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ANTIOXIDANT, ANALGESIC AND ANTIMICROBIAL ACTIVITIES OF DIFFERENT FRACTIONS FROM METHANOLIC EXTRACT OF *PSIDIUM GUAJAVA* L. LEAVES

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ABSTRACT: *Psidium guajava* Linn. the most popular and widely cultivated fruiting plant all over Bangladesh. The present study was aimed to investigate the antioxidant, analgesic and antimicrobial activities of n-hexane (HPG), chloroform (CLPG) and ethyl acetate fractions (ET APG) obtained from methanol extract of *Psidium guajava* (MPG) leaves. The *in-vitro* antioxidant activity of fractions HPG, CLPG and ET APG were determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and ascorbic acid was used as standard. The IC_{50} value of HPG, CLPG and ET APG were 29.96 µg/ml, 26.84 µg/ml and 24.29 µg/ml respectively whereas the IC_{50} value of ascorbic acid was 6.23 µg/ml. Analgesic activity of HPG, CLPG and ET APG extracts (400 mg/kg) were evaluated using acetic acid-induced writhing model of pain in mice and demonstrated significant reduction of pain in mice with the effect of 56.10%, 60.51% and 70.12% respectively ($p<0.05$) and were comparable to that of standard, diclofenac sodium (77.22%). Preliminary phytochemical screening of different fractions showed the presence of bioactive constituents like alkaloids, saponin, flavonoids, tannins and triterpenoids. Further, HPG, CLPG and ET APG fractions (500 µg/ml) showed antibacterial activity as measured by zone of inhibition on gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis* and gram-negative bacteria such as *Salmonella paratyphi*, *Salmonella typhi* and *Shigella dysenteriae* and compared with azithromycin (50 µg/ml) as a reference standard. The different fractions from *Psidium guajava* possesses antioxidant, analgesic and antimicrobial activities might be used as a therapeutic alternative in the treatment of infection and pain; and can protect against oxidative stress-induced tissue damage.

INTRODUCTION: *Psidium guajava* (common name-guava) is well known tropic tree which is abundantly grown for fruit.

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It belongs to phylum *Magnoliophyta*, class Magnoliopsida and Myrtaceae family which is originated from tropical South America [1]. Now, it is widely cultivated in many tropical and subtropical countries for its edible fruit.

Guava is a phytotherapeutic plant used in folk medicine containing bioactive constituents and might be helpful for the management of the various disease. Traditionally, different parts of the plant have been used to manage conditions like malaria,

gastroenteritis, vomiting, diarrhea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums and other conditions [2, 4]. Guava contains a high content of organic and inorganic compounds like secondary metabolites *e.g.* antioxidant polyphenols, antiviral and anti-inflammatory compounds [5]. Psidium fruits are a potential source of phytochemicals for which many bioactivities have been proved. Carotenoids, phenolic compounds and triterpenoids are the main phytochemicals characterized from leaves and fruits of this genus [6]. The plant has also been used for the control of life-changing conditions such as diabetes, hypertension and obesity [7]. The goal of the study was to investigate the antioxidant, analgesic and antibacterial activities of different fractions from *Psidium guajava* leaves.

MATERIALS AND METHODS:

Plant Materials: Fresh leaves from plants of *Psidium guajava* were collected from Tangail in July 2016 and the plant authenticity was confirmed from the Bangladesh National Herbarium, Mirpur, Dhaka, and a voucher specimen no. DACB-55126 was kept for future reference.

Preparation of Plant Extract: The collected leaves were cleaned and sun-dried for several days. The dried leaves were powdered (500 g) and soaked in 1.5 liters of methanol at room temperature for 7 days with occasional shaking and stirring. The extract was successively filtered through fresh cotton bed and filter paper (Whatman filter paper number 1). Then, the liquor was allowed to evaporate using rotary evaporator under reduced pressure to give a highly concentrated methanol extract of *Psidium guajava* (MPG). The semisolid mass (50 g) was then fractionated by the modified kupchan partitioning protocol [8] by three solvent such as n-hexane, chloroform and ethyl acetate in order of increasing polarity using separating funnel and the resultant fractions were evaporated to dryness to yield n-hexane, chloroform and ethyl acetate soluble fractions from MPG and designated as HPG, CLPG and ET APG respectively. The dried residue was then stored in a refrigerator until further investigation.

Drugs and Chemicals: DPPH (1, 1-diphenyl- 2-picrylhydrazyl -hydrate), diclofenac-Na and azithromycin disks were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals and solvents used were of analytical grade.

Animals: Nine weeks old male swiss albino mice (weight, 25-28 g) were purchased from ICDDRB, Dhaka, Bangladesh and housed in animal cages under standard environmental conditions (22-25 °C, humidity 60-70%) with food and water *ad. libitum*. The animals used in this study were cared in accordance with standard guidelines of animal experiments.

Phytochemical Screening: Phytochemical analysis was performed according to the methods described by Nayek and Pereira [9].

Antibacterial Activity Test by Disc Diffusion Assay: Disc diffusion method was used for the assessment of antibacterial activity of HPG, CLPG and ET APG against a number of Gram-positive strains such as *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative strains such as *Salmonella paratyphi*, *Salmonella typhi* and *Shegilla dysenteriae*. Test samples were prepared using sterile blank discs (5 mm) which were socked with the test samples dissolved in methanol at a concentration of 500 μg/ disc by a micropipette. Control discs (blank) were prepared using methanol. Both sample and control discs were dried in air. Samples containing discs, standard antibiotic discs (azithromycin 50 μg/ disc) and control discs were placed in petri dishes containing nutrient agar medium seeded with the test pathogens using sterile forceps. Then petri dishes were transferred into an incubator and kept at 37 °C for 20 h. After incubation, the zone of inhibition was measured using digital slide calipers [10, 11].

***In-vitro* Antioxidant Activity:** Antioxidant activity was estimated by using stable free radical 0.004% DPPH. 4 mg of DPPS was measured and dissolved in 100 ml of methanol thus 0.004% DPPH solution was prepared. For qualitative analysis stock solutions of HPG, CLPG and ET APG were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts.

The plates were dried at room temperature and sprayed with 0.02% DPPH in methanol. Bleaching of DPPH by the resolved bands was observed for 10 min on the plates and the color changes (yellow on purple background) were noted [12]. Further, for quantitative analysis **s**tock solutions of the three extracts were prepared by respective solvent from which a serial dilution carried out to obtain a concentration of 1, 5, 10, 50, 100, 500 µg/ml. Diluted solutions (2 ml) were added to 2 ml of a 0.004% solution of DPPH in methanol, mixed and allowed to stand for 30 min for reaction to occur. The absorbance was determined at 517 nm and from these values, the corresponding percentage of inhibitions (%) were calculated. Then % of inhibitions were plotted against log concentration and from the graph IC_{50} value was calculated. The experiment was performed 3 times and average absorption was noted for each concentration and fractions [13]. Ascorbic acid is used as standard and IC_{50} values were obtained followed by the same procedure as extracts.

Analgesic Activity by Acetic Acid-induced Writhing Test: The analgesic activity of the extracts was studied using an acetic acid-induced writhing test in mice [14]. The writhing was induced in mice by intraperitoneal (i.p) administration of 0.1 ml of 1% acetic acid. In the experiment, a total of 25 mice were divided into five groups and each group comprised of five mice. Group I served as control and mice received vehicles (1% Tween 80 in saline), group II served as standard group and received diclofenac-Na (10 mg/kg, i.p) as standard drug, group III, group IV and group V received HPG, CLPG, ET APG (400 mg/kg, p.o) extracts respectively. Extract and vehicle were administered orally 30 min before administration of 1% acetic acid in mice whereas diclofenac-Na was administered 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for a specific contraction of body referred to as "writhing" for the next 10 min.

Statistical Analysis: The results were expressed as mean ± standard error (SEM). Statistical analysis was performed by using ANOVA followed by Tukey's test using graph pad prism software version 5.03 and values $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION:

Phytochemical Analysis: The phytochemical analysis of mother extracts of leaves from *Psidium guajava* as well as different fractions (HPG, CLPG, and ET APG) revealed the presence of alkaloids, saponins, flavonoids, tannins and triterpenoids **Table 1**.

TABLE 1: RESULTS OF PRELIMINARY PHYTOCHEMICAL TESTS

Plant extract	Alkaloids	Saponins	Flavonoids	Tanins	Triterpenoids
MPG	+	+	+	+	+
HPG	+	+	+	+	+
CLPG	+	_	+	_	+
ET APG	+	+	+	+	+

+ = presence, - =Absence

Screening of Anti-bacterial Activity: The antibacterial activity of different fractions (HPG, CLPG and ET APG) of MPG was evaluated at the concentration of 500 µg/ml on the growth of pathogenic bacteria by the disc diffusion method. The fractions HPG, CLPG and ET APG exhibited antibacterial activity with zone of inhibitions ranges 13-18 mm against gram-positive bacteria and 7-11 mm against gram-negative bacteria, but not comparable to that of azithromycin standards (50 µg/ml) against both the strains. Among the fractions, CLPG showed the highest activity against *Staphylococcus aureus* and HPG exerted lowest activity against *Shigella dysenteriae*.

TABLE 2: ANTIBACTERIAL ACTIVITY OF DIFFERENT FRACTIONS

Type of sample	Diameter of the zone of inhibition in mm				
	Gram-positive		Gram-negative		
	S. aureus	*B. subtilis*	*S. paratyphi*	*S. typhi*	*S. dysenteriae*
Blank	-	-	-	-	-
Azithromycin 50 µg/ml)	30 ± 0.12	27 ± 0.44	22 ± 0.11	21 ± 0.12	24 ± 0.11
HPG (500 µg/ml)	14 ± 0.12	16 ± 0.17	10 ± 0.13	10 ± 0.21	7 ± 0.13
CLPG (500 µg/ml)	18 ± 0.23	17 ± 0.41	9 ± 0.33	8 ± 0.12	10 ± 0.11
ET APG (500 µg/ml)	15 ± 0.15	13 ± 0.50	11 ± 0.31	7 ± 0.44	10 ± 0.27

Values expressed in mm

***In-vitro* DPPH Free Radical Scavenging Activity:** The DPPH free radical scavenging activity of fractions from *Psidium guajava* and ascorbic acid represented in **Table 3**. The IC_{50} value of HPG, CLPG, ET APG, and ascorbic acid were 29.96 µg/ml, 26.84 µg/ml, 24.29 µg/ml and 6.23 µg/ml respectively **Fig. 1** and **2**. The inhibition of DPPH radicals by different fractions was as follows: ET APG > CLPG < HPG. Our results indicated that fractions of *Psidium guajava* possessed the strongest antioxidant activity (IC_{50}< 5 mg/ml); however, the values were not comparable to that of pure ascorbic acid used as the reference standard. So, the plant extract can be considered as possible good source of antioxidant compounds.

TABLE 3: IC_{50} VALUES OF DIFFERENT FRACTIONS FROM *PSIDIUM GUAJAVA* AND ASCORBIC ACID

Test Sample	Regression line	R^2	IC_{50} µg/ml
HPG	Y = 12.358 In (x) + 7.932	0. 8972	29.96
CLPG	Y = 8.1338 In (x) + 23.22	0.8533	26.84
ET APG	Y = 13.327 In (x) + 7.432	0.9586	24.29
Ascorbic Acid *	Y = 7.605 In (x) + 34.259	0.9004	6.23

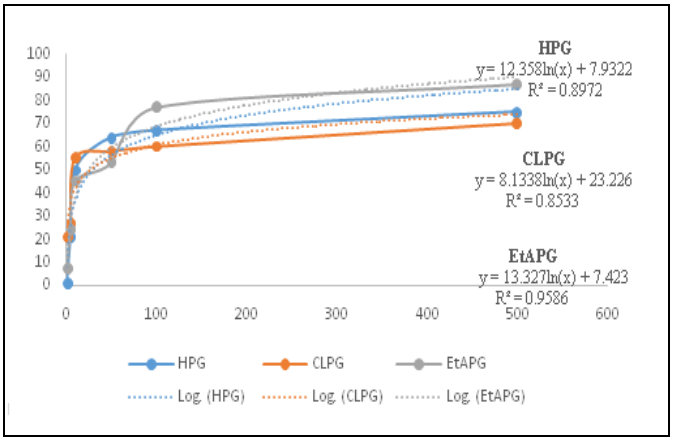

FIG. 1: CONCENTRATION AGAINST % OF INHIBITION OF HPG, CLPG AND ET APG FRACTIONS

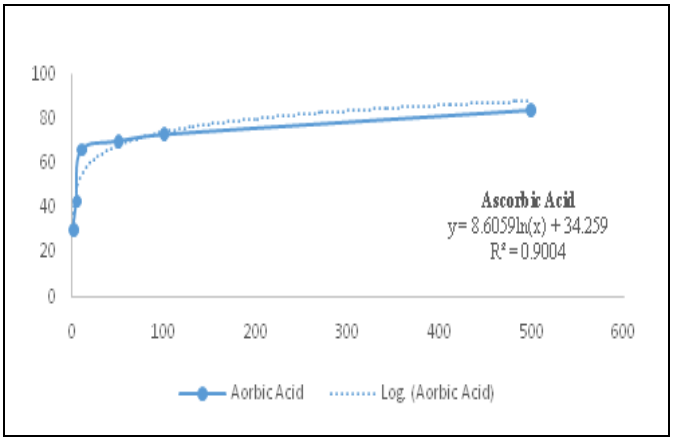

FIG. 2: CONCENTRATION AGAINST % OF INHIBITION OF ASCORBIC ACID

Evaluation of Analgesic Effect: The analgesic effects of different fractions from MPG were evaluated in acetic acid-induced writhing response to mice. The analgesic activity of HPG, CLPG and ET APG at the dose of 400 mg/kg showed 56.1%, 60.51% and 70.12% inhibitory response to writhing effects whereas diclofenac-Na showed 77.22% inhibition. So, HPG, CLPG, and ET APG fractions exerted significant reduction to pain induced by acetic acid when compared to the vehicle-treated control group ($p<0.05$). Among the fractions, ET APG demonstrated the most prominent analgesic action but less than that of diclofenac-Na standard **Table 4**.

TABLE 4: ANALGESIC EFFECT OF HPG, CLPG AND ETAPG FRACTIONSON MICE

Groups (n = 5)	Dose (mg/kg)	No. of Writhing	% of protection
Group I	Vehicle	38.5 ± .54	-
Group II	10	8.5 ± 0.23*	77.22
Group III	400	16.9 ± 0.22*	56.1
Group IV	400	15.2 ± 0.34*	60.51
Group V	400	11.5 ± 0.12*	70.12

Values were expressed in mean ± SEM. Group I received 1% Tween 80 in saline, group II received 10 mg/kg diclofenac-Na, group III, group IV and group V received HPG, CLPG, and Et APG extracts in saline respectively at a dose of 400 mg/kg body weight. *$p<0.05$ compared with vehicle-treated control.

DISCUSSION: The present study was designed to evaluate the antibacterial, antioxidant, and analgesic activities of different fractions from methanol extract of *Psidium guajava* (L) leaves. Infectious diseases are the major cause of morbidity and mortality worldwide. The appearance of the strains with reduced

susceptibility to antibiotics and the number of multidrug-resistant microbial strains are continuously increasing. The microbial resistance has been attributed to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agents, intravenous catheters, organ transplantation and human immunodeficiency virus (HIV) infections [15]. This situation provided the impetus to the search for new antimicrobial substances from various sources like medicinal plants.

In the present investigation, HPG, CLPG and ET APG extracts were evaluated for the exploration of their antimicrobial activity against certain gram-positive and gram-negative bacteria. Our results showed that different fractions have better bacterial growth inhibitory effects on gram-positive bacteria rather than gram-negative bacteria. It has been reported that the chemical compounds present in *Psidium guajava* such as anthocyagens, alkaloids, flavonoids, tannins and triterpenoids have *in-vitro* antibacterial activity [16].

Antioxidant properties, particularly radical scavenging activities, are very important to protect against the harmful effects of free radicals in foods and biological systems. DPPH method shows that the guava leaves has remarkable antioxidant contents and these antioxidants do not damage human neutrophils. Extracts in different solvents show that the antioxidant activity of guava depends upon phenolic compounds rather than flavonoids [17, 18]. DPPH is a stable free radical that can receive hydrogen or electron from an antioxidant to become a stable molecule. The reduction of DPPH absorption is indicative of the capacity of the extracts to scavenge free radicals, independently of any enzymatic activity.

The method widely used to predict the ability of flavonoids to transfer H-atoms to radicals is based on the free radical, in the DPPH assay [19]. The IC_{50} value of HPG, CLPG and ET APG fractions from *Psidium guajava* leaves were 29.96 µg/ml, 26.84 µg/ml and 24.29 µg/ml whereas IC_{50} value of ascorbic acid as a standard was 6.23 µg/ml. Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the fractions may be due to the presence of flavanoids in *Psidium guajava* leaves [20]. The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports [21] and the activity is mainly attributed to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [22]. The present study established the remarkable analgesic potential of HPG, CPG and ET APG fractions **Table 4**.

The acetic acid-induced writhing model represents pain sensation by triggering a localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from phospholipids by the action of phospholipase A2 and other acyl hydrolases [23]. The prostaglandins mainly prostacyclin and prostaglandin have been reported to be responsible for pain sensation in a localized area. Any agent that lowers the number of writhing will demonstrate analgesia preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition [24]. The response is thought to be mediated by peritoneal mast cells, acid-sensing ion channels and the prostaglandin pathway. Flavonoids being powerful antioxidants are reported to play a role in analgesic activity by targeting prostaglandins [25]. The possible mechanism of action of inhibition of prostaglandin synthesis is due to the presence of flavonoids in HPG, CLPG and ET APG fractions from *Psidium guajava*.

CONCLUSION: The results suggested that HPG, CLPG and ET APG fractions from *Psidium guajava* showed good antimicrobial, antioxidant and analgesic activities which suggested that the plant has the potential to be the source of alternative medicine due to its strong antioxidant properties.

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