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ORIGINAL ARTICLE

Evaluation of *In-vitro* anti-inflammatory property of novel polyherbal formulation containing *Sida cardifolia, Solanum xanthocarpum, and Psidium guajava*

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ABSTRACT
Inflammation is one of the most important immune reactions that may lead to arthritis, allergies, respiratory diseases, cancer, etc. Medicinal plants are recognized as one of the promising sources of novel anti-inflammatory drugs that can overcome the adverse side effects associated with conventional anti-inflammatory drugs. Solanum xanthocarpum, Sida cardifolia, and Psidium guajava are indigenous edible herbs that were extensively used to treat various diseases. The leaves of S. cardifolia and P.guajava with S.xanthocarpum fruits were mixed in 1:1:1 & 2:1:1 ratios respectively. The herbal mixture was subjected to the preparation of extract through a cold maceration technique using ethanol (Eth) and water (Aq) as solvents. The polyherbal formulations (PHF) were named Aq1:1:1, Aq2:1:1, Eth1:1:1, and Eth2:1:1. The total phenolic (TPC) and flavonoid content (TFC) were estimated using a biochemical method, and the anti-inflammatory activity was analyzed by inhibition of protein denaturation and SRBC membrane stabilization assays. The data were statistically analyzed by ANOVA and post hoc test (Bonferroni test). The results revealed that the Eth2:1:1 of PHF had the highest TPC (89.34 mg GAE/100g of Dry ext) & TFC (59.58 mg QE/100g of Dry ext). Similarly, the anti-inflammatory assays also showed that Eth2:1:1 had the highest percentage in SRBC membrane stabilization (66.7±1.2) and Protein denaturation inhibitory (60.9±1.7) assays than other PHF forms. Hence the current study exhibits the better anti-inflammatory effect of novel PHF with the 2:1:1 ratio of ethanolic extract. This finding may help to develop a new drug to treat various life-threatening inflammatory conditions.
Keywords: *Sida cordifolia, Solanum xanthocarpum, Psidium guajava, polyherbal, anti-inflammatory, total phenolic acid, Total flavonoid.*

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INTRODUCTION

Inflammation is one of the most important immune reactions protecting the body from harmful stimuli; however, prolonged and excessive inflammation exacerbates many diseases, such as arthritis, inflammatory bowel diseases, bronchial asthma, and cancer [1]. Pathologically inflammation can be defined as the critical response to any stimuli that causes tissue injury. Based on the exposure and persistence of signs and symptoms it can be categorized as acute and chronic inflammatory diseases [2]. In recent times, the drug development processes have predominantly depended on reverse pharmacology research which is multidisciplinary research based on the experiential knowledge of traditional medicines like herbals and other natural products[3,4].

Medicinal plants are recognized as one of the promising sources of novel anti-inflammatory drugs and nutraceuticals, which can overcome the adverse side effects associated with nonsteroidal anti-inflammatory drugs [5,6]. *Solanum xanthocarpum* (yellow-fruit nightshade (or) kandankattiri) belongs to

the family Solanaceae and is a traditional herbal plant that can be used to treat asthma and inflammations [7,8]. Similarly, *Sida cordifolia* (Family – Malvaceae) commonly known as country mallow or nilathuththi was documented as a CNS stimulant agent and a known herbal for pain and swelling [9-11]. *Psidium guajava* (family -Myrtacea) an eminent herbal plant known for its edible fruit that has valuable nutraceutical and pharmacological properties [12].
In our traditional medicine system, these three plants were extensively used for various disease conditions. The previous works of the literature showed that these plants have similar medicinal properties like Hypoglycemic, analgesic, anti-inflammatory, antioxidant, anti-microbial, wound healing, hepatoprotective, and anti-cancer effects[7-13]. However, the synergistic effects of these three plants have not been reported so far. Hence, the present study hopes to evaluate the anti-inflammatory activity of a novel polyherbal formulation containing Solanum Xanthocarpum, Sida Cardifolia, and Psidium Guajava.

MATERIAL AND METHODS

The chemicals used in this study were analytical grade and purchased from Sigma-Aldrich and SRL. Pvt Ltd, India. The complete study was done in the Central Research Lab, Rajas Dental College and Hospital, Kavalkinaru Jn, Tirunelveli, Tamil Nadu, India.

Collection and authentication of plant samples

Sida cardifolia (SC) leaves and *Solanum xanthocarpum* (SX) fruits were collected near kavalkinaru and vadakankulam village. Whereas, *Psidium guajava* (PG): leaves were collected from The garden of Rajas dental college campus. All collected plant samples were submitted to St.Xavier's Research Foundation, Palayamkottai to get botanical authentication.

Plant processing and extraction

The collected plants were allowed to shade dry with proper precautionary measures. Then the dried plant samples were powdered and the individual powders were mixed uniformly in 1:1:1 & 2:1:1 (2- SC; 1-SX; 1-PG) subjected to cold maceration and hot decoction method of extraction using ethanol distilled water as a solvent respectively. The extracts were named Ethanol extract (Eth. ext) 1:1:1, Ethanol extract (Eth. ext) 2:1:1, Aqueous extract (Aq.ext) 1:1:1, Aqueous extract (Aq.ext) 2:1:1.

Estimation of total phenolic content

Total phenolic contents were estimated using a Folin-Ciocalteau reagent-based assay as previously described with little modification [14]. To 0.5ml of each extract (100µg/ml) in methanol, 2.5ml of Folin-Ciocalteau reagent (diluted ten-fold), and 2ml (75g/l) of Na_2Co_3 were added. The mixture was allowed to stand at 20ºC for 30min and the absorbance of the developed color was recorded at 765nm using a UV-VIS spectrophotometer. 0.5ml aliquots of 20, 40, 60, 80, and 100 µg/ml methanolic Gallic acid solutions were used as standard for the calibration curve. The absorbance of the solution was compared with the gallic acid calibration curve. The total phenolic content was expressed as Gallic acid equivalents [14,15]. The absorbance of a solution is compared with the gallic acid calibration curve. The total phenolic content will be expressed as Gallic acid equivalents. From the linear regression equation of the Gallic acid calibration curve, the concentration of gallic acid (c) was found and the final Total phenolic acid content was calculated using the following formula.

$$C = \frac{Vc}{M}$$

C= total phenolic acid content; V= volume of sample; c= Concentration of Gallic acid obtained from standard curve; M weight of the dry sample (plant material)

Estimation of flavonoids

An aliquot of 0.5 ml of the sample (1mg/ml) was mixed with 0.1 ml of 10% aluminum chloride and 0.1 ml of potassium acetate (1M). In this mixture, 4.3 ml of 80% methanol was added to make a 5ml volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The absorbance of a solution is compared with the quercetin calibration curve. The total Flavonoid content will be expressed as quercetin equivalents. The quercetin calibration curve and its linear regression equation were derived to find the concentration of Quercetin (c) present in the polyherbal formulation [14,16]. The following formula was used to calculate the total flavonoid content of the various extracts.

In-vitro anti-inflammatory activity

Invitro anti-inflammation activity by sheep red blood cells (SRBC) membrane stabilization assay & protein denaturation assay.

Protein denaturation assay

The albumin denaturation assay was done by the Mizushima et al(1968) & Saket et al(2010) method using Aspirin as a standard drug [17,18]. 0.45ml of 5% BSA solution was added to the 0.05ml of various concentrations of polyherbal extracts (Eth ext1:1:1, Eth.ext 2:1:1, Aq.ext 1:1:1 & Aq 2:1:1) and Aspirin solutions to prepare 0.5ml of test and standard solutions. 0.5 ml of control solution was prepared with0.45 ml bovine serum albumin and0.05 ml distilled water. The pH of all the above solutions was adjusted to pH 6.3 by adding 1N HCl. All the test tubes were incubated at 370c for 20 minutes, and the temperature was raised to 570c for 3 minutes. After cooling the samples in running tap water, 2.5ml of phosphate buffer solution was added to all the above tubes. The absorbance was measured at 660nm in a UV-Vis spectrophotometer [19]. The mixture of 0.5 ml of distilled water with 2.5 ml of phosphate buffer solution was used as a test blank for absorbance reading. The experiment was performed in triplicate. Percentage inhibition of protein denaturation was calculated as follows:

$$\textbf{Percentage of inhibition} = \left\{\frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} x100\right\}$$

SRBC membrane stabilization assay:

SRBC membrane stabilization assay was carried out using the Gandhidasan et al. (1991) method [20] with slight modifications using Aspirin as a standard drug. The sheep's fresh blood was collected from a slaughterhouse in the Kavalkinaru village. Fresh whole blood was collected and mixed with equal sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in water). The blood sample was centrifuged at 3000 rpm for 10 min and the packed cells were washed three times with isosaline (0.85%, pH 7.2). The blood volume is measured and reconstituted as 10% v/v suspension with isosaline. The extracts' membrane stabilizing activity was assessed using hypotonic solution-induced human erythrocyte hemolysis. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extracts with concentrations (100-1000 μg/ml) and standard drug Aspirin. The control sample has 0.5 ml of RBC mixed with hypotonic buffered saline solution alone. The mixtures are incubated for 10 min at room temperature and centrifuged for 10 min at 3000 rpm, and the absorbance of the supernatant will be measured at 560 nm. The percentage inhibition of hemolysis or membrane stabilization was calculated using the formula [20-22].

RESULTS

Estimation of Total phenolic and flavonoid content

The total phenolic and flavonoid content of the polyherbal formulation (Table 1) was estimated in aqueous (Aq) and ethanolic (Eth) extracts with different ratios. The ethanolic extracts (Eth1:1:1 and Eth2:1:1) showed significantly higher phenolic and flavonoid content than the aqueous extracts. Eth2:1:1 exhibited the highest total phenolic content at 89.34 mg GAE/100g and flavonoid content at 59.58 mg QE/100g, indicating a more efficient extraction of these compounds in ethanol. In contrast, the aqueous extracts, particularly Aq1:1:1, displayed lower values, suggesting ethanol as a superior solvent for extracting phenolic and flavonoid compounds from the formulation

Table 1. Total phenolic and flavonoid content of Polyherbal formulation

	Total Phenolic acid content (mg GAE/100g of Dry ext)	Total Flavonoid content (mg QE/100g of Dry ext)
Aq1:1:1	31.8	9.58
Aq2:1:1	51.8	16.25
Eth1:1:1	61.29	45.42
Eth2:1:1	89.34	59.58

***In-vitro* anti-inflammatory activity**

Albumin Denaturation Assay

The in-vitro inhibition of albumin denaturation by polyherbal extracts (Table 2) was assessed at various concentrations, with results compared to the standard Aspirin (Figure 1). The Ethanolic extract in the 2:1:1 ratio demonstrated the highest inhibitory activity across all concentrations, reaching 60.9% inhibition at 1000 μg/ml, closely following the standard Aspirin, which exhibited 72.9% inhibition at the same concentration. The aqueous extracts showed relatively lower activity, with the 2:1:1 aqueous extract achieving 54.1% inhibition at 1000 μg/ml. Both the Ethanolic and aqueous extracts displayed a

concentration-dependent increase in inhibition, indicating potential anti-inflammatory properties, with the Ethanolic extracts proving more effective overall.

Table 2. In-vitro Inhibition of albumin denaturation

Concentration	1:1:1 Ethanol (%)	2:1:1 Ethanol (%)	1:1:1 Aqueous (%)	2:1:1 Aqueous (%)	Standard Aspirin (%)
100 µg/ml	12.8 ±6.1*	23.9±3.5*	10.3±8.1*	17.1±7.5*	22.9±8.9*
200 µg/ml	28.2±4.2*	36.1±6.9*	25.3±3.9*	29.6±2.8*	32.7±4.3*
400 µg/ml	42.3 ±2.8*	45.7±1.7*	34.2±2.1*	37.5±2.2*	52.4±1.4*
800 µg/ml	44.6±3.2	54.8±2.3*	41.6±2.8*	50.1±2.5*	54.7±1.1*
1000 µg/ml	51.7±2.2*	60.9±1.7*	52.7±2.1*	54.1±2.2*	72.9±1.4*

The values are expressed as Mean ± Standard deviation (n=3) p-value based on ANOVA followed by posthoc analysis using the **Bonferroni** test after adjusting for multiple comparisons ; * = Statistically Significant (**p < 0.05**)

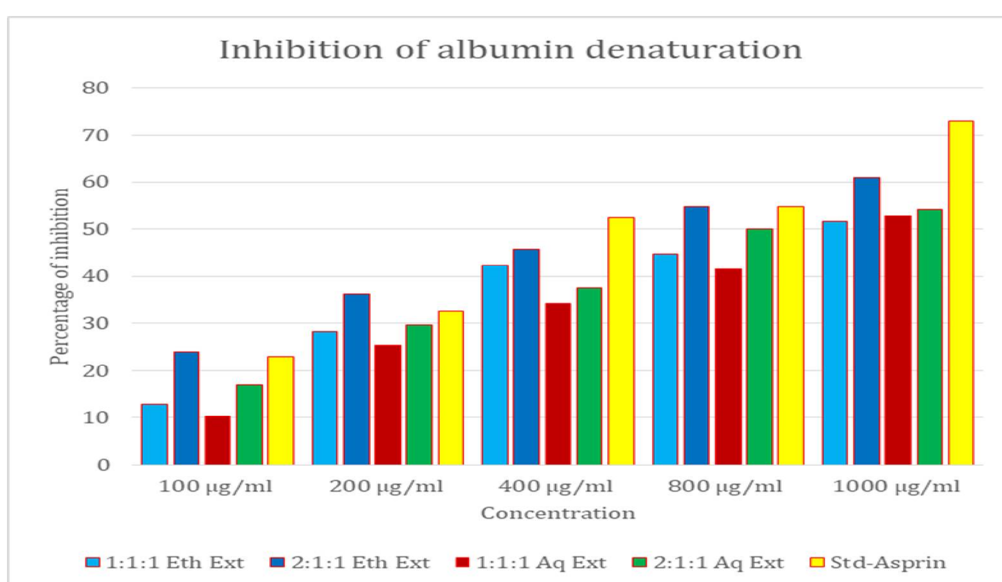

Figure 1: Effects of Polyherbal formulation on Inhibition of albumin Denaturation

SRBC Membrane stabilization activity

The SRBC membrane stabilization activity of polyherbal extracts (Table 3) was evaluated at various concentrations, with results compared to the standard Aspirin (Figure 2). Among the tested formulations, the 2:1:1 Ethanolic extract demonstrated the highest membrane stabilization effect, achieving 66.7% inhibition at 1000 µg/ml, although it remained lower than the standard Aspirin's 80.9% inhibition at the same concentration. The 2:1:1 aqueous extract also showed notable activity, with a 60.1% stabilization effect at 1000 µg/ml, while the 1:1:1 Ethanolic and aqueous extracts exhibited slightly lower activity. All extracts displayed a concentration-dependent increase in membrane stabilization, indicating their potential anti-inflammatory effects, with the Ethanolic extracts generally providing superior results compared to the aqueous formulations.

Table 3: SRBC Membrane stabilization activity

Concentration	1:1:1 Ethanol (%)	2:1:1 Ethanol (%)	1:1:1 Aqueous (%)	2:1:1 Aqueous (%)	Standard Aspirin (%)
100 µg/ml	26.8±1.8*	29.6±2.7*	26.7±2.3*	24±2.6*	30.6±3.9*
200 µg/ml	30.1±1.9*	37.8±1.0*	32.4±2.4*	37.6±1.5*	39.2±0.8*
400 µg/ml	41.2±0.8*	43.8±2.3*	38.3±2.1*	46.1±6.6	58.3±0.9*
800 µg/ml	46±2.9*	59±2.9*	43.9±1.4	50.7±2.7	69.2±0.8*
1000 µg/ml	49.6±3.1	66.7±1.2*	52.2±1.3*	60.1±2.8*	80.9±2.1*

The values are expressed in Mean ± Standard deviation (n=3) p-value based on ANOVA followed by posthoc analysis using the Bonferroni test after adjusting for multiple comparisons ; * = Statistically Significant (p < 0.05)

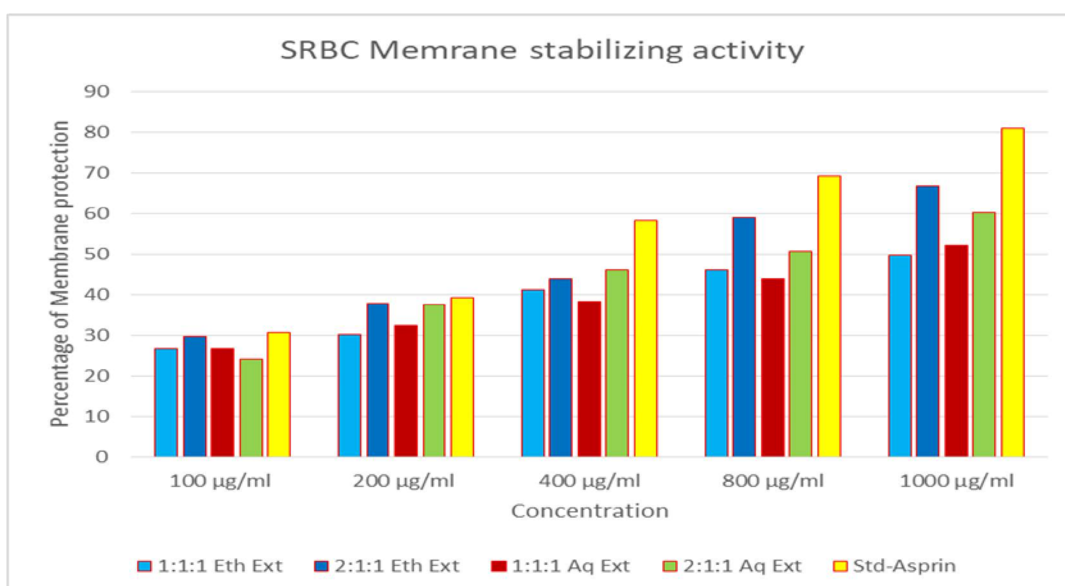

Figure 2: Effects of Polyherbal formulation on SRBC membrane stabilization property

DISCUSSION

Flavonoids and phenolic acids are the major bioactive compounds in the herbal industry and are responsible for their anti-inflammatory, anti-cancer, and anti-microbial properties [23,24]. Ethanol extract of 2:1:1 ratio of polyherbal formulation contains more flavonoids and phenolic acid than aqueous extracts. Phenolic acids like ferulic acid, coumaric acid & vanillin, and flavonoids like Rutin, Quercetin, Nariginin & kempferol were already reported in the selected herbal plants [25-27]. These compounds are majorly responsible for the herbal plants' analgesic, anti-inflammatory, and anti-microbial properties [27]. The SRBC membrane stabilization & Albumin Denaturation inhibition assay are the gold standard *in-vitro* models to evaluate a drug's or herbal product's anti-inflammatory efficacy. Usually, HRBC is used for the membrane stabilization assay, but in this study, we used SRBC (collected from a slaughterhouse) to avoid the unwanted wastage of human blood. RBC's membrane resembles the lysosomal membrane structure. The mediators released from the lysosomal are responsible for the inflammation and tissue destruction [28]. Hence instead of a lysosomal membrane the RBC's membrane stabilization study is employed. In addition, during the inflammatory process, the RBC membrane gets damaged, leading to the clearance of RBCs [29]. In this study the Eth. Ext 2:1:1 showed a greater percentage of SRBC membrane protection from hypotonicity-induced membrane damage. Albumin is a major protein in the plasma denatured due to immune and inflammatory reactions [30]. Protein denaturation during inflammation is an important phenomenon in many chronic inflammatory diseases especially with connective tissue damages. Protein denaturation is well-correlated with the pathology of inflammatory and autoimmune diseases. Since the protein molecule gets denatured due to physical and chemical stimulation, their biological functions may alter [19]. The denatured protein structures act as an autoantigen and stimulate the immune reaction, which further induces the release of immune and inflammatory mediators [31]. The overall analysis revealed that the 2:1:1 Ethanol extract & 2:1:1aqeous extracts have higher anti-inflammatory responses than 1:1:1 ratio extracts. The 2: 1: 1 ratio where the *Sida cordifolia* is higher than other plants showed a better anti-inflammatory effect and contained more flavonoids and phenolic acid levels [32,32]. This revealed the significance of *Sida cordifolia* over other plants.

CONCLUSION

The poly herbal formulation which contains more *Sida cordifolia* is proven with notable anti-inflammatory activity and its bioactive compounds are also high. These positive results may be helpful to take this formulation for the analysis of the *in-vitro* in-cell line and *in-vivo* anti-inflammatory and anti-microbial effects. This may also help to utilize the formulation for the prevention and treatment of various inflammatory conditions.

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Conflict of Interest
The authors declare they have no conflict of interest.

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