusion, studies on animal models of inflammation have suggested acute vascular responses: vasodilatation and increased vascular permeability resulting from the sequential release of low molecular weight mediators—histamines, serotonin and prostaglandins. The ability of the drug to reduce edema formation in adjuvant arthritic

rats is a hallmark of anti-inflammatory action due to its inhibitory action on prostaglandin synthesis.

The anti-inflammatory, anti-arthritis, antinociceptive and membrane stabilizing activity of fraction of the bark of Bridelia retusa primarily due to the phytosterols, triterpenoid (friedelin and lupeol) and tannins like gallic acid might be the key phytoconstituents for overall activities and support the use of this plant in traditional medicine. In addition, this study reveals that Bridelia retusa is a good candidate for a rich source of potent natural compounds. To the best of our knowledge, this is the first study evaluating the antinociceptive, anti-inflammatory and membrane stabilizing activity of Bridelia retusa.

The study may indicate the ethno pharmacological basis of the use of Bridelia retusa in traditional medicine for treating pain, swellings, rheumatoid arthritis etc. There is a need for further studies on the metabolism and toxic effects of B.retusa in order to elucidate the mechanism of action of the active fraction and phytochemical.

ACKNOWLEDGEMENTS

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