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EVALUATION OF TOOTHACHE ACTIVITY OF METHANOLIC EXTRACT AND ITS VARIOUS FRACTIONS FROM THE LEAVES *PSIDIUM GUAJAVA* LINN

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ABSTRACT

Psidium guajava L is commonly known as guava in English. The present investigation focuses on the toothache activity. The investigation is based upon the analgesic, anti-inflammatory and anti-microbial activity of water extract of *Psidium guajava* L. The study assessed the different solvent extracts of *Psidium guajava* leaf for thin layer chromatography (TLC) and also evaluated the analgesic activity by Hot plate and acetic acid induced writhing, anti-inflammatory action by *Invitro* HRBC membrane stabilization method and antimicrobial activity by Disc diffusion method, Agar well diffusion method and Minimum inhibitory concentrations (MIC) in animal model. The present preliminary study demonstrated marked toothache activity of *Psidium guajava* leaf. These results support the notion that plant flavonoids and extracts may have many roles as pharmaceuticals.

KEYWORDS

Psidium guajava L, analgesic, anti-inflammatory, anti-microbial, Toothache.

INTRODUCTION

This extensive flora has been greatly utilized as a source of many drugs in the Indian traditional system of medicine.[1] About 80% of the world population depends on herbal based alternative systems of medicine. The activities of these curative plants are evaluated by their chemical components. Indian Ayurveda utilizes about 2000 plants to cure different ailments.[2] *Psidium guajava* is a member of the Myrtaceae family, which contains at least 133 genera and more than 3,800 plant species. The ethanobotanical studies and folklore claiming reviewed that the leaves of the plant *Psidium guajava* Linn are used for antioxidant, hepatoprotective, anti-allergy, antimicrobial, antigenotoxic, anti plasmodial, cytotoxic, antispasmodic, cardioactive, anticough, antidiabetic, anti-inflammatory and anti nociceptive activities. Guava leaf tea is commonly used as a medicine against gastroenteritis (dysentry) and child diarrhea.[3]

Flavonoids and other plant phenolics have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory action, inhibition of platelet aggregation and antimicrobial activities. Quercetin belongs to the flavonoids and consists of three rings and five hydroxyl groups. It occurs in food as an aglycone. It is found in many common foods including apple, tea, onion, nuts, berries, cauliflower and cabbage. Quercetin has several pharmacological actions which possesses dose dependent antioxidant properties, Anti-inflammatory activity, Antiviral, antitumour activities. Toothache is pain typically around a tooth, teeth or jaws. In most instances, toothaches are caused by a dental problem, such as a dental cavity, a cracked or fractured tooth, an exposed tooth root, or gum disease.[4] Pain is produced by the excitation of particular receptors, the nociceptors or of their afferent fibres. The mouth contains a wide variety of oral bacteria, but only a few specific species of bacteria cause dental caries**.**

MATERIALS AND METHODS

(i) Collection and Authentication

The plant specimen (Leaves of *Psidium guajava* Linn.) for the proposed study was collected from the garden of Vels university, Pallavaram, Chennai during the month of July 2010. It was identified and authenticated by Dr. P. Jayaraman, Director Plant Anatomy research centre (PARC), Tambaram, Chennai. A voucher specimen No-PARC/2010/593 has been deposited in department of pharmacognosy.

(*ii) Extraction and Fractionation*[5]

The identified leaves of *Psidium guajava* Linn were shade dried and coarsely powdered. About 300 gm of powdered drug was extracted with methanol by cold maceration method. After 72 hrs of maceration it was filtered. After complete extraction the extract was concentrated by distilling off the solvent and concentrated extract was fractionated successively with solvents of increasing polarity petroleum ether, chloroform, ethyl acetate and water to yield its respective fractions. All the fractions were evaporated under reduced pressure using vacuum flash evaporator. The colour and consistency and percentage yield of all fractions were observed.

(iii) Phytochemical study

The methanolic extract and its fractions (petroleum ether, chloroform and ethyl acetate, and aqueous) were subjected to the following qualitative phytochemical test for identification of phytoconstituents.

(iv) Animals

Male *Swiss mice* weighing 20 - 30 gm and male *Sprague- Dawley rats* weighing 180 - 280 g were used. For the present pharmacological study the animals were kept in a room maintained on 12h/12h light/dark cycle, at 25°C constant temperature and 55% relative humidity. They had free access to food and water. Before testing, they were allowed to adapt in the test room for at least 12 h. The experiment was conducted as per CPSCEA form with approval of animal ethics committee and the registration number is (XII/VELS/PCOG/20/2000/CPCSEA/IAEC/22.02.11)

(v) Toxicity study

The acute toxicity study of methanolic extract of *Psidium guajava* Linn leaves as per OECD guidelines-423 was reported. It was found that the methanolic extract of leaf was nontoxic up to 2000 mg/kg dose level. So one tenth of the upper bound dose was decided (200 mg/kg body weight) for the present pharmacological experiments.

(vi) Thin Layer Chromatography (TLC)

Preparation of test sample- 10 mg of test sample of methanolic extract and its fractions (Petroleum ether, Chloroform, Ethyl acetate and aqueous) were dissolved in 5ml of 95% methanol. 1µl was applied as a spot.

Developing solvent system- A number of developing solvent systems were tried, but the satisfactory resolution was obtained in the solvent systems mentioned in the table-6. After development of plates, they were air-dried and number of spots and R_f values were noted. Eluted spots were visualized by UV light and sprayed with 10%v/v sodium hydroxide solution as detecting agent.

(vii) Animal groups for treatment

Group-1: 1 ml/kg of Carboxy methyl cellulose (1%CMC), Group-2: Methanolic extract of *Psidium guajava* Linn leaves (200 mg/kg P.o) suspended in 1%CMC, Group-3: Ethyl acetate fraction (200 mg/kg P.o) suspended in 1%CMC, Group-4: Total flavonoid (200 mg/kg P.o) suspended in 1%CMC, Group-5: Standard (Diclofenac sodium 45mg/kg P.o for anti-inflammatory activity) and Standard (Aspirin 200 mg/kg P.o for analgesic activity).

(viii) In vitro Anti-inflammatory activity

Invitro anti-inflammatory activity by HRBC membrane stabilization method[6]

The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline [0.36 %], 0.5 ml HRBC suspension[10 % v/v] with 0.5 ml of plant extract(methanolic extract, ethyl acetate fraction,) of various concentrations (31.25, 62.5, 125, 250, 500, 1000, 2000 µg/0.5ml), standard drug diclofenac sodium (250, 500 1000, 2000 µg/0.5ml) and control [distilled water instead ofhypo saline to produce 100 % hemolysis] were incubated at 37^o C for 30 min and centrifuged respectively . The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage hemolysis produced in the presence of distilled water was taken as 100 %. Percentage of HRBC membrane stabilization or protection was calculated using the formula,

$$\text{Percent stabilization} = 100 - \frac{\text{(Optical density of drug)}}{\text{(Optical density of control)}} \times 100$$

(ix) Anti nociceptive activity

Hot plate method[7]

Swiss albino mice of either sex weighing between 25-30 g were deprived of solid food for 10 hrs before the commencement of the experiment. Mice were individually placed on the hot plate maintained at constant temperature (55±0.1^{0}C). It produces behavior changes measured in terms of the reaction time of animals was noted. Animals whose basal reaction time was within 5 sec were selected for the study. A cut off period of 15 sec was allowed to avoid damage to the paw. The prolongation of latency time compared to control was noted. The

hot plate latencies of animals in each group at 30, 60 and 120 min after treatment were noted.

Acetic acid induced writhing test[8]

The anti-nociceptive effect was evaluated in mice by the writhing test induced by 0.6% acetic acid (0.1 ml/10 mg; I.P). The dose of the methanolic extract and its fraction (EAF) were administered orally 30 min before the acetic acid injection. 5 min after the administration of the writhing agent, the number of writhes and stretching movements (contraction of the abdominal musculature and extension of hind limbs) was counted over a 5 min for a period of 30 min. The strength of the elicited analgesic effect was compared to that of an effective dose of acetylsalicylic acid (ASA, 200 mg/kg) as reference standard.

$$\% \text{ Inhibition} = \frac{\text{Mean number of writhing in control} - \text{Mean number of writhing in test group}}{\text{Mean number of writhing in control group}}$$

(x) Antibacterial activity

Antibacterial activity using disc diffusion method[9]

Staphylococcus aureus , *S. mutans* was incubated individually in sterile nutrient broth for 24 h at 37°C,30° C and adjusted to yield approximately 1.0×10^{-7} CFU/ml. A prepared inoculum was added to molten agar, mixed and poured over the surface of the nutrient agar medium in sterile petri dishes and left to solidify. A sterile paper discs 6 mm in diameter were impregnated with specified concentrations (50µl/disc and 100µl / disc) of methanolic extract and its fractions (ethyl acetate, total flavonoid) (Ciprofloxacin 5 µl/disc was used as standard) the discs were placed on the surface of agar plates. Following the same procedure, sterile discs were impregnated with A disc without test material was used as control. The plates were left for 1hr at room temperature as a period of pre incubation diffusion to minimize the effects to variation in time between applications of the different solutions. The plates were incubated at 37°C for 24 h under aerobic conditions and observed for antibacterial activity. All disc diffusion tests were performed in four separate experiments and the antibacterial activity was expressed as the mean of inhibition diameters (mm).

Agar well diffusion Method[10]

The extract and its fractions were examined for their antimicrobial activities against the toothache bacteria named above using the micro dilution method. Each tested compound was added into a microtiter plate containing appropriate broth to obtain the concentration ranging from 10 to 200 microgram per ml. The bacteria to be tested were added to the wells containing the compound to obtain a final concentration of 104 CFU/ml. A positive control (without tested compounds) and a negative control (without tested bacteria) were included for each plate. After incubation at optimal temperature, bacterial growth was inspected at 24 h.

Determination of Minimum inhibitory concentrations (MIC) [11]

The MIC for the above organisms was found by Agar streak dilution method. Nutrient agar medium was used and the media were sterilized by autoclaving at 15 lbs/sq inch pressure. Stock solutions of the fractions were mixed with the known quantity of molten sterile agar media aseptically. After streaking all the plates were incubated at 37±1° for 24 h. Then the plates were observed for the growth of the microorganisms. The lowest concentration of the fraction required for inhibiting the growth was considered as the MIC of the fraction against bacterial strains. The MIC values of each fraction against the tested microorganisms done by agar streak dilution method.

(xi) Statistical analysis

For all the above methods, the results were expressed as mean ±SEM. Statistical analysis significant test for comparison was done by ANOVA, followed by Dunnett test.

RESULTS

The present work covers phytochemical study, antioxidant study, anti-inflammatory activity (*invitro*), anti-nociceptive activity and

antibacterial activity of methanolic extract, and bioactive fraction (EA) and isolated flavonoid (or) IF of the leaves of *Psidium guajava* Linn. The identified leaves of *Psidium guajava* Linn were shade dried and coarsely powdered. After complete extraction the extract was concentrated by distilling off the solvent and concentrated were observed.

extract was fractionated successively with solvents of increasing polarity petroleum ether, chloroform, ethyl acetate and water to yield its respective fractions. All the fractions were evaporated under reduced pressure using vacuum flash evaporator. The colour and consistency and percentage yield of all fractions

Table 1

Percentage Yield of methanolic extract and its fractions of the leaves of Psidium guajava Linn.

S.no	Extract/fraction	Colour	Percentage yield (%w/w)
1	Methanolic extract	Brownish black	5
2	Petroleum ether	Greenish yellow	0.37
3	Chloroform	Greenish yellow	0.75
4	Ethyl acetate	Brownish black	1.52
5	Aqueous	Brown	0.25

The extract found its fraction were by identified compare with standard Quercetin by TLC. It was performed using solvent system Toluene: Ethyl acetate: Formic acid (5:4:1) % v/v with sodium hydroxide as detecting agent. The result was observed which showed different R_f values methanolic extract showed 4 spots with R_f value of (0.21,0.51,0.78,0.82) and standard R_f value (0.82) , Ethyl acetate fraction R_f value (0.78).

Figure 1

TLC of ethyl acetate fraction of Psidium guajava Linn [S= Standard (Quercetin), EA= Ethyl acetate fraction]

Table 2

Thin layer chromatography of methanolic extract (ME) and its fractions (EAF, AF) of Psidium guajava Linn leaf

S.No	Test extract	Solvent system	Detecting agent	Number of spots	R_f value
1	ME	T.E.F (6:3:1)	NaoH	1	0.80 0.78 0.51 021
2	EAF	T.E.F (6:3:1)	NaoH	1	0.78

3	AF	T.E.F (6:3:1)	NaoH	1	0.51

R_f - Retardation factor, T:E:F- Toluene: Ethyl acetate: Formic acid, EAF – Ethyl Acetate, ME – Methanolic Extract of leaves of Psidium guajava Linn.

The methanolic extract and its fractions (petroleum ether, chloroform and ethyl acetate, and aqueous) were subjected to the following qualitative phytochemical test for identification of phytoconstituents.

Table 3

Phytochemical test of the Leaves of Psidium guajava (+) indicates present, (-) indicates absent

Chemical Test	Dried powder	Methanolic extract	Petroleum ether fraction	Chloroform Fraction	Ethyl acetate Fraction	Aqueous fraction
Alkaloids	-	-	-	-	-	-
Carbohydrates	-	-	-	-	-	+
Glycosides	+	+	-	-	-	-
Proteins	-	-	-	-	-	+
Amino acids	-	-	-	-	-	+
Saponins	-	-	+	-	-	+
Flavonoids	+	+	-	-	+	-
Phenolic compounds	+	+	-	-	+	-
Tannins	+	+	-	-	-	+
Terpenoids	-	-	-	+	-	-
Oil and fats	+	+	-	-	-	-
Steroids	+	+	+	+	-	-

The percentage protection of methanolic extract and its fraction (EAF) and IF was 98.34%, 98.35, 98.57 at 1000µg/ml It possesses significant activity comparable with that of the standard Diclofenac sodium. *Psidium guajava* has significant anti-inflammatory activity which may be due to presence of chemical profile such as Flavones, Flavonones and Phenols.

Table 4

Invitro Anti-inflammatory activity of Psidium guajava Linn leaf by HRBC membrane stabilization method

Concentration (mcg/0.5ml)	ME	EAF	IF	Diclofenac Sodium (std)
31.25	83.33±0.56**	84.22±0.77^{ns}	85.42±0.19**	-
62.5	88.37±0.78**	89.32±0.44 ^{ns}	89.64±0.55**	-
125	92.28±0.66**	93.56±0.76 ^{ns}	93.42±0.66**	-
250	93.10±0.34**	93.77±0.66 ^{ns}	93.98±0.77**	82.74 ±0.67
500	96.77±0.76**	97.66±0.54 ^{ns}	97.65±0.36**	88.39± 0.56
1000	98.34±0.77**	98.35±0.66 ^{ns}	98.57±0.66**	90.10 ±0.78
2000	98.66±0.44**	98.23±0/34 ^{ns}	98.86±0.68**	99.94 ±0.98

Values are mean ± SEM, **P< 0.01, ^{ns} P >0.05, Vs Standard (n=5)

Table 5
Analgesic activity of Psidium guajava Linn by Hot plate method

Treatment	Mean latency (s) before and after Drug administration(s)				% inhibition		
	0min	30min	60min	90min	30min	60min	90min
Control 1%CMC	2.26±0.219	2.45±0.225	2.16±0.197	2.58±0.261	---	---	---
ME 200mg/Kg	2.01±0.162	3.86±0.551**	5.82±0.670**	5.13±0.287**	35.23	58.14	54.78
EAF 200mg/kg	2.07±0.217	3.57±0.850**	6.34±0.517**	5.93±0.460**	31.50	65.89	56.56
IF 200mg/kg	2.34±0.088	5.62±0.624**	7.97±0.649**	11.67±1.007**	56.47	72.86	77.91
Standard 200mg/kg	1.96±0.217	4.56±0.281**	8.92±0.860**	11.53±1.159**	45.15	72.68	79.89

Values are mean ± SEM, **P< 0.01 Vs control (n=5)

Table 6
Analgesic activity of Psidium guajava Linn Acetic acid induced writhing method

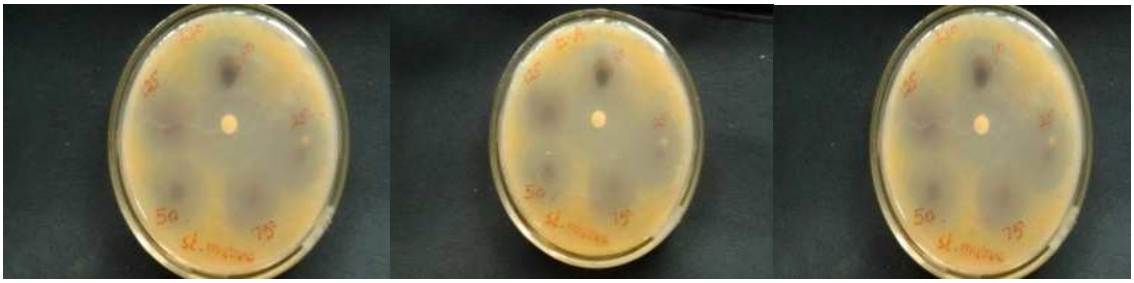
Group	Treatment	Dose mg/kg	Number of Writhing	% inhibition
I	Control	-	19.5±1.87	-------
II	ME	200	9.5±1.871**	51.28
III	EAF	200	7.166±1.169**	63.28
IV	IF	200	6.616±0.7528**	68.37
V	Standard	200	4.16±1.329**	78.63

Values are as mean ± SEM, **P<0.01 Vs Control (n=5)

The extract and the fraction and isolated flavonoid of plant were found to exhibit concentration dependent increase in latency time when compared with control. At 90 minutes, the percent inhibition of *Psidium guajava* methanolic extract and its fraction (EAF) and IF was 54.78%, 56.56%, 77.91% respectively.

The effect of the methanolic extract of *Psidium guajava* (L) and isolated compound on acetic acid induced writhing in mice. The extract and its fraction (EAF) and IF produced 51.28%, 63.28%, 68.37% writhing inhibition in test animals, respectively.

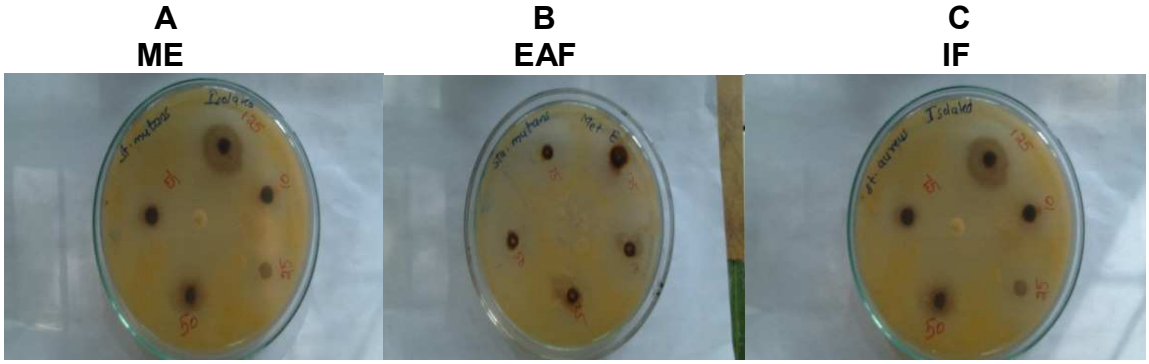
The antibacterial effects of methanolic extract of *Psidium guajava* Linn leaf and its fraction (EAF) and IF by disc diffusion method and agar well diffusion method against *Staphylococcus aureus, Streptococcus mutans* are the one of the etiologic factors for toothache. This extract and fraction isolated compound showed antibacterial activity in concentration dependent manner. The results of all tested samples were comparable with that of standard ciprofloxacin. The MIC of the extract and fraction, isolated flavonoid was calculated against tested organism.



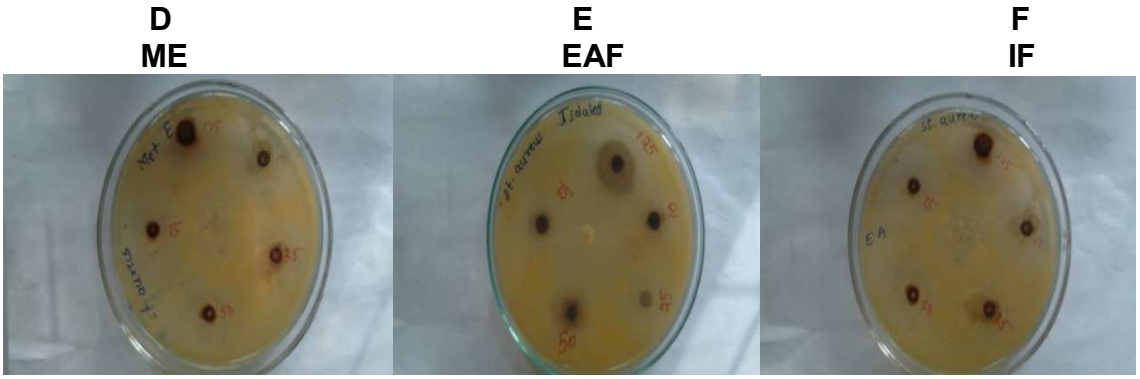
Inhibition zone of S.mutans for methanolic extract and its fractions(EAF,IF)

Inhibition zone of S.aureus for methanolic extract and its fractions(EAF,IF)

Figure 2
Antibacterial activity of Psidium guajava Linn by disc diffusion method



Inhibition zone of S.mutans for methanolic extract and its fractions(EAF,IF)



Inhibition zone of S.aureus for methanolic extract and its fractions(EAF,IF)

Figure 3
Antibacterial activity of Psidium guajava Linn by agar well diffusion method

Table 7
Antibacterial activity of *Psidium guajava* Linn leaves against *S. aureus* and *S. mutans* by disc diffusion method.

S. No	Treatment	Concentration (µg/ml)	Zone of Inhibition (in mm) for *S. aureus*	Zone of Inhibition (in mm) for *S. mutans*
1	ME	25 50 75 100	11 18 20 20.5	10.5 14 16 17.5
2	EAF	25 50 75 100	12 19 21 22	11.5 17.5 20 20.5
4	IF	25 50 75 100	14 20 23 24	12 16 18 19.5
5	Standard	25	25	25

Table 8
Antibacterial activity of Psidium guajava Linn leaves against S. aureus and S. mutans by Agar well diffusion method.

S. No	Treatment	Concentration (µg/ml)	Zone of Inhibition (in mm) for *S. aureus*	Zone of Inhibition (in mm) for *S. mutans*
1	ME	25 50 75 100	11 18 20 20.5	10.5 14 16 17.5
2	EAF	25 50 75 100	12 19 21 22	11.5 17.5 20 20.5

4	IF	25 50 75 100	14 20 23 24	12 16 18 19.5
5	Standard	25	25	25

Table 9
***MIC values of Psidium guajava Linn leaves for S. aureus*.**

S.no	Test drug	MIC
1	ME	625µg/ml
2	EAF	475µg/ml
3	AF	7.5mg/ml
4	IF	225µg/ml

DISCUSSION

Earlier phytochemical review reported that leaves of *Psidium guajava* contains high amount of flavonoids. Leaves of *Psidium guajava* was a rich source of quercetin. Quercetin was effective in free radical scavenging property, and has analgesic, anti-inflammatory, antibacterial properties. Phytochemical test was carried out to identify the phytoconstituents present in the methanolic extract and its fraction. Phytochemical screening of ethyl acetate fraction showed the presence of flavonoids and Phenolic compounds. The test extract exhibited membrane stabilization effect by inhibiting hypo tonicity induced lyses of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the ME and EAF, TF may as well stabilize lysosomal membranes. The test sample may inhibit the process, which may stimulate or enhance the efflux of these intracellular components. Since total flavonoid was found to be more effective in stabilizing the RBC membrane against hypo tonicity induced haemolysis in the control of inflammation, TF may inhibit the triggering of inflammation in Toothache.

The methanolic extract and its fractions (EAF, TF) of the *Psidium guajava* plant showed significant analgesic action compared to

standard drugs. The methanolic extract and its fractions of *Psidium guajava (L)* produced significant writhing inhibition. On the basis of this result it can be concluded that the methanolic extract and its fractions of *Psidium guajava (L)* might possess antinociceptive activity. The hot plate method is considered to be selective for the drugs acting centrally. Therefore, the methanolic extract and its fractions of the plant *Psidium guajava* Linn must have a central activity. The plant extract and fractions of *Psidium guajava* Linn exhibited both types of pain inhibition. The analgesic effect of the plant in both models suggests that they have been acting through central and peripheral mechanisms. *Psidium guajava* leaves have long been recognized for their antibacterial activity. They were shown to inhibit both Gram-positive and Gram-negative bacteria such as *S. aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Bacillus cereus*, *Proteus* spp., *Shigella* spp. and *E. coli.* The results showed that the flavonoids were able to inhibit all of the bacteria used in this study with different degree of inhibition *S. aureus* and *S. mutans* were the most and the least sensitive strains to the flavonoids, respectively. For each examined bacteria, MIC values of *Psidium guajava* Linn was measured.[12]

CONCLUSION

In Ayurveda the leaves of *Psidium guajava* found to be used for toothache. Earlier report revealed that the leaves were found to contain flavonoid quercetin. Flavonoids such as quercetin, rutin, keampferol are unique bioactive compounds that posess anti-inflammatory, analgesic property and free radical scavenging activity. All the fractions have protective effect against free radicals generated by nitric oxide. Flavonoid rich fraction and isolated compound were more effective in scavenging free radical which was reflected in tested inflammatory activity and anti-nociceptive activity may be one of the mechanisms by which drug was found to be effective in Toothache, which is used in traditional medicine. Antibacterial effects of methanolic extract and its fractions (EAF, TF) were evaluated by disc diffusion method against *Staphylococcus aureus,* and *S. mutans.* Zone of inhibition was observed for all tested sample. So finally it may be confirmed that flavonoid rich fraction (total flavonoid) have significant multiple activities against the biological events which are responsible for the pathogenesis of Toothache.

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