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WGMM Jayawikrama
Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Colombo, Sri Lanka

ARAMSSK Attanayake
Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Colombo, Sri Lanka

YAAB Yapa
Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Colombo, Sri Lanka

WD Ratnasooriya
Department of Basic Sciences, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Colombo, Sri Lanka

RN Pathirana
Department of Basic Sciences, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Colombo, Sri Lanka

Correspondence
WGMM Jayawikrama
Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Colombo, Sri Lanka

In vitro evaluation of antirheumatoid arthritic and anti-inflammatory activities of aqueous bark extract of *Bridelia retusa*

WGMM Jayawikrama, ARAMSSK Attanayake, YAAB Yapa, WD Ratnasooriya and RN Pathirana

Abstract
In Sri Lankan traditional medicine barks of Bridelia retusa (Family: Euphorbiaceae) is used to treat the rheumatoid arthritis and inflammation. However, this claim has not been scientifically validated or refuted yet. The aim of this study was to evaluate the antirheumatoid arthritic and anti-inflammatory activities of aqueous bark extract (ABE) of Bridelia retusa *in vitro* using heat induced protein (egg albumin) denaturation test which is widely used to access *in vitro* antirheumatoid arthritic activity and anti-inflammatory activity. ABE was made as described in Sri Lankan traditional medicine: 60g of dried bark was cut into small pieces and was boiled slowly in 1920 mL of distilled water approximately for 6 hrs until the volume was reduced to 240 mL. The solution was filtered using a muslin cloth. Different concentrations (31.25, 62.50, 125,250 and 500 µg/mL) of the ABE and reference drug diclofenac sodium (2500, 1250, 625, 312.50, 156.25, and 78.125µg/mL) were made and percentage inhibition of protein denaturation was accessed (N= 4). Further, phytochemical analysis was carried out using standard phytochemical techniques. The results revealed for the first time, that ABE of Bridelia retusa has marked and dose dependent (r^2= 0.9653; P < 0.05) antirheumatoid arthritic and anti-inflammatory activities with an IC50 value of 197.8 µg/mL.
Diclofenac sodium also showed marked and dose dependent (r^2 = 0.9678; P< 0.05) antirheumatoid arthritic and anti-inflammatory activities with an value of IC50 603 µg/mL. Phytochemical analysis of the ABE of Bridelia retusa showed the presence of flavonoids, phenols, tannins, alkaloids, saponins, phytosterols, glycosides and diterpenes. It is concluded that ABE of Bridelia retusa possess *in vitro* antirheumatoid arthritic and anti-inflammatory activities providing scientific justification for its claimed activities. These activities are likely to be mediated via synergistic action of flavonoids, phenols, tannins, alkaloids, saponins, phytosterols, glycosides and diterpenes.

Keywords: Bridelia retusa, anti-inflammatory activity, antirheumatoid arthritic activity, heat induced protein denaturation

Introduction
Bridelia retusa belongs to Family Euphorbiaceous. It is called as "Ketakala" in Sinhala and "Parsam" in Tamil language [1]. The plant body is erect and a large deciduous tree, usually growing up to 10 meters tall but occasionally to 20 meters. The tree is harvested from the wild for local use as a medicine and source of a good quality wood. The root is cylindrical in shape. Their length ranges from 30cm-61cm and 15cm-31cm in breadth [2]. It is usually monoecious, simple, symmetric, basally attached, margin entire to somewhat crenate [3]. Leaves of this plant are oval shape and distichously. The red and purple color flowers which have five petals, can be seen as bunches in June and July [1]. In Sri Lanka, *Bridelia retusa* can be seen in wet zone up to 2000 feet. In addition to Sri Lanka it is distributed in India, Myanmar, Indonesia, Tropical Africa, Madagascar, Yemen, China., Australia, the Solomon's and Vanuata and Thailand [3]. *Bridelia retusa* bark traditionally used to treat rheumatism, wound healing, diarrhea, dysentery, snake bite and fractures. The bark of *Bridelia retusa* is used with gingili oil in painful condition of musculoskeletal systems [4]. The bark is used as liniment in rheumatism [5]. In pharmacological trials of *Bridelia retusa* shown to exhibit antiviral, hypoglycaemic and hypotensive properties. Rheumatoid arthritis is a chronic inflammatory autoimmune disorder due to immunomediated responses. Currently, to reduce the inflammatory symptoms NSAIDs (Non-Steroidal Anti Inflammatory drugs) are widely used. However, use of these drugs induces serious side effects [6]. Hence there is a demand for novel and potent antirheumatoid and anti-inflammatory drugs with lesser side effects, preferably from plant sources. In Sri Lankan traditional medicine, bark of *Bridelia retusa* is used to treat the inflammation and rheumatoid arthritis [1].

However, this claim has not been scientifically validated or refuted yet. So, in this study, aqueous bark extract (ABE) of *Bridelia retusa* was investigated *in vitro* for antirheumatoid arthritic and anti-inflammatory properties using the heat induced protein (egg alumin) denaturation test. In this test, percentage of inhibition of denaturation of egg albumin is used as an index of antirheumatoid arthritic activity and anti-inflammatory activity. In addition, phytochemical profile of the ABE was investigated.

Methodology

Few branches from the mature plant of *Bridelia retusa* was collected form Pahala Yagoda, Gampaha, Western province (7.0679'N, 79.9780'E) Sri Lanka in January 2016.The air dried pieces of mature bark of *Bridelia retusa* plant was taxonomically identified by the herbarium of the National Botanical Garden in Peradeniya Sri Lanka and a voucher specimen of bark was deposited in the herbarium.

The bark of the *Bridelia retusa* was cut off from the plant and was thoroughly washed in pure running tap water. Then, the bark was air dried in shade for 5-6 days and was cut into small pieces. The 60g of cut pieces were boiled slowly in 1920 mL of distilled water approximately for 6 hrs until the volume was reduced to 240mL. The solution was filtered using a muslin cloth. This ABE was diluted appropriately to obtain the required concentrations 31.25, 62.50, 125, 250 and 500 µg/mL. The reaction mixture (5 mL) was consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of freshly prepared phosphate buffered saline (PBS, pH 6.4) and 2mL of varying concentration of ABE (31.25,62.50,125,250 and 500 µg/mL). Four series of solutions of ABE of the plant *Bridelia retusa* (N=4) were prepared using these specific concentrations. Similar volume of distilled water was served as negative control. The specific concentrations of 2500, 1250, 625, 312.50, 156.25, and 78.125µg/mL of Diclofenac sodium was used as the positive control. Four series of solutions for the reference drug Diclofenac sodium (N=4) were prepared using above specific concentrations. Then, the mixture was incubated in a water bath at 37^0C for 15 minutes and the temperature was gradually increased up to 70^0C at which the samples were retained for 5 minutes. Then the samples were allowed to cool down to room temperature (30^0C) and the absorbance was measured at 660nm using a spectrophotometer (model: OPTIZEN 3220uv) and vehicle as blank [7].

The percentage of inhibition of protein denaturation was calculated using the absorption readings according to following formula and statistical analysis was done in order to get dose response curve

Percentage of inhibition = 100 * [Vt / Vc- 1]

Vt = absorbance of test sample

Vc= absorbance of control sample [7].

Phytochemical analysis was made using standard phytochemical techniques [8].

Results

The results obtained for *in vitro* antirheumatoid arthritic and anti-inflammatory activities are summarized and depicted in Tables 1 and 2. As shown, in Table 1 ABE of *Bridelia retusa* induced profound inhibition of protein denaturation activity (ranging from 188% to 802% inhibition).This effect was concentration dependent (r^2 = 0.96, P<0.05) with an IC_{50} value of 197.8mg/ml. The reference drug, diclofenac sodium also displayed a marked inhibition of protein denaturation activity (ranging from 534 to 1057%inhibition).This effect too was concentration dependent (r^2=0.96,P<0.05) with an IC_{50} value of 603 mg/ml. Phytochemical analysis of ABE of *Bridelia retusa* revealed the presence of flavonoids, tannins, phenols, alkaloids, saponins, sterols, diterpenes and glycosides.

Table 1: Effect of *Bridelia retusa* aqueous bark extract on *in vitro* heat induced denaturation of egg albumin protein (n=4)

Concentration (µg/ml)	Absorbance Mean ± Sem	% Inhibition Mean ±Sem
31.25	0.200±0.020	188±30.3
62.50	0.175±0.007	171±8.83
125.00	0.296±0.012	358±15.1
250.00	0.488±0.013	666±18.7
500.00	0.624±0.026	802±31.3

Table 2: Effect of Diclofenac Sodium on *in vitro* heat induced denaturation of egg albumin protein (n=4)

Concentration (µg/ml)	Absorbance Mean±Sem	% Inhibition Mean±Sem
78.125	0.456±0.004	534±6.55
156.25	0.533±0.026	655±37.2
312.50	0.563±0.002	681±3.47
625.00	0.689±0.002	856±3.78
1250.00	0.758±0.003	953±4.79
2500.00	0.833±0.003	1057±3.97

Discussion

This study investigated the *in vitro* antirheumatoid arthritic and anti-inflammatory activities of ABE of *Bridelia retusa*. This was assessed *in vitro* using the inhibition of heat induced denaturation of egg albumin protein, which is an index of antirheumatoid arthritic and anti-inflammatory activities [9, 10]. This bio assay technique is widely used, simple, inexpensive, reliable, reproducible, well established validated *in vitro* technique to evaluate antirheumatoid and anti-inflammatory activities of pharmacophores [9, 10]. Another reason for selecting this *in vitro* technique was to avoid the use of live animals and ethical issues related to use of live animals. Water extract was selected for this study since in Sri Lankan traditional medicine, water extract (decoction) is used in the treatment of rheumatoid arthritic and inflammation. The results conclusively show that ABE of *Bridelia retusa* possess marked antirheumatoid arthritic and anti-inflammatory activities *in vitro* (in terms of inhibition of heat induced denaturation of egg albumin protein).This is a novel finding for ABE of Sri Lankan *Bridelia retusa*. Inhibition of heat induced denaturation of egg albumin protein was concentration dependent. This indicates that the observed effect is genuine, causal and specific and not a random phenomenon. Further the antirheumatoid arthritic and anti-inflammatory activities of ABE of *Bridelia retusa* was threefold higher than that of reference drug, diclofenac sodium a potent non-steroidal anti-inflammatory agent: IC_{50} for ABE of *Bridelia retusa* was 197.8µg/ml. This is therapeutically important finding.

The denaturation of tissue proteins is one of the major causes of inflammation [11, 12]. Inflammation plays vital role in rheumatoid arthritis which is chronic inflammatory autoimmune disorder [11, 12]. In some rheumatic arthritic diseases the production of auto antigens is due to denaturation of proteins. Further anti-inflammatory and antiarthric drugs therapeutically used in the management of inflammation and rheumatoid arthritis has the capacity to inhibit protein denaturation, possibly by interacting with the aliphatic region

around the lysine residue in the albumin protein as revealed by 1D, 2DH NMR studies (one dimensional and two dimensional protein Nuclear Magnetic Resonance) [7, 13]. This mechanism is likely to play a vital role in inducing anti-inflammatory and antirheumatoid arthritic activities by ABE of *Bridelia retusa*.

Phytochemical analysis showed the presence of flavonoids, tannins, phenols, alkaloids, diterpenoids, saponins, sterols in the ABE of *Bridelia retusa*. Flavonoids [14, 15, 16], tanins [17, 18], phenols [19], alkaloids [20] have been shown to induce anti-inflammatory and antirheumatoid arthritic activities. Thus, it is likely that anti-inflammatory and antirheumatoid arthritic activities of ABE of *Bridelia retusa* is mediated via synergistic activities of the above mentioned phytochemicals by inhibiting the denaturation of proteins: denaturation of proteins is one of the main causes of inflammation and certain rheumatoid arthritic conditions [21, 22]. Production of auto antigens are due to denaturation of proteins.

In conclusion this *in vitro* study show for the first time, that ABE of *Bridelia retusa* possess marked antirheumatoid and anti-inflammatory activities and also scientifically justifies the use of *Bridelia retusa* bark in Sri Lankan traditional medicine as a treatment modality for rheumatoid arthritics and inflammatory conditions. The results also indicate that bark of Sri Lankan grown *Bridelia retusa* offer high promise to be develop a novel, safe, efficacious and cost effective antirheumatoid arthritic and anti-inflammatory agent.

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