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Original Article

Phytochemical Screening, Biochemical Estimations and Spectroscopic analysis of Various Extracts of *Piper betel* Leaves

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ABSTRACT

Piper betel called 'paan' has known ethnomedicinal properties and has been used as traditional herbal medicine in several countries. In the present study, an effort has been made to screen the most potent extract of *Piper betel* leaves which can show its ability as an antioxidant and antihaemolytic agent for alternative medicine in managing the disease. Results revealed that petroleum ether extract had high phenolic and flavonoids content followed n-butanol and aqueous extract.TLC of various extracts of *Piper betel* leaves depicted that the RF values of petroleum ether, n-butanol and aqueous extract was quite close to quinine standard thus representing high alkaloids content. Several concentrations of various extract ranging from 200-1000 µg/ml were tested for their reducing ability. It was observed that n-butanol fraction showed highest reducing power ability. Highest NO scavenging activity was observed in aqueous and n-butanol extracts at 1mg/ml concentration. *In vitro* antihaemolytic was performed by hypotonic induced method. Highest antihaemolytic activity was observed by petroleum ether and n-butanol extract. FTIR spectrum profile was generated and reported the presence of characteristic functional group.

Key words:*Piper Betel*, Antioxidant, Antihaemolytic, FTIR.

1. INTRODUCTION

Piper betel called 'paan' is green heart shaped very famous leaf belongs to the family piperaceae. It is rich in nutrient, minerals, vitamins, antioxidants. *Piper betel* leaves are rich in many nutrients like water, protein fats, fiber, calcium, iron etc. and helps in curing various diseases like diabetes, hypertension, brain toxin, halitosis, boils, abscesses, obesity, wound healing, voice problems, conjunctivitis, constipation, itches, ringworm, swelling of gum, rheumatism, abrasion,

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cuts and injuries etc[1]. *Piper betel* leaves contains various biologically active compounds which are responsible for antioxidant activity[2]. The leaves have an essential oil composing of terpene-4-ol, safrole, allyl pyrocatehalmonob acetate, eugenol acetate hydroxyl chavicol, eugenol.*Piper betel*oil contains cadinene carvacrol, chavicol, P- cymene, caryophyllene, chavibetol, cineole and estragol as the major components[3,4]. Antioxidant compounds can donate electrons to reactive radicals, reducing them into more stable and unreactive species. Free radicals have been claimed to play a vital role in affecting human health by causing several diseases including cancer, hypertension, heart attack and diabetes. Dietary intake of phenolic compounds correlates with reduced coronary heart disease, cancer mortality and protective in many health-related properties, such as antioxidant, anticancer, antiviral and anti-inflammatory activities[5,6]. Phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activities[7,8].

Anomalies in RBCs makes it fragile leading to severe anaemia[9]. Many researchers have been carried out the study of the mechanism of haemolytic actions of many agents on RBCs and it has been reported that RBCs lysis is related with oxidative stress. Haemolyticanaemia have been reported to cause membrane lipid peroxidation and denaturation of cytoskeleton[10].

In the present study, an effort has been made to screen the most effective extract of *piper betel* leaves which can show antioxidant andantihaemolytic activity and prove to be beneficial in treating haemolytic anemia caused due to various diseases such as malaria.

2. MATERIALS AND METHODS

Qualitative phytochemical screening of *Piper betel*leaves in various extracts

Preparation of plant extract

Fresh *piper betel* leaves were collected from village Sandalpure, Antri of Gwalior, M.P. 1kg of *piper betel* cut in to small pieces and shade dried. The dried *piper betel* leaves were ground to a fine powder. 50g of powder was weighed and extracted with soxhelet apparatus using various solvent according to their polarity i.e. petroleum ether, chloroform, methanol, n-butanol, ethyl acetate and water. After solvent extraction, it was evaporated to obtain a powdered extract for various biochemical analysis.

Preliminary phytochemical screening of the extracts was performed for the presence of alkaloids, flavonoids, steroids, tannins, saponin, phenol and by the standard procedures.

Alkaloids: To 1 ml of extract, 2-3 drops of Wagner's reagent were added. The appearance of pale or white precipitate indicated the presence of alkaloids[11].

Steroids: To 2 ml of extract, 2 ml of chloroform and 2 ml of concentration sulphuric acid was added. Tubes were shaken and allowed to stand. Formation of red colour chloroform layer indicates the presence of steroids[12].

Tannins: 3 ml of extracts was treated with 1% lead acetate solution. A red or yellow colour precipitate was formed, indicating the presence of tannins[11].

Saponins: To 3 ml of extracts, few drops of sodium bicarbonate was added and shaken vigorously for 3 min. Honey comb froth was formed, showing the presence of saponins[11].

Phenolic: To 1 ml of extracts, 2 ml of distilled water and few drops of 10% ferric chloride solution were added. Formation of blue or green colour indicates the presence of phenols[11].

Flavonoids: To2 ml of each extract was added few drops of 20% sodium hydroxide.Formation of intense yellow colour is observed, by adding 70% hydrochloric acid which disappeared. Disappearance of yellow colour indicate the presence of flavonoids in the extract[13].

Quantitative Estimation of Secondary Metabolites

Total Phenolic Content

The total phenolics in the extract were determined using Folin-Ciocalteu method as described by[14]. To each sample solution (1.0 ml) and the standard (gallic acid) was added 5 ml of Folin-Ciocalteu (sigma-aldrich) and 4 ml sodium carbonate (7% w/v) and shaken. The solution could stand for 30 min in the dark at room temperature, after which absorbance was measured at 765 nm using a spectrophotometer. The phenolic content was calculated from the standard curve of gallic acid[15].

Total Flavonoid Content

A known volume of extract was placed in a 10ml volumetric flask add distilled water to make final volume 5 ml followed by adding 0.3 ml $NaNO_2$(1:20). Add 3ml AlCl3 (10%) 5 min later. After 6 min, 2 ml 1 M NaOH was added and the total volume was made up to 10ml with distilled water. The solution was mixed well again, and the absorbance was measured against a blank at 510 nm with a (UV-VISIBLE Parkin Elmer Lambda 23 with win lab N6.0software.). The flavonoid content was calculated with quercetin as standard[16].

Reducing Power assay

2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml 1% potassium ferrocyanide was mixed in 1 ml of different fraction of plant extract at various concentration (200 - 1000 µg/ml) diluted in distilled water. The test tubes were incubated at 50ºC in water bath for 10 min. followed by addition of 2.5 ml 10% TCA and centrifuged1 at 3000 rpm for 10 min. 2.5 ml of upper layer was collected, and 2.5 ml distilled water was added followed by 0.5 ml 0.1% $feCl_3$ (freshly prepared). Increase in absorbance was measured at 700 nm against a suitable blank[17].

NO radical Scavenging activity

NO (Nitric oxide) radical scavenging activities of plant extract in different fraction were examined by Royer *et al*.[18]. To 200 µl sodium nitroprusside (5Mm), 800 µl extracts (0.1-1 mg/ml) dissolved in PBS (25 mM, pH 7.4)was added. The mixture was incubated for 2.5 hrs. at 37ºC under normal

light followed by incubation in dark for 20 min. 600 µl Griess reagent was added and incubated for 40 min. at room temperature and absorbance was measured at 540 nm against a suitable blank (2ml H_2O and 0.6 ml Griess reagent). Control (1.6 ml H_2O, 400µl SNP and 600µl Griess reagent) was prepared and percent of inhibition was calculated by using this equation.

Percentage inhibition = OD of control – (OD of extract/ OD of control) x 100

TLC (Thin layer chromatography)

Thin layer chromatography (TLC) is a chromatographic method which is employed to separate mixtures. It is performed on aluminum or plastic foil, which is covered with a thin layer of adsorbent substance, generally silica gel aluminum oxide, or cellulose. This film of adsorbent is identified as the stationary silica phase. 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 was separated[19].

Antihaemolytic activity/ membrane stabilizing method

5 ml of whole blood of a healthy person in heparinized tube was collected. The blood was centrifuged at 3000g for 10 min. supernatant was removed and RBCs were washed thrice with sodium chloride isotonic solution (154 mM NaCl) in 10 mM Sodium phosphate buffer (pH 7.4) through centrifugation using the same volume as supernatant. Finally, RBCs were resuspended in the same volume of isotonic buffer solution. 0.5 ml of RBCs suspension was mixed with 5 ml of hypotonic solution (50 mM NaCl in 10 mM sodium phosphate buffer pH 7.4) containing 0. 5 ml plant extracts (10mg/ml). The control sample was prepared by 0.5 ml suspension mixed with hypotonic buffered saline. The mixture was incubated for 10 min. at room temperature, centrifuged at 3000g for 10 min. and the optical density of supernatant at 540nmwas measured[20,21].

Fourier Transform Infrared Spectroscopy

FTIR spectra for the *Piper betel* leaf extracts were analysed in the present study[22,23]. FTIR is used as a tool for the characterization and identification of compounds or functional group (chemical bonds) present in an unknown mixture of plant extract. The FTIR from 4000 to 390 cm^{-1} was recorded on Perkin Elmer (spectrum 2) spectrophotometer.

Statistical analysis

Results were expressed mean+ standard deviation from three replicate (n=3) experiments. The free radical scavenging activity was calculated by using GraphPad Prism 7 ink. software

3. RESULTS AND DISCUSSIONS

Table 1 show the qualitative preliminary phytochemical screening of extract. The results revealed that the petroleum extract had high tannin content followed by alkaloids, steroids and saponins. The petroleum ether depicted high phenolic and flavonoids content on the contrary it was less detected in the chloroform and methanolic extract which can be correlated to their antioxidant activity.

Table 2 depicts quantitative analysis of flavonoids and phenolic content and revealed that highest flavonoid content was found in petroleum ether, chloroform, extract followed by aqueous extract. The phenolic content was also high in petroleum ether followed by chloroform, methanol and lower in aqueous extract. Flavonoids and related compounds whichare widely distributed in the form of flavonoid, flavones and flavanols are reported to possess strong antioxidative characteristics. The antioxidant activity of flavonoids could be related to the hydroxyl groups[24].

Phenolic compounds are known to be a powerful chain breaking antioxidants as they possess scavenging ability due to their hydroxyl group. The medicinal plants could inhibit oxidative stress by antioxidant mechanism. Hydrogen donation is the main mechanism of phenolics as antioxidants. The lower strength of the O–H bond present in phenolics corresponds to a higher scavenging activity. Thus, high phenolic content in the n-butanol followed by petroleum ether and aqueous represent a powerful antioxidant activity.

Table 2 shown the TLC pattern of various extracts of *piper betel* leaves the retention factor (RF) of aqueous, n-butanol and petroleum ether extract was quite close to quinine standard thus representing high alkaloids content.

Several concentrations of various extract ranging from 200-1000 µg were tested for their reducing ability. Fig 1 shows the reducing power of *Piper betel* in various extracts. The pattern of reducing ability in *piper betel* was concentration dependent. It was observed that n-butanol fraction had highest reducing power ability followed by ethyl acetate. N-butanol exhibited reducing power capability was 3.2 at 1000 µg/ml.In the present study highest reducing power capability was observed at 1000µg/ml which exert antioxidant action by breaking the free radical chain through donating a hydrogen atom[25]. In this assay, Fe3+/ferricyanide complex is reduced to the ferrous form by antioxidants and can be monitored by measuring the formation of navy blue color at 700 nm[26]. The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of Fe^{3+} $(CN^-)_6$-$Fe^{2+}(CN^-)_6$.

Fig. 2 shows the NO scavenging activity of *Piper betel*in various extracts. Highest scavenging activity was found in n-butanol and aqueous extract which was 55.7 and 51.3 respectively at 1 mg/ml concentration. *P. betel* reduced efficiently the generation of NO radical from sodium nitroprusside[27].

Table 4 shows the antihaemolytic activity of *Piper betel*. Control shows 100% haemolysis in absence of extract. Petroleum ether and n-butanol extract shows minimum percent of haemolysis was 25.05% and 35% respectively at

10 mg/ml concentration. These two extracts have more membrane protecting capability[28].

FTIR (Fourier Transform Infra-Red) spectral data interpretation is as follows fig. 3 (a-d)

Petroleum Ether Extract: The extract of *piper betel* represented a characteristic band at 2851 cm^{-1} indicating the presence of C-H Stretch and 1734 cm^{-1} indicating presence of carbonyl group C=O, 1318 cm^{-1} C-N Stretch, 834 =C-H bending and 723 cm^{-1} for C-Cl Stretch group presented petroleum ether extract.

Ethyl acetate extract: It showed a characteristic band at 3276 cm^{-1} indicating the presence of O-H Stretch, 1736 cm^{-1} C=O stretch, 1241 cm^{-1} for C-O stretch, 889 cm^{-1} =C-H bending and 725 cm^{-1} C-Cl stretch group presented in the chloroform extract.

n-butanolic extract: It exhibited characteristic band at 2919 cm^{-1} C-H Stretch, 1736 cm^{-1} C=O Stretch, 1241 cm^{-1} C-O Stretch, 889 cm^{-1} =C-H bending and 778 cm^{-1} C-Cl stretch.

Aqueous Extract. A characteristic absorption band were exhibited at 3284 cm^{-1} for O-H stretch, 2922 cm^{-1} for C-H Stretch, 1617 cm^{-1} for C=O Stretch, 1369 cm^{-1} for C=C stretch, 664 cm^{-1} for C-Br stretch.

In FTIR the various functional groups were observed in the different extracts. The FTIR analysis revealed presence of carbonyl and aldehyde group hence rich in primary metabolites as well as secondary metabolites. Our results agree Kumar et al (2014)[29]. Where functional group components of amino acid, amides, amines, carboxylic acid, carbonyl compounds, organic hydrocarbons and halogens were analyzed in the methanolic leaf extract of *Ampelocissuslatifolia* by FTIR and reported that the transition metal carbonyl compounds and aliphatic fluoro compounds were only present in the extract.

Table 1: Qualitative phytochemical screening of various extracts of *piper betel* leaves

Fractions	Alkaloids	Steroids	Tannins	Saponins	Phenolics	Flavonoids
Petroleum ether	++	++	++	++	+++	+++
Chloroform	+	+	+	+	+	+
Ethyl acetate	+	+	-	+	+	+
Methanol	+	+	+	+	+	+
n-butanol	+	+++	++	+	+	++
Aqueous	++	+	+	++	++	++

(+) indicates presence of compounds, (-) indicates absence of compounds

Table 2: Quantitative secondary metabolite estimations of various extract of *Piper betel* leaves

Fractions	Flavonoids	Phenolics
Petroleum ether	10.92 mg/g ± 0.9	38.52 mg/g ± 3.5
Chloroform	10.26 mg/g ±0.87	21.72 mg/g ± 1.9
Ethyl acetate	7.39 mg/g±2.1	21.32mg/g±3.8
n-butanol	10.15mg/g±0.9	45.12mg/g±0.92
Methanol	7.715 mg/g±0.7	12.48 mg/g±1.2
Aqueous	10.97 mg/g ± 1.1	31.27 mg/g ± 1.1

Values are expressed as mean ±SE (n=3)

Table 3: Thin-layer Chromatography (TLC) of various extracts of *Piper betel*leaves for the presence of alkaloids.

Sample Name	RF values
Quinine Standard	0.53
Petroleum Ether Extract	0.50
Chloroform Extract	0.72
Ethyl acetate	0.85
n-butanol	0.56
Methanolic Extract	0.65
Aqueous Extract	0.52

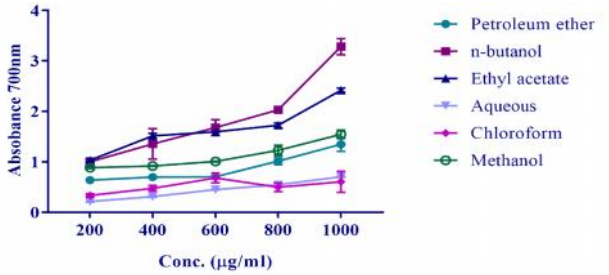

Fig1: Reducing power of *Piper betel* leaves in various extracts

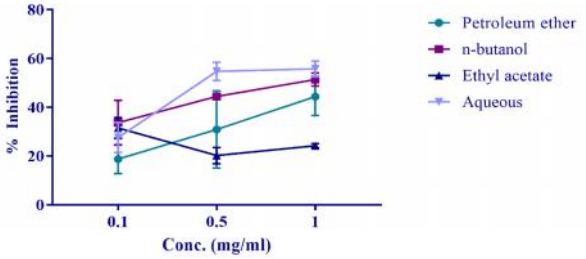

Fig 2: NO radical scavenging activity of *Piper betel* leaves

Table 4: Antihaemolytic activity of *Piper betel* leaves in various extracts

Sample	% haemolysis
Control	100%
Piper betel-petroleum ether	25.05%
Piper betel-ethyl acetate	99.6%
Piper betel--n-butanol	35%

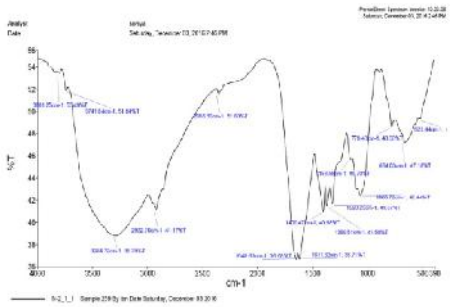

Fig. a

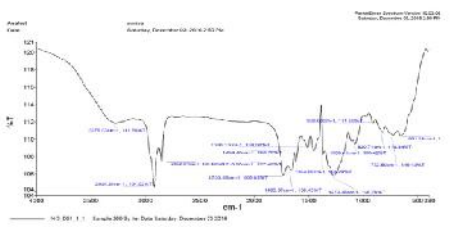

Fig. b

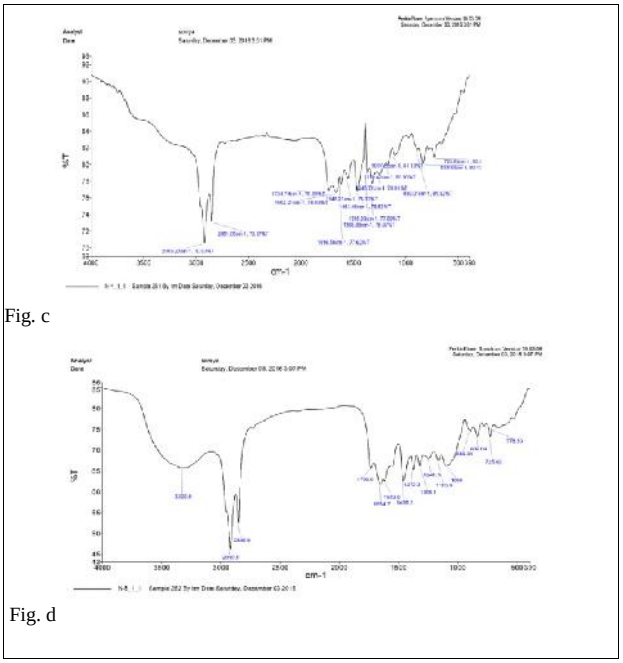

Fig. c

Fig. d

Fig 3: FTIR spectra showing functional groups of various extract of *Piper betel* leaves(a) petroleum ether extract (b) ethyl acetate extract (c) n-butanol extract (d) aqueous extract

4. CONCLUSION

Thus, it can be concluded from the present study that out of six extracts i.e. petroleum ether, chloroform, ethyl acetate, n-butanol, methanol, aqueous of *Piper betel* leaves three extracts such as n-butanol, aqueous, petroleum ether contains high flavonoid. These three extracts efficiently reduced the Fe^{3+} and NO radicals and reduced percentage of haemolysis. The butanolic, petroleum ether and aqueous extract showed the potential to reduce free radical and membrane stabilizing ability thus exhibiting more antioxidant and anti haemolytic properties. Ethyl acetate and chloroform extracts did not give satisfactory results. Hence further *in vivo* studies are needed for evaluating the potential of the extracts.

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