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In vitro investigation of anti-inflammatory activity and evaluation of phytochemical profile of *Syzygium caryophyllatum*

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Abstract

This study examined the anti-inflammatory potential of an aqueous root extract of *Syzygium caryophyllatum* (Family: Myrtaceae) and reference drug Diclofenac sodium, *invitro* using heat induced egg albumin denaturation bio assay technique. The concentrations of the root extract used were 781.25, 1562.5, 3125, 6250 and 12500 µg/mL. While diclofenac sodium were 78.12, 156.25, 312.5, 625, 1250 and 2500 µg/mL. Both the extract ($r^2 = 0.97$; $P < 0.05$) and Diclofenac sodium ($r^2 = 0.87$; $P < 0.05$) displayed marked and concentration dependent inhibition of heat-induced protein denaturation with IC_{50} value of $6.229 \times 10^3 \mu\text{g/ml}$ and $\sim 1.704 \times 10^8 \mu\text{g/mL}$ respectively. Phytochemical analysis of the extract revealed the presence of the flavanoids, phenols, tanins, alkaloids, saponins and amino acids. It is concluded that the aqueous root extract of Sri Lankan grown *Syzygium caryophyllatum* possess marked *in vitro* anti-inflammatory activity which is mediated possibly by flavanoids, tanins and alkaloids. This is a novel finding. Further the results scientifically justify the use of roots of *Syzygium caryophyllatum* in Sri Lankan traditional medicine to treat inflammation.

Keywords: *Syzygium caryophyllatum*, anti-inflammation, inflammation, egg albumin denaturation assay, Sri Lankan, traditional medicine

Introduction

Inflammation which is characterized by warmth, redness, pain and swelling is an important nonspecific defense reaction to tissue injury, such as that caused by a pathogen or wound (Willey, Sherwood, & Woolverton, 2009) [22]. In addition denaturation of proteins which leads to the formation of antigens associate with type III hypersensitivity can produce inflammation (Agrawal & Paridhavi, 2007) [11]. Inflammation is of two types chronic inflammation, which is a long term dysregulated form of inflammation whereas acute inflammation which is a short term regulated form of inflammation (Murakami & Hirano, 2012) [12].

A wide array of human diseases are associated with inflammation (Eggleton, 2001) [9]. And currently, there is a high demand for anti-inflammatory drugs. In western medicine, most commonly used anti-inflammatory drugs belong to a class known as non-steroidal anti-inflammatory drugs (NSAIDs) which includes forms like ibuprofen, diclofenac and mefenamic acid (Al-Shidhani, Al-Rawahi, Al-Rawahi, & P, 2015) [2]. These drugs although efficacious are relatively expensive, particularly in developing countries, and also induce considerable and harmful adverse effects such as gastric ulcers, gastric pain, cardiovascular, hepatic and renal dysfunctions, increased risk of bleeding, fluid retention, hypertension, headache and dizziness and skin rashes (Ong, Lirk, Tan, & Seymour, 2007) [13].

Accordingly there is a dire need and demand for development of novel, cheap, efficacious and safe anti-inflammatory drugs preferably from natural source.

The roots of *Syzygium caryophyllatum* plant is used in an ointment known as "Waatha Widuranga thailaya" (වාත විදුරාච්චිත තේලය) used to treat inflammation in Sri Lankan traditional medicine (Somasiri, 1963). *Syzygium caryophyllatum* is an endangered plant which is native to Sri Lanka. *Syzygium caryophyllatum* is a member of Myrtaceae family restricted to forests in the wet zone (World Conservation Monitoring Centre, 1998). It's a small tree with ascending branches and a pale green bark with leaves which are generally sub-opposite with dry red brown matt beneath (Dassanayake & Fosberg, 1981) [7]. There are about 1200 species present in the *Syzygium* genus which exhibits pharmacological characteristics ranging from anti-fungal, anti-inflammatory, anti-bacterial and anti-oxidant. It is shown that *Syzygium caryophyllatum* possess antimicrobial properties specially against pathogens like *Staphylococcus aureus* (Annadurai *et al.*, 2012) [4].

However, yet, anti-inflammatory properties of *Syzygium caryophyllatum* is not scientifically proven or refuted. Hence, the main aim of this study was to scientifically investigate the anti-

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inflammatory potential of roots of *Syzygium caryophyllatum*. This was evaluated *invitro* using an aqueous root extract and heat induced egg albumin denaturation test. Anti-inflammatory drugs (eg indomethacin, ibufenac, flufenamic acid and salicylic acid) has the ability to stabilize heat treated bovine serum albumin by preventing it from denaturation, anti-denaturation effect of the drug in heat treated bovine serum albumin was used as an assay (Williams *et al.*, 2008) [23]. The principle effect of the underlying protein denaturation assay mentioned above using bovine serum as compound was also observed when solutions of egg albumin, and human serum albumin were used (Huggins & Jensen, 1949). The rationale behind implementing this assay is that the denaturation of albumin protein leads to formation of antigens which initiate type III hypersensitive reaction leading to inflammation (Agrawal & Paridhavi, 2007) [1]. The other objective of this study was to investigate the phytochemical profile of the aqueous root extract of *Syzygium caryophyllatum* using well established standard procedure.

Materials and Methods

Collection of plants

Sri Lankan traditional medical books were referred, and indigenous medical professionals were consulted in order to investigate a viable plant for the experiment. Then a plant list was prepared which was checked in correlation with research data base to determine a plant which wasn't researched before. Based on the natural availability of the plant as well as acquiring of the root samples *Syzygium caryophyllatum* plant was selected.

Identification and authentication

303.56g of *Syzygium caryophyllatum* roots were collected from Ilimba, Horana, Sri Lanka (GPS Coordinates: 6°43'32.0"N 80°05'28.1"E). The taxonomic authentication was conducted at the herbarium of department of plant Sciences at the University of Colombo.

Preparation of the aqueous extract

The root portion of the plant was removed and washed under running tap water to remove the adhering soil particles. Then the root sample was air dried for a week and the weight was measured. After which the sample was stored in air tight containers at Laboratory of British College of applied studies. The air dried roots were cut into small pieces and 60g of root sample was boiled in 1920ml of distilled water for 5 hours and 40 minutes till the volume of the solvent was reduced to 240ml and an aqueous root extract (ARE) was prepared. Thus the prepared ARE was transported to the Industrial institute of Sri Lanka for freeze drying. The yield produced after freeze drying for *Syzygium caryophyllatum* was 3g. Then ARE was prepared again following the same procedure which was to be used in the phytochemical analysis.

Phytochemical analysis

The extract was then subjected to qualitative analysis for the presence of flavonoids, polyphenols, tannings, alkaloids, steroids, terpenoids and amino acids as (Harry, Maung, & Fransworth, 1996) [11]; (Tiwari, Kumar, Kaur, Kaur, & Kaur, 2011); (Gautam, 2007) [11].

Assessment of *in vitro* anti-inflammatory activity by protein denaturation technique

A 5ml solution was made which was comprised of 2.8ml of freshly prepared phosphate buffered saline of pH - 6.3, 2 ml

of specific concentration of ARE and 0.2 ml of egg albumin extracted from hens egg. Specific concentrations were prepared separately for *Syzygium caryophyllatum* as 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 1.5625mg/ml, 0.78125mg/ml, 0.39062 mg/ml. Four series of solutions (n=4) were prepared using these specific concentrations. 2 ml of distilled water was used as negative control while specific concentrations of 2500 µg/ml, 1250 µg/ml, 625 µg/ml, 312 µg/ml, 156.25 µg/ml, 78.125 µg/ml, Diclofenac sodium was used as the positive control. Four series of solutions for the reference drug diclofenac sodium (n=4) was prepared using the above specific concentrations. Then the mixtures were heated in water bath at 37°C for 15 minutes and the temperature was gradually increased upto 70°C at which the samples were retained for 5 minutes. After which the samples were allowed to cool down to room temperature and absorption was measured at 660 nm. Solution was prepared using the vehicle which was considered as reagent blank while 2 sample blanks were prepared for each concentration.

Statistical analysis

The results are depicted as mean± Standard error of mean (SEM). The half maximal inhibitory concentration (IC50) value and concentration dependencies were calculated using non-linear regression with Graphpad prism 5 software.

Results

Phytochemical analysis

Plants contain bioactive compounds known as phytochemicals which are responsible for anti-inflammatory properties of the plant extract. These compounds are investigated to establish a conclusion which phytochemicals present in the plant is responsible for the anti-inflammatory properties displayed by the plant extract.

Table 1: Phytochemical analysis results of Aqueous root extract of *Syzygium caryophyllatum*

Phytochemicals	Type of test	<i>Syzygium caryophyllatum</i>
Flavonoids	Alkaline reagent test	+
	Lead acetate test	+
Phenols	Ferric chloride test	+
	Gelatin test	+
Alkaloids	Mayer's test	-
	Wagner's test	+
	Dragendroff's test	+
	Hager's test	-
Saponins	Froth test	+
Amino acids	Xanthoproteic test	+
	Ninhydrin test	+
Phytosterols	Salkowski's test	-

Presence (+) or absence (-) of phytochemicals.

As shown in the table 1 the extract contained flavanoids, phenols, tannings, alkaloids, saponins and amino acids.

In vitro anti-inflammatory assay

Under non-linear fit of transform of concentration vs response analysis it was found that marked denaturation response was observed in *Syzygium caryophyllatum* which is concentration dependent ($r^2 = 0.9709$, $p < 0.05$) with an IC50 value of 6.229×10^3 µg/ml. Best fit nonlinear dose response curve was developed using GraphPad Prism 5 software. The dose response natures of the results are displayed in figure 1 below. In which you could observe the positive and concentration response nature of the *Syzygium caryophyllatum* while the absorbance and percentage inhibition results of samples

which contain *Syzygium caryophyllatum* was recorded in table 2.

Table 2: Mean absorbance and percentage inhibition of heat induced denaturation of proteins by *Syzygium caryophyllatum* root extract

Drug Concentration (µg/ml)	Mean absorbance ±SEM	Percentage Inhibition
781.25	0.49 ± 0.0107	35.1766
1562.5	0.69825 ± 0.0061	24.2319
3125.0	0.81575 ± 0.0075	42.5883
6250.0	0.607 ± 0.0235	109.3701
12500.0	0.39425 ± 0.0272	176.9201

The results obtained are with aqueous root extract of *Syzygium caryophyllatum* is summarized in table 2. As shown, the aqueous root extract inhibit the heat denaturation of proteins by 24.23% to 176.92%. This effect was concentration dependent ($r^2 = 0.9709$, $P < 0.05$). The IC₅₀ value for by *Syzygium caryophyllatum* was 6.229×10^3 µg/ml.

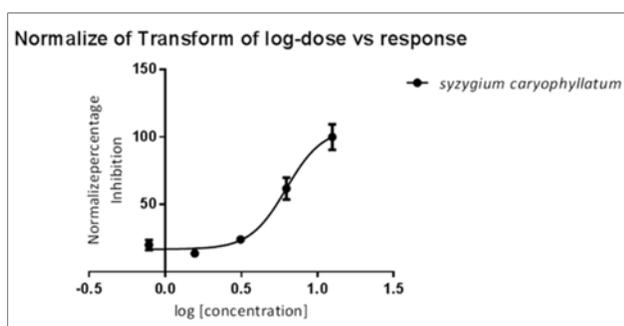


Fig 1: Concentration response curve for anti-inflammatory properties displayed by *Syzygium caryophyllatum*

Table 3: Concentration Absorbance and percentage inhibition of samples using as anti-inflammatory compound Diclofenac sodium

Drug concentration (µg/mL)	Mean absorbance ±SEM	Percentage Inhibition
78.125	0.371 ± 0.0062	59.2274
156.25	0.4575 ± 0.0047	96.3519
312.5	0.46 ± 0.0047	97.4248
625	0.4715 ± 0.0046	102.3605
1250	0.52475 ± 0.0041	125.2145
2500	0.643 ± 0.0036	175.9656

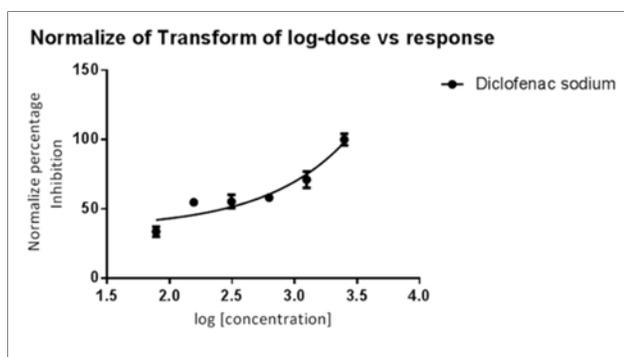


Fig 2: Concentration response curve for anti-inflammatory properties displayed by Diclofenac sodium

The positive control diclofenac sodium which was used as the positive control gave a result of IC₅₀ ~ 1.704×10^8 µg/ml ($r^2 = 0.8749$, $p < 0.05$). Figure 2 displays the dose response curve of diclofenac sodium while mean absorbance and percentage

inhibition of sample containing the reference drug diclofenac potassium recorded in table 3.

Discussion

This study assessed the anti-inflammatory potential of the roots of *Syzygium caryophyllatum* which is used in traditional medicine of Sri Lanka to treat inflammation (Somasiri, 1963). This was tested *in vitro* using an aqueous root extract and heat induced egg albumin denaturation bio assay. This is widely used, validated, sensitive, quick and a reliable *in vitro* technique to investigate anti-inflammatory activity of natural products (Chandra, Chatterjee, Dey, & Bhattacharya, 2012)^[6]. The rationale behind implementing this assay is that denaturation of albumin proteins leads to the formation of antigens which initiates type III hypersensitive reaction leading to inflammation (Agrawal & Paridhavi, 2007)^[1]. And therefore inhibition of its denaturation process by an agent indicates its anti-inflammation properties; higher the degree of inhibition greater would be its anti-inflammatory potential. The results clearly showed that the aqueous root extract of *Syzygium caryophyllatum* exhibit marked and concentration dependent anti-inflammatory activity *in vitro* with an IC₅₀ value of 6.229 µg/ml. This is a novel finding. The lowest concentration of the extract tested was 781.25 µg/ml (due to lack of sensitivity of the spectrophotometer available) and the highest was 2500 µg/ml (due to colour interference). Further, during the execution of the bioassay we used gradual heating to increase the temperature to 70°C from 37°C (instead of rapid heating (Chandra *et al.*, 2012)^[6] to prevent formation of irregular clumps resulting from protein coagulation resulting from evaporation of water molecules from egg white and thermal denaturation of egg protein (Pelegri, 2012)^[15]. The reference drug diclofenac sodium also showed profound and concentration-dependent *in vitro* anti-inflammatory activity, as expected. However, IC₅₀ obtained (~ 1.704×10^8 µg/ml) was different to what is reported by some other investigators and this may be attributed to differences in bioavailability of different brands of the generic drug used (Al Ameri *et al.*, 2012)^[3].

In phytochemical analysis, both the alkaline reagent and the lead acetate tests gave positive results indicating the presence of flavanoids in the extract. Flavanoids are known to induce anti-inflammatory activity by impairing and quenching of free radicals (Sasikumar, Subramaniam, Aneesh, & Saravanan, 2015)^[18] and by inhibiting the cyclooxygenase enzyme which produce inflammatory mediator, prostaglandin (Dillard & German, 2000)^[8], (Berkey *et al.*, 2011)^[15]. Certain flavanoids have the ability to prevent platelet aggregation thus reducing the effect of inflammation (Dillard & German, 2000)^[8]. Such modes of actions are possible with this extract under *in vivo* conditions but unlikely to operate in the heat-induced egg albumin denaturation test. The extract contained tannins and tannins are shown to have anti-inflammatory activity by inhibiting the production and the effect of the inflammatory mediators such as NO, TNF-α, IL6 or PGE2 (Park, Cho, Jung, Lee, & Hwang, 2014)^[14]. Although, such mechanisms is possible in the body it is unlikely to be operative in heat induced egg albumin test. Positive results with Wagners' test and the Dragondroff's test indicate the presence of purine type of alkaloids in the extract and the negative results to the Mayer's test and the Hager's test indicates the presence of pseudoalkaloids on the extract (Purohit, Kokate, & Gokhale, 2008)^[16]. These alkaloids can produce anti-inflammatory properties *in vivo* as several alkaloids have been shown to possess anti-inflammatory properties (Souto *et al.*, 2011)^[20].

The exact mechanism through which the extract mediated its anti-denaturation effect in the heat induced protein denaturation test is unknown at present. But, it may be due to interaction of its flavanoids, tanins and alkaloids with aliphatic region around the lysine residue on the albumin protein (Williams *et al.*, 2008) [23]. 1D and 2D ¹H NMR (one dimensional and two dimensional protein nuclear magnetic resonance) studies have shown that agents which have albumin anti-denaturation action binds/interacts at these two sites (Williams *et al.*, 2008) [23]. However, as heat induced protein denaturation bioassay indicates anti-inflammation activity of the results of this study shows the anti-inflammatory properties of the extract.

Conclusions

In conclusion, this study shows, for the first time, *in vitro* anti-inflammatory activity of aqueous root extract of Sri Lankan, *Syzygium caryophyllatum* and scientifically justify its use in Sri Lankan traditional medicine to treat inflammatory conditions. Its anti-inflammatory action is mediated by the synergistic action of flavanoids, tannins and purine and pseudo-alkaloids. Furthermore, some studies have shown that the heat-induced protein denaturation test also indicated anti-rheumatoid arthritic activity (Ranaweera, Pathirana, Ambalenduwa, Jayakody, & Ratnasooriya, 2014) [17] in which case the results also suggest that *Syzygium caryophyllatum* may be useful to treat rheumatoid arthritis as well.

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