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In Vitro Evaluation of Anti Inflammatory Activity of Methanolic And Ethanolic Leaf Extract of *Psidium Guajava*

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ABSTRACT

The objective of the current research work was to determine the anti inflammatory activity of *Psidium guajava* by the Human Red Blood Cell membranes stabilization method. The methanol and ethanol leaf extract of plant *Psidium guajava* were investigated for their *in vitro* anti-inflammatory property by Human red blood cell membrane stabilization method. The prevention of hypotonicity induced HRBC membrane lysis was taken as a measure of a anti-inflammatory activity. The anti inflammatory activity of methanol and ethanol leaf extract of *psidium guajava* were compared to that of standard drug Diclofenac.

Key words:

Psidium guajava,
anti-inflammatory activity,
Human Red Blood Cell, Diclofenac.

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INTRODUCTION

Medicinal plants are source of drugs from many centuries and used extensively as crude materials or as pure compounds for treating various diseases. Relatively 1-10% of plants are used by humans out of estimated 250,000 to 500,000 species of plants on earth (borris1996). The chemical diversity of natural products is complementary to the diversity found in synthetic libraries. However, natural products are more complex and have greater ring system diversity because of the long evolutionary selection process. Therefore strategies to exploit natural sources and to develop methodologies for preparation of natural product libraries by combinatorial biosynthesis and related techniques are possible (Lewis & Lewis 1995). Mainstream medicine is increasingly receptive to use than plants derived drugs, as current day therapeutic agents are becoming ineffective possibly due to indiscriminate use (Schultes, 1978). Information on medicinal plant database (Rastogi & Mahotra 1990) More than 13,000 secondary metabolites have been isolated from the medicinal plants which are less than 10% of the total (Rubbia et al, 1999). In many cases these secondary metabolites serve as plant defense compounds or perform specialized mechanisms. These secondary metabolites were found to be endowed with medicinal properties including anti-inflammatory¹⁻⁴. Although synthetic oral hypoglycemic agents /insulin are the mainstream treatment of inflammation The plant *psidium guajava* has been used traditionally as a medicinal plant throughout the world for a number of ailments (Kaner & Chanda 2011). The present work represents the influence of anti-inflammatory activity of methanol and ethanol extract of leaves *psidium guajava* by human red blood cell membrane stabilization method⁵⁻⁹.

PLANT PROFILE

Psidium guajava, which is considered a native to Mexico (Riosetal, 1977) extends throughout the South America, European, Africa and Asia. Based on archaeological evidence. It has been used widely and known in Peru since pre-Columbian times. It grows in all the tropical and subtropical areas of the world, adapts to different climatic conditions but prefers dry climates (Stone, 1970). The main traditional use known is as an anti-diarrhoeal. Other reported uses include gastroenteritis, dysentery, stomach, antibacterial colic pathogenic germs of the intestine.



Fig 1: *Psidium guajava* Plant

SPECIES

Psidium guajava L. belongs to Myrtaceae.

SYNONYMS

Psidium guajavillus Burn, *Psidium pomiferum* L., *Psidium pumilum* Vahl, *Psidium pyrifolium* L.

DESCRIPTION

Guava (*Psidium guajava* L.) is an important tropical tree cultivated for its fruits. The fruit processing by-products, the leaves and the fruits themselves can be used to feed livestock though their nutritional value is low.

MORPHOLOGY¹⁰⁻¹⁵

Guava is a fast growing evergreen shrub or small tree that can grow to a height of 3-10 m. It has a shallow root system. Guava produces low drooping branches from the base and suckers from the roots. The trunk is slender, 20 cm in diameter, covered with a smooth green to red brown bark that peels off in thin flakes. Young twigs are pubescent. The leaves grow in pairs, opposite each other. The leaf blade is elliptic to oblong in shape, 5-15 cm long x 3-7 cm broad, finely pubescent and veined on the lower face, glabrous on the upper face. The flowers are white in colour, about 3 cm in diameter, solitary or in 2-3 flower clusters borne at the axils of newly emerging lateral shoots. The fruit is a fleshy, pyriform or ovoid berry that can weigh up to 500 g. The skin colour is yellowish to orange. The flesh can be white, yellow, pink or red, sour to sweet, juicy and aromatic. The fruit contains a variable number of seeds (about 3-5 mm long) and its mesocarp is characterized by the presence of small (0.1 mm) and hard fibrous structures called stone cells (sclereids), which may cause damage to processing machinery¹⁶⁻¹⁹.

USES IN TRADITIONAL MEDICINE

Psidium guajava is used in anti-inflammatory, for diabetes, hypertension, caries, wounds, pain relief and treatment of many diseases.

Collection of plant material

- The leaves of *Psidium guajava* were collected from the college of Avanthi Institute of Pharmaceutical Sciences, Tagarapavalasa.
- The plant was taxonomically identified and authenticated by the botanist of department of botany Andhra University.
- Leaves were dried and made into a coarse powder.
- Initial weight of the dried leaves = 1130 g
- Weight of powdered leaves = 880 g



Fig 2: *Psidium guajava* powder material

MATERIALS AND METHODS

PREPARATION OF THE EXTRACT^{20,21}

Soxhlet extraction: Different components which are used in Soxhlet extraction like thimble, water cooling system, and reservoir, by pass tube, siphon tube and condenser can be seen in fig. The leaves of *Psidium guajava* were air dried and pulverized into coarse powder. About 34 g of the powdered sample of each medicinal plant were weighed and 100 ml of solvent was added and extracted in a Soxhlet apparatus separately and the process is carried out for 7 days at 40-50°C. The filtrate was evaporated to dryness at 40°C in a rotary evaporator. And the above process was repeated for several times, until the sufficient amount of extract is produced. The concentrated extract of each plant was stored at 4°C until when required for use.



Fig 3: Soxhlet extract of plant material

EXTRACTION OF PLANT MATERIAL

The plant material was subjected for shade drying, then the shade dried plant material was subjected to pulverization to get coarse powder and it was extracted in Soxhlet apparatus using various solvents according to their polarity.

- Petroleum ether
- Chloroform
- Ethyl acetate
- Methanol
- Ethanol

MATERIALS REQUIRED

Shade dried coarse powder of *Psidium guajava*, petroleum ether, chloroform, ethyl acetate, methanol and ethanol.

Preparation of extracts

Petroleum Ether Extract

The crude powder was extracted with 2-3 liters of petroleum ether (60°- 80°C) using Soxhlet apparatus by continuous hot percolation method. After extraction it was filtered and then the removal of solvent was done under reduced pressure by

distillation process. Then the (10gm) extract was stored in a desiccators.

Chloroform Extract

After extraction, the marc left out was dried and then it is extracted with 2-3 liters of chloroform by continuous hot percolation method using Soxhlet apparatus. After extraction it was filtered and the removal of solvent was done under reduced pressure by distillation process. Then the extract was stored in a desiccators.



Fig 4:

Chloroform extract

ETHYL ACETATE EXTRACT

After extraction, the marc left out was dried and then it is extracted with 2-3 litres of ethyl acetate (69.0°C) by continuous hot percolation using Soxhlet apparatus. After extraction it was filtered and the removal of solvent was done under reduced pressure by distillation process. Then the extract was stored in a desiccators.

METHANOL EXTRACT

The marc remains after acetone extraction was dried and then it is extracted with 2-3 litres of alcohol 95 % using Soxhlet apparatus by continuous hot percolation method. After extraction, it was filtered and the removal of solvent was done under reduced pressure by distillation process. Then the extract was stored in a desiccators.

ETHANOL EXTRACT

The marc remains after extraction was dried and then it is extracted with 2 - 3 litres of ethanol using Soxhlet apparatus by continuous hot percolation method. After extraction, it was filtered and the removal of solvent was done under reduced pressure by distillation process. Then the extract was stored in a desiccators.



Fig.5: Etahnolic extract of plant powder

The above extracts are used for

- Identification of constituents by Phytochemical studies
- Separation and isolation of plant constituents by Thin Layer Chromatography
- Pharmacological studies

PHYTOCHEMICAL EVALUATION

IDENTIFICATION OF CONSTITUENTS BY PHYTOCHEMICAL STUDIES

The extracts were subjected to qualitative tests for detection of phytoconstituents present in it viz. alkaloids, carbohydrates, glycosides, phytosterols, fixed oils & fats, phenolic compounds & tannins, proteins and free amino acids, gums & mucilage's, flavanoids, lignin's and saponins.

TEST OF ALKALOIDS

A little fraction of the solvent free petroleum ether, chloroform, ethyl acetate, methanol and ethanol extracts were mixed individually with a small amount of drops of dilute hydrochloric acid and it is filtered. The filtrate was evaluated carefully with different alkaloid reagents such as Mayer's reagent - Cream precipitate Wagner's reagent - Reddish brown precipitate

TEST FOR CARBOHYDRATES & GLYCOSIDES

The least amount of extracts was dissolved in 5ml of distilled water and it is filtered. The filtrate was subjected to analysis for the presence of carbohydrates and glycosides.

a) Molisch's Test

The filtrate was mixed with 2-3 drops of 1% alcoholic alpha naphthol and along the Sides of the test tube; 2ml of concentrated sulphuric acid was added and appearance of purple colour ring at the junction of two liquids.

b) Fehling's Test

The filtrate was heated with 1ml of Fehling's A solution. Orange precipitate was obtained indicates the presence of carbohydrates. Another portion of the extracts was hydrolysed with hydrochloric acid for few hours on a water bath and the hydrolysate was subjected to Legals, Borntrager's test to detect the presence of different glycosides.

c) Legal's Test

The Hydrolysate was mixed with chloroform and then the chloroform layer was separated. To this identical quantity of dilute ammonia solution was added. Purple colour in ammonical layer was observed.

d) Test for Phytosterol (Liebermann Burchard Test)

One gram of the extract was dissolved in few drops of dry acetic acid, to this 3 ml of acetic anhydride and few drops of concentrated sulphuric acid was added. Bluish green colour appears which shows the presence of phytosterol.

e) Salkowski's test :

Treat extract in chloroform with few drops of concentrated sulphuric acid, shaken well and allow to stand for some time, red colour appear in the lower layer indicate the presence of sterols and formation of yellow coloured lower layer indicate the presence of triterpenoids

Test for Tannins and Phenolic Compounds

Small quantity of extracts were dissolved individually in water and tested for the presence of phenolic compounds and tannins with

- i. Dilute Ferric chloride solution 5% - Violet colour
- ii. 1% solution of gelatin containing 10% NaCl -White precipitate
- iii. 10% Lead acetate solution - White precipitate. About 10ml of extract were added individually to 25ml of absolute alcohol with continuous stirring and it is filtered. The precipitate was air dried and evaluated for its swelling properties and for the presence of carbohydrates.

TEST FOR FLAVANOIDS

- a) When mixed with aqueous sodium hydroxide solution, Colour changes from blue to violet colour (Anthocyanins), yellow colour (Flavones), yellow to orange (Flavonones).
- b) When mixed with concentrated sulphuric acid, yellowish orange colour (Anthocyanins), yellow to orange colour (Flavones), orange to crimson (Flavonones).

Tab 1: Preliminary Phytochemical Screening of Leaves Extract of *Psidium guajava*

Phytochemical Constituents	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Ethanol
Alkaloids	(-)	(-)	(-)	(-)	(-)
Carbohydrates	(-)	(-)	(-)	(-)	(-)
Glycosides	(+)	(+)	(-)	(-)	(-)
Flavonoids	(+)	(+)	(-)	(-)	(+)
Tannins	(+)	(+)	(-)	(-)	(-)

Separation and Isolation of Plant Constituents by Chromatographic techniques

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a chromatographical method which is employed to separate mixtures. It is performed on a glass sheet, aluminium or plastic foil, which is covered with a slim layer of adsorbent substance, generally silica gel, aluminium oxide, or cellulose (Blotter paper). This film of adsorbent is identified as the stationary silica phase³. After the sample has been filled on the plate, a solvent or

solvent mixture (mobile phase) is drained up the plate via capillary action. Because dissimilar analytes rise in the TLC plate at different rates, finally the mixture is separated.

Plate Preparation

TLC plates are generally commercially obtainable, with usual particle size ranges to develop reproducibility. They are prepared by adding the adsorbent, such as silica gel, with least quantity of inert binder like calcium sulphate (gypsum) and water. Thick slurry was prepared by spreading the mixture on a sheet, generally made up of thick aluminum foil, glass, or plastic. Heat the resulting plate for thirty minutes at 110 °C for drying and activation. The width of the adsorbent layer is usually around 0.1 – 0.25 mm for investigative purposes and about 0.5 – 2.0 mm for preparative TLC⁵.

Analysis

If the chemicals which are separated are colourless, several methods are available to visualize the spots. Repeatedly a little quantity of a fluorescent compound, generally manganese activated zinc silicate, is mixed with the adsorbent that allows the visibility of spots under a black light at 254 nm. The adsorbent layer will appear in fluorescence light green by self; however this fluorescence is 58 quenched by the spots of analyte. Iodine vapors are a common unspecific colour reagent. The TLC plate is dipped into the specific colour reagents or which are sprinkled on top of the plate, Potassium permanganate – oxidation, Iodine. Once visible, the *R_f* value of each spot can be calculated by dividing the distance travelled by the product by the total distance travelled by the solvent. These values which are calculated depend on the nature of solvent used and the nature of TLC plate and will not come under physical constants. The thin layer contains the eluent at the top of the plate.

Application of Extracts for Separation

The various diluted extracts spotted on a TLC plate 2cm above its bottom using capillary tube. Most solutions for application were between 0.1 - 1% strength. The starting point was equally sized as far as possible and spots had diameter ranging from 2-5mm.

Method

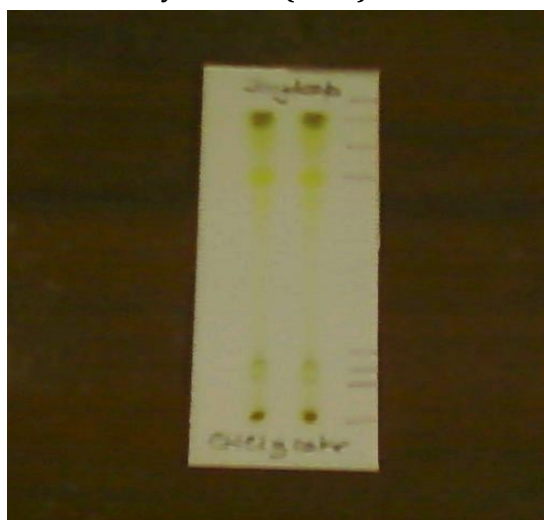
The extracts of *Psidium guajava* of petroleum ether, chloroform, ethyl acetate, methanol and ethanol extracts were subjected to Thin Layer Chromatography using different solvent systems and observed for characteristic spots under UV light and Iodine chamber.

The different solvent systems used were

1. Petroleum ether extract - Benzene: Chloroform: ethylacetate (4:3:3) Hexane: Ethyl acetate (5:4)
2. Chloroform extract - Benzene: Chloroform: Ethylacetate (3:4:3) Chloroform: Ethyl acetate (4:6)
3. Ethyl acetate extract - Toluene: Ethyl acetate (8:2) Toluene: Ethylacetate: Formic acid (7:2:1)
4. Methanol extract - Ethyl acetate: Methanol (7:3) Ethyl acetate: Formic acid: Acetic acid: Water (100:11:27)



Fig 6: TLC Profile of the Pet ether extract of P. guajava leaf. Solvent system: benzene: chloroform:ethyl acetate (4:3:3)



Rf values 0.10,0.74,0.87,0.93.

Fig 7: TLC profile of chloroform extract of P. guajava leaf. Solvent system: benzene : chloroform :ethyl acetate (3:4:3) Rf values 0.10,0.15,0.21,0.73,0.84,0.93



Fig.8: TLC profile of the methanol extract of P. guajava leaf. Solvent system: ethyl acetate: methanol (7: 3). Rf values: 0.34, 0.44, 0.50, 0.68, 0.84.

IN VITRO ANTI-INFLAMMATORY ACTIVITY BY HUMAN RED BLOOD CELL MEMBRANES STABILIZATION METHOD : EXTRACTION PROCEDURE

Ten grams of plant coarse powder of P. guajava plant weighed in to a 250 ml conical flask and 100 ml of methanol and ethanol was added separately for each plant powder then on a rotary shaker at 190 – 220 rpm for 24 hours. This was filtered with whatman No1. Filter paper, the residue discarded, and the filter were evaporated to dryness in a water bath temperature at 80⁰C.

PREPARATION OF STOCK SOLUTION FOR EACH EXTRACT OF LEAVES SELECTED INDIGENOUS PLANTS POWDER

stock solution was prepared by weighing 10 mg of each dried solvent extract dissolved in 1 ml of dimethyl sulphoxide (DMSO) giving a final concentration of 10,000 µg/ml. The stock solution was kept in screw capped bottles for subsequent use Anti-Inflammatory Activity of Indigenous plant Extract by HRBC Method.

Human red blood cell membrane method was used for the invitro estimation of anti-inflammatory activity. Blood was collected from healthy volunteers and was mixed with equal volume of sterilized Alsevers solution (equal volume of (2% dextrose, 0.8% sodium citrate, 0.05% citric acid & 0.42% sodium chloride in water). This blood solution was centrifuged at 3000 rpm and the packed cells were separated. The packed cells were washed with isosaline solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of extract, reference sample and control were separately mixed with 1ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of HRBC suspension. Instead of hyposaline 2 ml of distilled water used in the control. All the assay mixtures were incubated at 37⁰c for 30 minutes and centrifuged at 3000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by spectrophotometer at 560 nm. The percentage of hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

$$\% \text{ Percentage Protection} = 100 - \frac{\text{Optical Density of sample}}{\text{Optical Density of Control}} \times 100$$



Fig.9: Blood collection for activity

RESULTS AND DISCUSSION

Methanol and Ethanol leaf extract of *Psidium guajava* L. exhibited membrane effect by inhibiting hypo tonicity induced Lysis of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membrane.

Stabilization of lysosomal membrane is important in reducing the inflammatory response by preventing the release of lysosomal constituent of activated neutrophil such as bacterial enzymes and proteases which cause further tissue inflammation and damage.

Anti-inflammatory activity all plant showed anti-inflammatory activity by preventing lysis of RBC membrane. The results of the anti-inflammatory activity of plant extracts tested by Human Red Blood Cell membranes stabilization method are shown in Table -1 and 2. The methanol and ethanol extract of *Psidium guajava* L., show significant anti-inflammatory activity. From the above study it was concluded that the Methanol and ethanol leaf extract of *Psidium guajava* has significant membrane stabilization property.

Inflammation is a dynamic and multifactorial process involving with many systems in the body. Rheumatic diseases are common inflammatory diseases in the world. There is a great disadvantage of present anti-inflammatory synthetic drugs like Steroidal and non-steroidal drugs lie in their toxicity and reappearance of symptoms after discontinuation of treatment. Plant based traditional medicine system play a vital role in healthcare and various plant ext *Psidium guajava* Linn, belonging to the Family Myrtaceae.

More recent ethno pharmacological studies show that *Psidium guajava* is used in many parts of the world for the treatment of a number of diseases, e.g. as an anti-inflammatory, for diabetes, hypertension, caries, wounds, pain relief and reducing fever. Some of the countries with long history of traditional medicinal use of guava include Mexico and other Central American countries including the Caribbean, Africa and Asia. Some of these uses will be outlined here.

Medicinal plants are an important element of the indigenous medical systems in Mexico (Lara and Marquez, 1996). These resources are part of their traditional knowledge. The phytoconstituents present in leaf extract are viz. alkaloids, carbohydrates, glycosides, phytosterols, fixed oils & fats, phenolic compounds & tannins, proteins and free amino acids, gums & mucilage's, flavanoids, lignin's and saponins.

- Initial weight of the dried leaves = 1130g
- Weight of powdered leaves = 880g
- Weight of powder used for exp. = 34g
- Weight of petroleum ether extract = 2.5g
- Weight of chloroform extract = 2.3g
- Weight of ethyl acetate extract = 2.3g
- Weight of methanol extract = 2.5g
- Weight of ethanol extract = 2.4g

Table 2: Anti-inflammatory activity of Standard Diclofenac at various concentrations

S.No.	Plant Extract and Standard Drug	Concentration $\mu\text{g/ml}$	% of Activity (Prevention of lysis)
01	Standard Diclofenac		58%
			100%

Table 3: Anti inflammatory activity of Methanol and Ethanol extracts of *P. guajava* leaf at 100 and 200 $\mu\text{g/ml}$ concentrations

S.No.	Plant Extract	% of Activity (Prevention of lysis) At 100 $\mu\text{g/ml}$ Concentration	% of Activity (Prevention of lysis) At 200 $\mu\text{g/ml}$ Concentration
01	Methanol	93.28%	100%
02	Ethanol	60%	100%

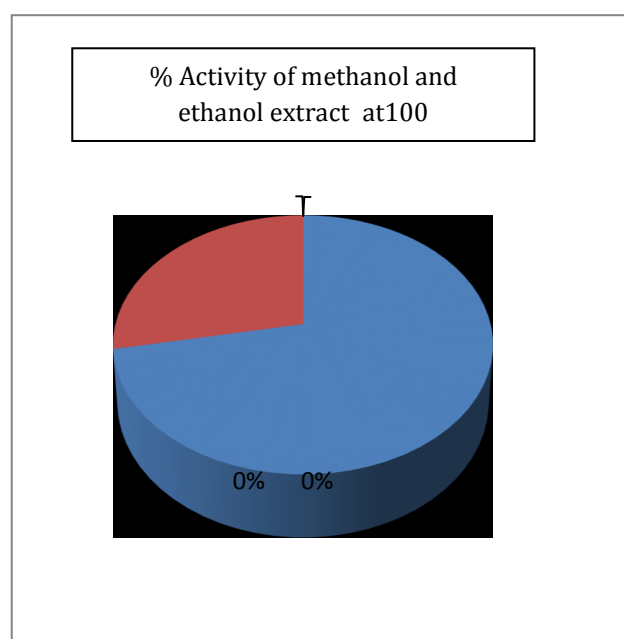


Fig 10: % activity of methanolic and ethanolic extract

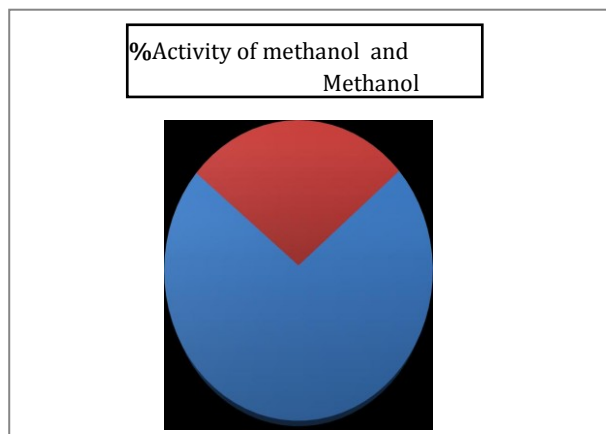


Fig 11: % activity of methanolic and ethanolic at 200µg/ml conc

CONCLUSION

The present work provide evidence that methanol and ethanol extracts of leaves of *Psidium guajava* exhibited anti inflammatory activity by invitro evaluation tests through HRBC method this effect may be due to presence of phyto constituents like flavonoids, steroids, glycosides, tannins, terpenoids. These constituents individually or together help in anti inflammatory activity. Methanol leaf extract of *Psidium guajava* exhibited highest anti inflammatory activity and ethanol leaf extract exhibited lowest activity. The results give a justification for the use of these plants in anti-inflammatory medicine.

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