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Phytochemical profile and *in vitro* anti-inflammatory activity of aqueous leaf extract of Sri Lankan variety of *Psidium guajava* L

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Abstract
The leaves of *Psidium guajava* L. (Common name: Guava/Yellow Guava/ Lemon Guava) have proven medicinal values. The aims of this study were, to evaluate the phytochemical constituents and the anti-inflammatory properties of freeze-dried aqueous leaf extract Sri Lankan variety of *P. guajava* L. Phytochemical investigation was done with standard qualitative tests and revealed the presence of terpinoids, alkaloids, carbohydrates, flavonoids, tannins, phenols, glycosides, and proteins. The anti-inflammatory activity was determined by two *in vitro* models: inhibition of heat induced denaturation (which is an index of anti-inflammatory activity) of egg albumin and Bovine serum albumin. The percentage inhibition of denaturation of egg albumin (R^2 = 0.612, p = 0.01) and Bovine serum albumin (R^2 = 0.621, p = 0.01) were dose dependent. The maximum inhibition was observed at 125 µg/ml (IC_{50} = 15.625 µg/ml) for egg albumin and at 500 µg/ml (IC_{50} = 50 µg/ml) for Bovine serum albumin. The anti-inflammatory effect obtained in Bovine serum albumin denaturation test was comparable to reference drug, Diclofenac sodium and in egg albumin denaturation test it was higher approximately 30 fold higher than the reference drug. It is concluded that the aqueous leaf extract of the Sri Lankan variety of *P. guajava* L. possesses marked anti-inflammatory activity *in vitro* and this is a novel finding.

Keywords: *Psidium guajava* Linn, aqueous leaf extract, phytochemicals, egg albumin denaturation test, Bovine serum albumin denaturation test, anti-inflammation, Sri Lanka

1. Introduction
Sri Lanka is a country with a rich assortment of medicinal plants and a rich history of Ayurveda and traditional medicine. The earliest reference of Ayurveda medicine in Sri Lanka dates back to prehistoric times and it was the main method of medication for decades [1]. The plant products have been the back bone of this traditional system of healing throughout the globe. Although, the herbal drug preparation dates back thousands of years ago, their application on modern drug development started only in 19th century. Even though, with the time, popularity of synthetic products increased due to their time effectiveness, production cost, easy quality control and quick effect, their safety and efficacy are always remained questionable [2]. As World Health Organization data indicates, the value of worldwide annual market for herbal medicinal products approaches US$60 billion [3]. With this assembly researchers are keen to produce bio-drugs for future.

Psidium guajava Linn is a tropical plant grown in Sri Lanka, which comprises of numerous medicinal values. The plant is widely available in tropics and within the reach of local populace. It belongs to family Myrtaceae and originated from South America [4]. In traditional medicine, it has been used as a hyperglycaemic, antioxdant, hepatoprotective, anti-allergic, antimicrobial, antigenotoxic, antiplasmodial, cyototoxic, antispasmodic, cardioactive, anticough, antidiabetic, anti-inflammatory and antinociceptive agent [5, 6]. Extracts and metabolites obtained from the plant, especially, from leaves and fruits acquire pharmacological activities. The leaves of *P. guajava* L. have several medicinal values. There are proven scientific evidence on antimicrobial [7], antidiarrhoeal [8], hypoglycaemic [9-13] antioxidant [12], anticough [14], hepatoprotective [15], analgesic and anti-inflammatory (in rats and mice) [16-19], hypotensive [20], anti-tumor [21] and anti-mutagenic [22, 23] activities of leaves of *P. guajava* L.

Different preparations of leaves had scientifically validated for their phytochemical constituents [24], antimicrobial properties, antioxidant properties, anti-diabetic properties, antimicrobial properties, anticough properties, hepatoprotective properties, anti-diarrhoeal properties, spermatoprotective properties, antimutagenic properties, ionotropic effect, spasmolytic effect, anti-cancer, immunomodulatory activity, treatment of acne, antiproliferative activity, antipyretic, contractile affects, hypotensive, anti-malaria,

CNS activity, vaginal disorders and rheumatism [25].

These findings and traditional applications of *P. guajava* L., leaves made a strong incentive for further research on phytochemical constituent and their anti-inflammatory activity of leaves. Considering the facts that, the plant varies in nutrient content across cultivars [26] and there are no studies conducted on aqueous extracts of leaves of the Sri Lankan variety and the current study was conducted on freeze dried aqueous extract (FALE) of the leaves of the local variety of *P. guajava* L.. This study done on evaluated the phytochemical constituents and *in vitro* anti-inflammatory activity of FALE of Sri Lankan variety of *P. guajava* L. using two widely used bio assay models.

2. Materials and Methods

2.1 Chemicals

Phosphate Buffered Saline (PBS), Diclofenac sodium, Bovine Serum Albumin (BSA), chloroform, concentrated sulfuric acid, acetic anhydride, mercuric chloride, potassium iodide, iodine, bismuth nitrate, glacial acetic acid, copper sulphate, potassium sodium tartrate, sodium hydroxide, ferric chloride, 90% ethyl alcohol, ammonia, ammonium chloride, alcoholic alpha-napthol, ethyl acetate, 45% ethanol, led sub acetate, sodium nitrate, nitric acid were purchased from Sigma Aldrich, USA. Diclofenac sodium was purchased from Sate Pharmaceutical Corporation, Sri Lanka. All the chemicals and reagents were of analytical grade. A fresh hen's egg was purchased from a local supermarket. The plant extract was freeze dried at Industrial Technology Institute, Malabe, Sri Lanka. Phytochemical analysis was performed at Faculty of Medicine, General Sir John Kotalawela Defence University, Ratmalana, Sri Lanka. The tests for anti-inflammatory activity were performed at Institute of Biochemistry, Molecular Biology and Biochemistry (IBMBB), University of Colombo, Sri Lanka.

2.2. Plant identification and preparation of FALE of *P. guajava* L.

Fresh mature leaves of *P. guajava* L. were collected from Koralaima (Latitude 6.7731948, Longitude 80.0017854) in Kalutara district, Sri Lanka, during the month of November, 2016. The identification and authentication of the plant was done at the Department of Botany, University of Colombo, Sri Lanka. A voucher specimen (JCK/001) was deposited at herbarium, University of Colombo, Sri Lanka.

The leaves of *P. guajava* L. were washed and shade dried for 4-6 hrs. Hundred grams of the dried leaves were boiled in 1.5 L of distilled water for 4 hours. The boiled extract was filtered using Whatman filters paper No.4. The filtrate was concentrated in a rotary evaporator. The concentrated decoction was freeze dried (Christ-Alpha 1-4 Freeze dryer, Biotech International, Germany) and stored at -20 ºC until use [24]. The freeze dried product was brown in color and the percentage of yield was 6.37 (W/W).

2.3. Phytochemical analysis of FALE of *P. guajava* L.

FALE of *P. guajava* L. was subjected to following phytochemical screening for qualitative analysis. Salkowski and Libermann-Burchard tests were performed to determine the presence of sterols. The presence of terpinoids was examined by Salkowski test. The qualitative analysis for alkaloids was performed using Meyer's, Wagner's, Dragendroff's and Hager's tests. Test for phenols was achieved via ellagic acid test. Ammonium and aluminum chloride tests were carried out for flavonoids. Ferric chloride test and lead sub acetate tests were executed for tannins. The investigation of carbohydrates was performed by Molish and Fehling's tests. Xanthoproteic test was performed for proteins. The examinations for glycosides were done by Keller-Kiliani test and concentrated sulphuric acid test. Further, foam test was performed for the analysis of saponins [27].

2.4. Anti-inflammatory activity

2.4.1. *In vitro* egg albumin denaturation method

A mixture of 0.2 ml of egg albumin, 2.8 ml of PBS (pH 6.4) and 2 ml of varying concentrations (31.25, 62.50, 125, 250, 500 and 1000 µg/ml) of FELE of *P. guajava* L. was used as the test sample. The same mixture replacing the plant extract with Diclofenac sodium (78.125, 156.25, 312.5, 625, 1250 and 2500 µg/ml) was used as the reference drug. A similar volume of double distilled water served the control (represents 100% denaturation). The above mixtures were incubated at 37±2 ºC, for 15 min and then heated at 70 ºC for 5min. The test procedure was repeated 6 times. After cooling the absorbance was measured at 660 nm using multi-mode micro plate reader (Synergy Biotech, USA). The percentage inhibition of denaturation, which is an index of anti-inflammatory activity, was calculated using the following formula.

% Inhibition = 100 X (V_t/V_c - 1)

Where, V_t = absorbance of the test sample

V_c = absorbance of the control

The plant extract concentration for 50% inhibition (IC_{50}) was determined by dose-response curve [28].

2.4.2. *In vitro* BSA denaturation method

The test was performed as described by Rahman *et al.*, 2015 [28]. A concentration series of 50, 100 and 250 µg/ml of FELE of *P. guajava* and Diclofenac sodium were taken as the test sample and the reference drug respectively. The test was performed using six wells for each sample. The absorbances were measured at 255 nm using multi-mode micro plate reader (Synergy Biotech, USA). The control represents 100% protein denaturation. The test procedure was repeated 6 times. The results were compared with the reference drug. The percentage of inhibition was calculated using the following formula.

% Inhibition = 100 X (V_t/V_c - 1)

Where, V_t = absorbance of the test sample

V_c = absorbance of the control

The plant extract concentration for 50% inhibition (IC_{50}) was determined by dose-response curve.

3. Statistical analysis

The results are given as mean ± SEM. Statistical comparisons were made using Mann-Whitney U- test. Significance was set at $P<0.05$. Dose dependencies were investigated using Pearsons's correlation analysis.

4. Results

4.1 Phytochemical Screening

According to the results obtained by the phytochemical screening of FALE of *P. guajava* L. the leaves contained, phenols, flavonoids, tannins, alkaloids, terpinoids, saponins, glycosides, carbohydrates, proteins and steroids (Table 1).

Table 1: Preliminary phytochemical screening of aqueous leaf extracts of *P. guajava* L.

Phytochemical	Test	Results
Phenols	Ellagic test	+ve
Flavonoids	Ammonia test	+ve
	Aluminium Chloride test	+ve
Tannins	Ferric chloride test	+ve
	Led sub Acetate test	+ve
Alkaloids	Mayer's test	+ve
	Wagner's test	+ve
	Dragendroff's test	+ve
	Hager's test	+ve
Terpinoids	Salkowiski test	+ve
Saponin	Foam test	+ve
	Haemolytic test	+ve
Glycosides	Keller-Kiliani test	+ve
	Con. H_2SO_4 test	+ve
Carbohydrates	Molisch test	+ve
	Fehling's test	+ve
Proteins	Xanthoproteic test	+ve
Steroids	Salkowiski test	+ve
	Libermann-Burchard test	+ve

4.2 Anti-inflammatory activity

4.2.1 *In vitro* denaturation of egg albumin

The results of *in vitro* denaturation of egg albumin of FALE of *P. guajava* L. are summarized in Tables 2 and 3. FALE of *P. guajava* L. showed a marked inhibition in protein denaturation with an IC_{50} value of 15.625 µg/ml (IC_{50} value of Diclofenac sodium is 379.375 µg/ml). As shown in Table 2 and 3, FALE of *P. guajava* L. had shown a higher inhibition of protein denaturation than Diclofenac sodium indicating that FALE of *P. guajava* L. is a more potent anti-inflammatory agent. Maximum percentage of inhibition was expressed at 125 µg/ml and the inhibition was dose-dependent (R^2= 0.612, p= 0.01 (2-tailed)).

Table 2: Effect of FALE of *P. guajava* L. on *in vitro* heat induced denaturation of egg albumin protein (n = 36)

Concentration (µg/ml)	Mean absorbance at 660 nm ± SEM	% Inhibition
00	0.103±0.003	0000.0
31.25	2.545±0.002	2366.9
62.50	3.449±0.008	3243.1
125	4.002±0.000	3778.8

Table 3: Effect of Diclofenac sodium on *in vitro* heat induced denaturation of egg albumin protein (n = 36)

Concentration (µg/ml)	Mean absorbance at 660 nm ± SEM	% Inhibition
00	0.089±0.006	00.0
78.125	0.125±0.008	39.6
156.25	0.131±0.006	46.6
321.5	0.161±0.013	79.9
625	0.303±0.006	238.8
1250	0.601±0.016	573.1
2500	0.930±0.008	941.0

4.2.2. *In vitro* denaturation of BSA

As shown in the Table 4, FALE of *P. guajava* L. indicated a significant inhibition of BSA protein denaturation. The highest inhibition was observed at 500 µg/ml and the inhibition was dose-dependent (R^2 = 0.621, p = 0.01 (2-tailed)). The IC_{50} value for FALE of *P. guajava* L. was found to be 50 µg/ml (IC_{50} value of Diclofenac sodium is 50 µg/ml).

Table 4: Effect of FALE of *P. guajava* L. and Diclofenac sodium on *in vitro* heat induced denaturation of BSA (n = 6)

	Concentration (µg/ml)	Mean absorbance at 255 nm ± SEM	% Inhibition
FALE of *P. guajava* L.	00	0.041±0.002	0
	100	3.403±0.002	8166
	250	3.621±0.001	8695
	500	3.838±0.007	9223
Diclofenac sodium	00	0.040±0.004	0
	100	1.043±0.004	2432
	250	2.063±0.001	4910
	500	3.088±0.001	7402

5. Discussion

The analysis of the phytochemical constituents of plants aids the screening of their biological activities [29] and has great interest in pharmaceutical companies for the production of new drugs. The phytochemicals are the plants' secondary metabolites that help the plant to combat competitors, predators or pathogens [30].

The therapeutic activity, of *P. guajava* L. leaves is attributed to these phytochemicals present in the leaves. The previous phytochemical studies showed that leaves of *P. guajava* L. are rich on flavonoids especially Quercetin. Quercetin relaxes intestinal smooth muscle and inhibits the bowel contraction leading to anti-diarrheal effect [25] and reduces the capillary permeability in the abdominal cavity [4] which promotes medicinal applications of *P. guajava* L. leaves. Further, Quercetin will contribute to anti-inflammatory activity [31]. An active flavanoid; Quercetin-3-O-alpha-1-arabinopyanoside (Guajaverin) has shown antiplaque activity by inhibiting the growth of *Staphylococcus* mutants [28]. A flavonoid glycoside; morin-3-O-alpha-L-lyxopyranoside and two known flavonoids, guaijavarin and quercetin found in leaves of *P. guajava* L. have shown antibacterial activity against *Salmonella enteritidis* and *Bacillus cereus* [31, 32].

Other than that, flavonoids have biological activities such as anti-oxidant, anti-apoptotic, anti-aging, anti-carcinogenic, anti-inflammatory, anti-artherosclerosis, cardiovascular protection and improvement of endothelial function, inhibition for angiogenesis and cell proliferation activities [28, 32]. Phenolic compounds contribute to analgesic, anti-inflammatory, anti-microbial, hepatoprotective and anti-oxidant activities [4]. Moreover, phenols such as catechin and epichatechin decrease the cholesterol level, prevent type 2 diabetes and act as anti-oxidants [32]. Terpenoids found in *P. guajava* L. leaves have shown anti-microbial, anti-glycemic and phytotoxic properties [32]. The presence of steroids increases the pharmaceutical value of Guava leaves in such a way the steroids increase the protein synthesis and thus promotes the growth of muscle and bones [26]. Further, Glucosides such as Saponins can reduce the cholesterol levels in the body [33].

In the present study, the FALE of *P. guajava* L. of Sri Lanka was subjected to broad phytochemical screening using standard laboratory testing. The phytochemical tests confirm the presence of phenolic compounds, flavonoids, tannins, alkaloids, terpinoids, saponins, glycosides, carbohydrates, proteins and steroids which are known to exhibit medical and physiological activities. These findings evidenced that the local variety of *P. guajava* L. can also be used for treating different ailments and further studies would enhance the potential of applying these phytochemicals in production of effective and safe drugs.

The denaturation of proteins is one of the causes of inflammation. In certain rheumatic diseases, the production of

auto-antigens may be due to denaturation of proteins [34]. Anti-inflammatory drugs are known to inhibit the denaturation of proteins [35, 36]. Nonsteroidal anti-inflammatory drugs are the major pharmacological agents used for the anti-inflammatory and pain-relief management due to their capacity in inhibiting protein denaturation [37]. Therefore, agents that can prevent the denaturation could be used for the development of anti-inflammatory drugs. In 1986, Mizushima and Kobayashi [38] used *in vitro* screening models to assess the protein denaturation. In the current study, heat induced denaturation of egg albumin (EA) and Bovine serum albumin (BSA) were selected for the *in vitro* assessment of anti-inflammatory properties of FALE of *P. guajava* L.. These two bioassay techniques are widely used validated, reliable and sensitive tests to evaluate *in vitro* anti-inflammatory potential of pharmacophores. The increase of the absorbance of FALE, with respect to the reference drug indicated the stabilization of proteins in a dose-dependent manner and thus a genuine anti-inflammatory action. This is a novel finding for aqueous extract of leaves of Sri Lankan variety of *P. guajava* L. The IC_{50} value obtained at the inhibition of EA denaturation, indicated that FALE of *P. guajava* L. has a higher inhibitory effect than Diclofenac sodium, even at a very low concentration. However, the IC_{50} value for the inhibition of denaturation of BSA was similar to the reference drug.

Obviously, the phytochemicals found in FALE of *P. guajava* L. contribute to the anti-iflammatory activity of this plant. Phytochemicals sush as Quercetin has shown an increase secretion of bifidobacterial anti-inflammatory agent(s) capable of suppressing LPS-induced nitric oxide production in macrophages [39]. Further it has been reported that flavonoids and saponins exerted profound stabilizing effect on lysosomal membrane both *in vitro* and *in vivo* while tannins and saponins possess ability to bind cations there by stabilizing erythrocyte membranes and other macromolecules [40]. Further, tissue protein denaturation due to auto antigens may be the cause of arthritis [34]. Hence, agents that can prevent protein denaturation, therefore, would be possible candidate for anti-inflammatory and anti-arthritic drug development [41].

6. Conclusion

In conclusion, the results of this study conclusively show that, the water extract of leaves of Sri Lankan variety of *P. guajava* L. possess potent and dose-dependent anti-inflammatory activity *in vitro*. This is a novel finding, which scientifically justifies its use as an anti-inflammatory drug in Ayrurvedic and Sri Lankan traditional medicine. Possibility also exists for the development of a safe and efficacious anti-inflammatory drug and perhaps anti-arthritic drug based on Sri Lankan variety of *P. guajava* L.

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