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Research Article

ANTIMICROBIAL, ANTI-OXIDATIVE AND ANTI-HEMOLYTIC ACTIVITY OF *PIPER BETEL* LEAF EXTRACTS

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ABSTRACT

Piper betel L. belongs to family *Piperaceae* commonly known as *Paan*. It is extensively grown in Srilanka, India, Thailand, Taiwan and other Southeast Asian countries. The leaves are pungent, bitter, sweetish, acrid in nature. It has got large number of biomolecules which show diverse pharmacological activity along with carminative, stomachic, antihelminthic, tonic, aphrodisiac, laxative activities. The leaves are used for treating cough, foul smelling in mouth, ozoena, bronchitis, clears throat, vulnery and styptic. In the present experiment four different extracts (water, methanol, ethyl acetate and petroleum ether) of *Piper betel* leaves were tested against four different pathogenic bacteria namely *Streptococcus pyogenes, Staphylococcus aureus, Proteus vulgaris* and *Escherichia coli*. Further few known and unknown metabolites were isolated from these extracts. Structural elucidations of new metabolites were done by different analytical techniques like NMR, Mass and IR spectroscopy. Later on anti-oxidative and anti-haemolytic activities were determined. Anti-oxidative studies were done by TBARS and DPPH method. Anti-haemolytic activity was determined using erythrocytes model and the extent of lipid peroxidation of the same was also determined.

Keywords: *Piper betel* leaves extract, Antimicrobial, Antioxidative and Antihemolytic.

INTRODUCTION

The family of *Piperaceae* belonging to superorder *Nymphaeiflloraea*, order *Piperales* and genus *Piper* of family *Piperaceae* commonly known as pan comprises about 10 genera, 2000 species. The genus *Piper* (*Piperaceae*) is largely distributed in tropical and subtropical regions of the world[1]. Over 700 species of *Piper betel* has been distributed in both of the hemispheres of world. Of these, 30 species have been recorded in India, 18 in Srilanka and 3 are endemic. *Piper betel* is cultivated in India, Srilanka, Malaysia, Indonesia, Phillipine Islands and East Africa[2]. The parts of *Piper betel* utilized, are leaves, roots, stems, stalks and fruits. *Piper betel* has light yellow aromatic essential oil, with sharp burning taste. Leaf posses activity like antidiabetic, antiulcer, antiplatelet aggregation, antifertility, cardiotonic, antitumour, antimutagenic, respiratory depressant and antihelminthic[3-11]. *Piper betel* is used to treat alcoholism, bronchitis, asthama, leprosy and dyspepsia. Earlier, anti-ulcerogenic activity of *Piper betel* was attributed to its antioxidative property. A preliminary study has reported *Piper betel* leaves extracts contains large numbers of bioactive molecules like polyphenols, alkaloids, steroids, saponins and tannins[12]. The leaves extract of *Piper betel* have also been reported to exhibit biological capabilities of detoxication, antioxidation, and antimutation that suggested the chemopreventive potential of extracts against various ailments including liver fibrosis and carcinoma[13].

Free radicals play a vital role in most major health problems like cancer, rheumatoid arthritis, cardiovascular diseases, alzeihmer's disease and other neurodegenerative disorders. Antioxidant that scavenges these free radicals proves to be beneficial for these disorders as they prevent damage against cell proteins, lipids and carbohydrates[14]. Antioxidant activity includes free radical scavenging capacity, inhibition of lipid peroxidation, metal ion chelating ability and reducing capacity. *Piper betel* leaves are also known to contain significant amount of anti-oxidants like hydroxychavicol, eugenol, ascorbic acid and b-carotene[15-16].

In the present experiment different extract of *Piper betel* leaves were checked against four different microorganisms, namely *Streptococcus pyogens, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherechia coli*. *Streptococcus pyogens* is a spherical gram positive bacterium which causes various human diseases ranging from mild superficial skin infections to life-threatening systemic diseases. *Staphylococcus aureus* is a facultative anaerobic, gram positive coccus and it causes range of illness from minor skin infections like pimples, boils to life threatening diseases like pneumonia, meningitis etc. *Escherichia coli* is a gram negative rod-shaped bacterium, its virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis etc[17]. These micro organisms secretes different types of toxins, mainly enterotoxins and exotoxins and cause various wild type of diseases in human beings. Further, antihemolytic and antioxidative effects were studied and bioactive components from the extracts were isolated and their structural elucidation were done.

MATERIALS AND METHODS

Plant material

Piper betel (paan) leaves were purchased from local market, Ahmedabad and identified and authenticated from Department of Botany, Gujarat University, Ahmedabad, Gujarat, India.

Preparation of plant material

Fresh leaves were collected and dried at room temperature. Dried leaves were powdered mechanically. Powdered leaves were then packed in soxhelet apparatus and extraction was done[18].

Ethyl actetate: 15 gm of dry powder was subjected to soxhelet extraction with 300 ml ethyl acetate as solvent, extraction was carried out for 3hrs, 9 cycles and temperature was maintained at 65°C. Colour of extract was dark green.

Methanol: 15gm of dry powder was subjected to soxhelet extraction with 300 ml methanol as solvent, extraction was carried out for 3hrs, 10 cycles and temperature was maintained at 65°C. Colour of extract was dark green.

Petroleum ether: 15gm of dry powder was subjected to soxhelet extraction with 300 ml petroleum ether as solvent, extraction was carried out for 3hrs, 9 cycles, temperature was maintained at 65°C. Colour of extract was green.

Water: Powdered leaves were stirred using magnetic stirrer for 4 hrs, it was then filtered using whatmann filter paper and filtered solution was then dried at room temperature and dry extract was collected.

Preparation of bacterial suspension

The bacterial sample of *Streptococcus pyogens* (MTCC No. 1923), *Proteus vulgaris (*MTCC No.426), *Staphylococcus aureus* (MTCC No.737) and *Escherichia coli* (MTCC No. 448) were obtained from institute of Microbial Technology, Chandigarh, India. Cells were grown and stored at 37°C on agar slant[19].

Chemicals

2, 2 diphenyl-1picrylhydrazylhydrate (DPPH) and nitroblue tetra-zolium (NBT), 2 Deoxy 2 –ribose ascorbic acid and potassium ferricyanide, were obtained from Himedia. Other chemicals used in these experiments were also of analytical grade.

Primary screening of secondary metabolites

The condensed extracts were utilized for primary screening of different phytochemicals such as alkaloids (wagner's test, hager's test), flavanoids (shinoda test), catechins (phloroglucinol test), tannins (Fecl3 test), sterols (Salkowski method) and phenols (Folin's test) [20].

Separation of secondary metabolites by thin layer chromatography (TLC)

Three solvent systems were standardized for separation of secondary metabolites from various extracts for thin layer chromatography. Solvent system – A; Chloroform : methanol : glacial acetia acid (90:10:1), Solvent system – B; Chloroform : ethyl acetate : glacial acetic acid (50:50:1), Solvent system – C; Butanol : Ethyl acetate : HCL (1:1:1). Of these solvent systems, choloform : methanol : glacial acetic acid (90:10:1) gave the best separation of the metabolites and was considered for further processing. Retention factor (Rf) of isolated fractions were also measured. Isolated fractions were once again subjected to 2D – TLC and preparatory TLC for further purification and isolation of the metabolite in bulk.

Scrapped matter of TLC was isolated and then re-dissolved in methanol, later supernatant was collected and λ max was found out to check the purity of the isolated compound and maximum absorbance was recorded at 280 and 640nm. λ max of all the isolated components were compared and studied thoroughly. Later, flavonoids as well as phenol estimations were carried out from the isolated components in order to determine their presence and concentration. Further, NMR, IR and Mass spectroscopy of these isolated spots were also performed [21-22].

Isolation and elucidation of the bioactive components

Instruments

The infrared (IR) spectra were recorded on a Hitachi model 270 – 50 spectrometer as KBr discs. ^{1}H-NMR (500 MHz) spectra were recorded on a Avance III 400 MHz FT NMR Liquid with solid multi nuclei probe in DMSO solutions, Mass Spectra of the compounds were analyzed by Thermo Scientific, USA, LCQ Fleet and TSQ Quantam Access with Surveyor Plus HPLC System Instrument and UV spectra was analyzed by using UV Spectrothermo Spectronic UV-1 Spectrophotometer Instrument.

Determination of antimicrobial activity

Zone of inhibition

Four different extracts of *Piper betel* were checked against four different bacteria, namely *Streptococcus pyogens, Staphylococcus aureus, Escherichia coli* and *Proteus vulgaris.* Five different concentrations of plant extract were tested for anti-microbial activity using agar well diffusion method, standardized by National Committee for Clinical Laboratory Standards (2002). The microorganisms were inoculated in 100 ml flask containing nutrient broth. These flasks were incubated at 37°C, for 24 hrs. Media was prepared using N-agar, test microorganisms were then spread over the solidified plates and wells were bored using sterile cup borer of 1mm diameter. These wells were then filled with different concentrations of plant extract. A bacterial positive control and antibiotic control were kept for comparative study. Antibiotic used was Gentamycin (1 mg/ ml) [23]. These plates were incubated at 37-48°C according to optimum temperature required for bacterial species. Antibacterial activity was obtained by determining the zone of inhibition around the well.

Determination of anti-oxidative activity

DPPH photometric assay

The hydrogen donating ability of *Piper betel* extracts was determined in presence of 2, 2 diphenyl-1-picrylhydrazylhydrate (DPPH) radical. It produced violet colouration in ethanol[24]. Stock solution (1mg/ml) was diluted to final concentration of 5, 10, 25 and 50 µg /ml in respective solvents, 1 ml of 0.3 mM DPPH ethanol solution was added to 2.5 ml of extracts of different concentrations and allowed to react at room temperature. After 30 minutes, absorbance values were measured at 518 nm. Respective solvent (1ml) plus plant extract solution was used as blank. DPPH solution (1ml: 0.3mM) plus ethanol (2.5ml) was used as negative control. Ascorbic acid was taken as a positive control.

Measurement of reductive ability

For the measurement of reductive ability, the Fe^{3+} - Fe^{2+} transformation in the presence of the extract was investigated. 1 ml of plant extract (1 mg/ml), 2.5 of 0.2M phosphate buffer (pH 6.6), 2.5 ml of 1% potassium fericynide [$K_3Fe(CN)_6$] were incubated at 50^{0}C for 20 minutes. 2.5 ml of 10% Trichloacetic acid (TCA) were added to the mixture and centrifuged for 10 minutes at 3000 rpm. 2.5 ml of the supernatant were diluted with 2.5 ml water and shaken with 0.5 ml of freshly prepared 0.1 % Ferric chloride. The absorbance was measured at 700 nm. The reference solution was prepared as above, but contained water instead of extract. Increased absorbance of reaction mixture indicates increased reducing power[25].

Measurement of hydroxyl radicals

Hydroxyl radicals (OH^-) were generated by the reaction of ferric EDTA together with H2O2 and ascorbic acid to attack the substrate deoxyribose. The resulting products of the radical form pink chromogen when heated with TBA in acid solution[26].

Measurement of superoxide radicals

Assay is based on the capacity of the sample to inhibit blue formazon formation by scavenging the superoxide radicals generated in riboflavin – light – NBT system. Reaction mixture contains 50 mM phosphate buffer pH – 7.6, 20 µg /ml H2O ribolflavin, 12 mM EDTA, NBT 0.1 mg / 3ml phosphate buffer added in sequence. Reaction was initiated by illuminating reaction mixture with different concentration of samples for 150 secs and absorbance was measured at 590 nm. Ascorbic acid was used as positive control[27].

Determination of antihaemolytic activity

In a set of experiment, human venous blood samples collected in EDTA bulbs from well nourished healthy adults (25 – 30 yrs of age) were diluted with saline (0.9% NaCl). It was centrifuged at 1,000 g for 10 min. Obtained RBC pellets were washed twice and finally diluted with saline to have a cell density of 2 X 10^4 RBC/ml suspensions and processed for studying H_2O_2-induced cytotoxicity. The prophylactic effects of test compound (new metabolites) in amelioration of H_2O_2-induced toxicity on human RBC were also studied. The volume of each tube was made up to 4 ml with additional saline (0.9% NaCl) in order to have the required concentrations of test compound accordingly, which were also prepared in normal saline. The tubes were mixed gently and incubated at 37^0 C for 1 h with intermittent shaking. In another set, vitamin–C was taken as a reference compound. Required concentrations of different test compounds were prepared in saline[28]. Thereafter the tubes were centrifuged at 1,000 g for 10 min and the colour density of the supernatant was measured spectrophotometrically at 540 nm on a Systronics -Visiscan 167 colorimeter[29]. To achieve 100 percent haemolysis, 2 ml of distilled water was added to 2 ml of RBC suspension. Further lipid peroxidation assay was performed in terms of malonaldehyde production to check the efficacy of extract on H_2O_2–induced lipid peroxidation of RBC cells.

RESULTS

Table 1 Explains the preliminary screening of secondary metabolites obtained in four different extracts (methanolic, petroleum ether, aqueous and ethyl acetate) of *Piper betel* leaves by TLC. It was deduced that methanolic extract possess high concentration of sterols, phenols and flavonoids, also tannins were found to be present in moderate concentration and tannins were present in scarce concentration. Aqueous extract was found to possess high concentrations of flavonoids and tannins, phenols were present in

scarce quantity. Ethyl acetate extract fraction was found to have very high concentrations of phenols, tannins and sterols, whereas flavonoids were found in moderate concentration.

Table 2 Explains Retention Factor (Rf) in cm, obtained in TLC of four crude extracts (Methanolic, Petroleum ether, Aqueous and Ethyl acetate). Three solvent systems were tested Chloroform: methanol : glacial acetic acid (90:10:1), Chloroform : ethyl acetate : glacial acetic acid (50:50:1), Butanol : Ethyl acetate : HCL (1:1:1). Of these solvent systems, Chloroform : methanol : glacial acetic acid (90:10:1) was found to be appropriate for separation of maximum secondary metabolites from different extracts.

Table 3 Explains maximum absorbance (λ max in nm) of the fractions isolated from four different extracts (methanolic, petroleum ether, aqueous and ethyl acetate). The isolated extracts were scrapped from TLC plate and dissolved in methanol, centrifuged and supernatant was collected and was subjected to UV-Vis spectrophotometer, where base line scanning was done from 200 to 700 nm. Maximum peaks were obtained at 200-300 nm, confirming the presence of flavonoids and phenols in maximum amount. Further preparatory TLC was performed.

Table 4 Explains total phenol content and total flavanoid content of isolated compounds of four different extracts. Phenols were estimated by Folin-ciocalteu reagent. High phenol content was found in ethyl actetate as well as methanol extract, petroleum ether extract was found to have fewer amounts of phenols. Flavanoids were estimated by aluminium chloride colorimetric technique. Ethyl acetate extract and petroleum ether extract were found to have high concentration of flavanoids. Methanolic extract was also found to posses high concentration of flavanoids.

Fig 3 represents Infra Red Spectra of catechol (Fig 2) which was isolated from one of the spot in TLC.

Table 5A, 5B, 5C, 5D shows the antibacterial activity of four different extracts of *Piper betel* leaves (aqueous, methanolic, ethyl acetate and petroleum ether). Five concentrations of extracts were taken, (5, 10, 25, 50 and 100 mg mL^{-1}) and tested against four different bacteria, namely *Streptococcus pyogens, Staphylococcus aureus, Proteus vulgaris, Escherichia coli*. Zone of inhibition for the following was also measured in mm. Significant increase in the zone of inhibition was observed on increasing the concentration of extracts. In case of aqueous extract maximum inhibition was obtained against *Escherichia coli* at the concentration of 100 mg / ml. Whereas, in case of methanolic extract maximum inhibition was obtained against *Staphylococcus aureus*. Ethyl acetate gave clear zone of inhibition against *Escherichia coli* and ether extract against *Proteus vulgaris* respectively (Fig 1).

Table 6A Refers to DPPH photometric assay. DPPH was reduced in presence of extracts in concentration dependant manner. Ascorbic acid was taken as positive control. Petroleum ether extract showed highest amount of antioxidative activity followed by ethyl acetate extract, methanol extract and aqueous extract respectively.

Table 6B Refers to measurement of reductive ability, the reductive ability of extract is checked by ability of extract to reduce Fe^{+2} ions. Increased absorbance of reaction mixture indicates increased reducing power. Maximum activity was seen In case of water extract at 25 µg/ml.

Table 6C Refers to antioxidative activity of extracts by superoxide radical scavenging method. Based on the capacity of the sample to inhibit blue formazon formation by scavenging the superoxide radicals generated in riboflavin – light – NBT system. High activity was observed in water extract, followed by ethyl acetate extract and petroleum ether extract.

Table 6D refers to antioxidative activity of Piper betel extracts by hydroxyl radical scavenging assay. Hydroxyl radicals (OH^-) were generated by the reaction of ferric EDTA together with H_2O_2 and ascorbic acid to attack the substrate deoxyribose. The resulting products of the radical form pink chromogen when heated with TBA in acid solution. DMSO was used as positive standard. High activity was observed in case of methanolic extract followed by petroleum ether extract, water extract and ethyl acetate extract.

Table 7 explains the antihaemolytic activity of the extract in the presence of toxicant H_2O_2, where ascorbic acid was taken as a positive control. It was found out that when RBC cells were treated with H_2O_2 along with four different extract, marked reduction in the haemolysis was observed than that of cells treated with the toxicant alone. When cells were treated with the extract alone, no haemolysis was obtained explaining the non toxic behavior of the extracts on human RBC. In case of lipid peroxidation, simultaneous addition of plant extract to H_2O_2 treated cell decreased the extent of lipid peroxidation. Out of all extract ethyl acetate showed the best result.

DISCUSSION

Natural products are in great demand owing to their extensive biological properties and bioactive components which have proved to be useful against large number of diseases. It is proved that present extracts of *Piper betel* leaves showed wide array of activities like antibacterial, antioxidative and antihaemolytic.

The bioactive molecule thought to be responsible for antibacterial activity is sterol which has been obtained in large quantities in *Piper betel* extracts. The mode of action may be due to surface interaction of sterol molecules present in the extracts with the bacterial cell wall and membrane leading to alteration in the primary structure of cell wall and membrane, ultimately leading to pore formation and degradation of the bacterial components. It has been reported that sterol works through the disruption of the permeability barrier of microbial membrane structures[17]. It has been reported earlier that *Piper betel* extracts containing high concentration of fatty acids like palmitic acid, stearic acid and hydroxy fatty acid esters shows potent antimicrobial activity against diverse pathogenic microorganism[30]. All the extracts showed clear zone of inhibition against all the bacteria taken in the present study, is may be because of high concentration of sterols.

The extracts also contained high concentration of flavonoids and polyphenols. The antioxidative and antihaemolytic activity of the extract is may be because of the combined activity of the above mentioned bioactive components. It is well documented that flavonoids are the polyphenolic compounds which showed potential beneficial effects on human health and posses antiviral, anti-inflammatory, antitumour, antihaemolytic and antioxidative activity[31]. When purified spots isolated from the extracts were subjected to infrared, nuclear magnetic resonance and mass spectroscopy the noble molecule which was found to be in maximum concentration was catechol. Catechol is a well known component, which shows high degree of antioxidative property[32]. The basic nature of flavonoids, polyphenols and catechols can be explained by oxidative antioxidative mechanism of various toxicants and antioxidants interaction. When red blood cells were treated with H_2O_2 (toxicant), % hemolysis was found to be increased. This may be because of the oxidizing nature of H_2O_2 with respect to cell membrane degradation and release of haemoglobin from the cell. H_2O_2 also cause mobilization of Fe^{2+} by Ca^{2+} via Fenton reduction stimulating the production of OH^- radicals[33]. All these factors combinedly cause destabilization of cell membrane, which is probably the key event of the lysis of the cell. Lipid peroxidation is regarded as one of the primary rationale in cellular damage[34]. The erythrocyte membranes are susceptible to peroxidation because they are rich in polyunsaturated fatty acids. They contain haemoglobin, which may catalyze the oxidation as they are continuously exposed to high concentration of oxygen. The oxidation of erythrocytes serves as good models for the oxidative damage of biological membranes[35]. It has been found that certain chemicals, having ability to generate radicals attack the erythrocyte membrane, inducing the chain oxidations of lipids and proteins and eventually causing membrane damage leading to haemolysis[36]. When red blood cells were treated with extracts along with H_2O_2 marked reduction in haemolysis was found. This may be because of radical scavenging activity of the bioactive components present in the extracts showing potent antihaemolytic nature of the extracts.

Table 1: Phytochemicals screening of different extracts of leaves of *Piper betel*

Sec. Met	Test	Extract 1	Extract 2	Extract 3	Extract 4
Alkaloids	Hager's	-	-	-	-
Flavanoids	Shinoda	+ +	++	++	++
Catechins	Phloroglucinol	-	-	-	-
Tannins	$Fecl_3$	++	+	++	+++
Sterols	Salkowski	+++	+++	-	+++
Phenols	Phenol	++	++	+	++++

Extracts: 1: Methanolic extract, **Extract 2:** Petroleum ether extract, **Extract 3:** Aqueous extract, **Extract 4:** Ethyl acetate extract.

+: Low concentration, **++**: Moderate concentration, **+++**: High concentration, **++++**: Very high concentration

Table 2: Retention factor (Rf) of isolated metabolites in different solvent systems

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1) Solvent-A														
Water	0.5	0.6	-	-	-	-	-	-	-	-	-	-	-	-
Methanol	0.9	1.8	3.8	7	9.7	10.4	12.8	14.2	16	-	-	-	-	-
Pet ether	3.4	5.2	8.8	9.2	11	13.7	13.8	-	-	-	-	-	-	-
Ethyl acetate	0.9	2.4	3.1	3.7	4.2	4.7	6.3	7.2	9.2	9.5	9.7	10	13	15
2) Solvent-B														
Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methanol	0.9	8.9	12.3	15.5	-	-	-	-	-	-	-	-	-	-
Pet ether	8.2	11	12.5	14.3	-	-	-	-	-	-	-	-	-	-
Ethyl acetate	8.3	12	14	15	-	-	-	-	-	-	-	-	-	-
3) Solvent-C														
Water	1	2.3	3.2	12.9	16	-	-	-	-	-	-	-	-	-
Methanol	1.9	8.0	13.3	14.8	-	-	-	-	-	-	-	-	-	-
Pet ether	8.9	13	14.6	-	-	-	-	-	-	-	-	-	-	-
Ethyl acetate	7	9.5	11	14.5	-	-	-	-	-	-	-	-	-	-

Solvent system – A; Chloroform: methanol: glacial acetic acid (90:10:1); **Solvent system – B;** Chloroform: ethyl acetate: glacial acetic acid (50:50:1); **Solvent system – C;** Butanol : Ethyl acetate : HCL (1:1:1)

Table 3: Maximum absorbance (λ max in nm) of the isolated fractions

Solvent-A	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methanol	270	275	275	280	285	286	270	275	270	-	-	-	-	-
Pet ether	449	280	290	290	290	280	270	-	-	-	-	-	-	-
Ethyl acetate	270	270	280	280	280	280	270	290	290	295	295	290	280	270

Solvent system – A**;** Chloroform : methanol : glacial acetic acid (90:10:1)

Table 4 Total phenol and flavonoid estimation of isolated fractions

	Total phenol content (µg/mg)	Total flavanoid content (µg/mg)
Petroleum ether Extract		
1	0.07 + 0.05	0.28 + 0.05
2	0.85 + 0.10	0.18 + 0.02
3	0.01 + 0.05	0.86 + 0.03
4	0.75 + 0.05	0.50 + 0.01
5	0.81 + 0.05	1.24 + 0.05
6	0.91 + 0.10	2.19 + 0.04
Methanol Extract		
1	0.13 + 0.02	0.69 + 0.05
2	0.007 + 0.03	0.36 + 0.01
3	0.51 + 0.01	0.28 + 0.02
4	1.10 + 0.02	0.17 + 0.03
5	0.82 + 0.05	1.89 +0.04
	0.90 + 0.02	1.87 + 0.05
Ethyl acetate Extract		
1	0.53 + 0.05	0.54 +0.02
2	1.24 + 0.03	2.96 + 0.01
3	0.98 + 0.04	1.18 + 0.03
4	0.64 + 0.02	0.20 + 0.04
5	0.72 + 0.01	0.89 + 0.05
6	0.41 + 0.03	2.05 + 0.03

Values are means + S.E.M.; n=5.

Table 5: Zone of inhibition (mm) of bacterial growth by varying concentration of extracts of *Piper betel* by agar well diffusion method

A. AQUEOUS EXTRACT

	Antibiotic	5 mg/ml	10mg/ml	25mg/ml	50mg/ml	100mg/ml
Streptococcus pyogens	2.3 + 0.05	1.2 + 0.02[a]	1.3 + 0.025[a]	1.4 + 0.035[a]	1.5 + 0.02[a]	2 + 0.05[a]
Staphylococcus aureus	2.5 + 0.02	1.25 + 0.03[a]	1.3 + 0.03[a]	1.7 + 0.04[a]	2 + 0.025[a]	2.2 + 0.02[a]
Proteus vulgaris	2.5 + 0.03	1.2 + 0.04[a]	1.35 + 0.05[a]	1.5 + 0.03[a]	1.8 + 0.03[a]	2.5 + 0.03[a]
Escherichia coli	1.4 + 0.04	1.24 + 0.05[a]	1.5 + 0.04[a]	1.6 + 0.02[a]	1.75 + 0.04[a]	3 + 0.04[a]

B. METHANOLIC EXTRACT

	Antibiotic	5 mg/ml	10mg/ml	25mg/ml	50mg/ml	100mg/ml
Streptococcus pyogens	3 + 0.05	1.2 + 0.02[a]	1.5 + 0.01[a]	1.7 + 0.03[a]	2 + 0.03[a]	2.5 + 0.02[a]
Staphylococcus aureus	3.5 + 0.02	1.3 + 0.05[a]	1.45 + 0.02[a]	1.6 + 0.05[a]	1.75 + 0.02[a]	2.7 + 0.03[a]
Proteus vulgaris	3.5 + 0.03	1.4 +0.03[a]	1.5 + 0.03[a]	1.7 + 0.04[a]	1.85 + 0.01[a]	2.65 + 0.04[a]
Escherichia coli	3.3 +0.05	1.4 +0.04[a]	1.6 + 0.04[a]	1.8 + 0.03[a]	1.95 + 0.03[a]	2.9 + 0.05[a]

C. ETHYL ACETATE EXTRACT

	Antibiotic	5 mg/ml	10mg/ml	25mg/ml	50mg/ml	100mg/ml
Streptococcus pyogens	3.5 + 0.03	1 + 0.05[a]	1.2 + 0.05[a]	2 + 0.05[a]	2.5 + 0.03[a]	3 + 0.05[a]
Staphylococcus aureus	3 + 0.02	1 + 0.02[a]	1.2 + 0.04[a]	1.4 + 0.02[a]	2 + 0.02[a]	3.3 + 0.04[a]
Proteus vulgaris	3 + 0.04	1.3 + 0.03[a]	1.5 + 0.03[a]	1.7 + 0.04[a]	2.3 + 0.04[a]	4 + 0.03[a]
Escherichia coli	2.8 + 0.05	1.2 + 0.04[a]	1.5 + 0.05[a]	1.7 + 0.05[a]	2.2 + 0.05[a]	4.2 + 0.04[a]

D. ETHER EXTRACT

Antibiotic	5 mg/ml	10mg/ml	25mg/ml	50mg/ml	100mg/ml
3 + 0.03	1.1 + 0.02[a]	1.3 + 0.03[a]	2 + 0.02[a]	2.2 + 0.03[a]	2.5 + 0.05[a]
3.2 + 0.02	1.3 + 0.03[a]	1.5 + 0.02[a]	1.73 + 0.05[a]	2 + 0.02[a]	2.45 + 0.05[a]
3 + 0.05	1.28 + 0.03[a]	1.34 + 0.05[a]	1.4 + 0.03[a]	1.8 + 0.04[a]	2.6 + 0.03[a]
3.4 + 0.03	1.3 + 0.05[a]	1.5 + 0.04[a]	1.5 + 0.03[a]	1.9 + 0.02[a]	2.4 + 0.02[a]

Values are means + S.E.M.; n=5; Significant at the level: As compared to antibiotic control: [a] **p** <0.001 (Student's 't' test)

Table 6: Antioxidative activity of different extracts of *Piper betel* leaves

Concentrations	Water Extract	Methanolic Extract	Ethyl acetate Extract	Ether Extract
Positive Control Ascorbic acid (10 mg/ml)	84.00 + 0.28	84.03+0.26	86.66+2.68	84.00+1.73
Positive Control H_2O_2 (0.1 %)	04.00 + 0.28	04.00+0.28	04.00+0.28	04.00+0.28
5 µg/ml	08.03 + 0.31	60.06+0.23	84.06+0.23	93.23+0.14
10 µg/ml	19.93 + 0.34	55.83+0.44	79.40+0.30	92.56+0.29
25 µg/ml	24.06 + 0.23	47.83+0.44	68.13+0.18	92.70+0.55
50 µg/ml	24.16 + 0.16	44.06+0.23	52.46+0.29	89.46+0.29

A. DPPH (DIPHENYL PICRYL HYDRAZINE) PHOTOMETRIC ASSAY

Concentrations	Water Extract	Methanolic Extract	Ethyl acetate Extract	Ether Extract
Positive Control Ascorbic acid (10 mg/ml)	84.00 ± 0.28	84.03±0.26	86.66±2.68	84.00±1.73
Positive Control H_2O_2 (0.1 %)	04.00 ± 0.28	04.00±0.28	04.00±0.28	04.00±0.28
5 µg/ml	08.03 ± 0.31	60.06±0.23	84.06±0.23	93.23±0.14
10 µg/ml	19.93 ± 0.34	55.83±0.44	79.40±0.30	92.56±0.29
25 µg/ml	24.06 ± 0.23	47.83±0.44	68.13±0.18	92.70±0.55
50 µg/ml	24.16 ± 0.16	44.06±0.23	52.46±0.29	89.46±0.29

B. REDUCTIVE TEST ACTIVITY

Concentrations	Water Extract	Methanolic Extract	Ethyl acetate Extract	Ether Extract
5 µg/ml	0.361+0.005	0.110+0.005	0.707+0.001	0.251+0.006
10 µg/ml	0.375+1.732	0.114+0.002	0.984+0.003	0.248+0.024
25 µg/ml	0.392+0.001	0.140+0.002	1.149+0.006	0.297+0.008
50 µg/ml	0.325+0.002	0.117+0.002	1.297+0.008	0.345+0.002

C. SUPEROXIDE ANION SCAVENGING ACTIVITY

Concentrations	Water Extract	Methanolic Extract	Ethyl acetate Extract	Ether Extract
Positive Control	74.53+0.29	74.53+0.29	74.53+0.29	74.53+0.29
Ascorbic acid (10 mg/ml)				
5 µg/ml	78.50+0.28	76.78+0.14	74.89+0.20	75.98+0.21
10 µg/ml	72.75+0.14	74.37+0.18	75.40+0.20	74.47+0.28
25 µg/ml	74.24+0.14	72.94+0.53	76.26+0.14	76.85+0.17
50 µg/ml	72.70+0.14	69.69+0.15	74.83+0.44	77.52+0.28

D. HYDROXYL RADICAL SCAVENGING ACTIVITY

Concentrations	Water Extract	Methanolic Extract	Ethyl acetate Extract	Ether Extract
Positive Control	88.62+0.29	88.62+0.29	88.62+0.29	88.62+0.29
DMSO				
5 µg/ml	51.38+0.20	82.61+0.19	39.76+0.14	41.55+0.23
10 µg/ml	42.54+0.29	92.61+0.31	47.48+0.28	48.24+0.14
25 µg/ml	49.27+0.14	44.20+0.15	36.99+1.52	38.24+0.14
50 µg/ml	44.63+0.31	16.55+0.29	14.17+0.16	32.38+0.14
100 µg/ml	38.24+0.90	11.55+0.28	15.56+0.29	31.29+0.15
200 µg/ml	45.5+0.28	01.82+0.16	0.76+0.14	29.14+0.44

Table 7: Antihaemolytic activities of four extracts of *Piper betel* leaves

Tubes	Incubate the tubes in dark for 1 hr at 37ºC and OD was taken at 540 nm	**% Haemolysis**	**% Lipid peroxidation** (Nmoles Mda/ Mg protein/ 60 Min)
Control Tube		2.7 %	3.54 %
100 % Haemolysis		100 %	25.6 %
Toxin (H_2O_2 - 0.1 %)		93.4 %	80.4 %
Positive control : Ascorbic acid (5 mg/ml)		7.83 %	2.56 %
Toxin (H_2O_2 - 0.1 %) + Ascorbic acid (5 mg/ml)		40.6 %	53.2 %
Methanolic Extract (0.1mg / ml)		8.26 %	4.32 %
Aqueous Extract (0.1mg / ml)		10.06 %	5.67 %
Ether Extract (0.1mg / ml)		11.14 %	6.54 %
Ethyl acetate Extract (0.1mg / ml)		4.28 %	3.43 %
Toxin (H_2O_2 - 0.1 %) + Methanolic Extract (0.1mg / ml)		21.04 %	16.12 %
Toxin (H_2O_2 - 0.1 %) + Aqueous Extract (0.1mg / ml)		23.16 %	14.23 %
Toxin (H_2O_2 - 0.1 %) + Ether Extract (0.1mg / ml)		17.29 %	15.34 %
Toxin (H_2O_2 - 0.1 %) + Ethyl acetate Extract (0.1mg / ml)		6.35 %	8.25 %

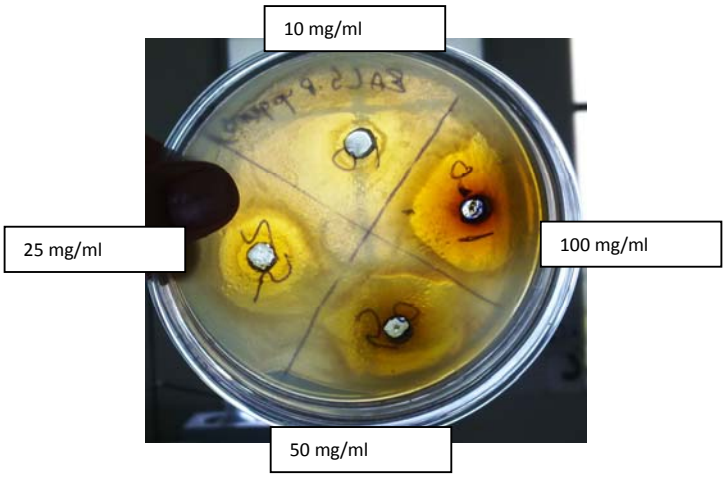

Fig. 1 Zone of inhibition (mm) of ethyl acetate extract on *Streptococcus pyogenes*

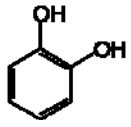

Fig. 2; Catechol structure

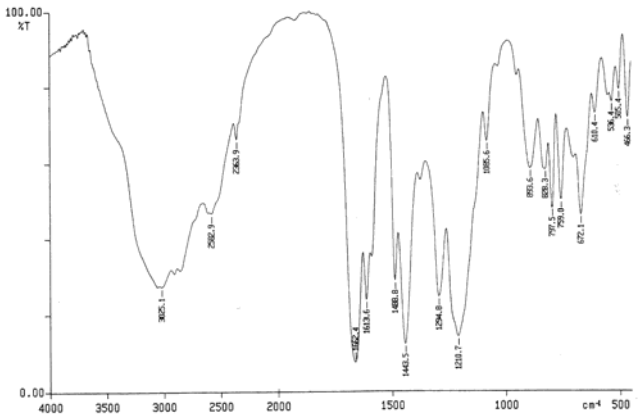

Fig. 3: Infra red spectra of Catechol

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