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Anti-Oxidant and Anti-Inflammatory Activities of Different Varieties of Piper Leaf Extracts (*Piper Betle L.*)

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Abstract

Piper betle L. (locally known as Paan) is a widely distributed plant in the tropical and subtropical regions, its leaves being largely consumed as a masticator and mouth freshener. It is valued as a mild stimulant and also has its use in Ayurvedic medicine.

The present study investigates on the free radical scavenging potential as well as total phenolic and aflavanoid contents of methanolic extract of 9 different varieties of *Piper betle* leaves (A,B,C,D,E,F,G,H&I). These extracts from diverse localities and varying on treatment, maturation at plucking etc. were used to evaluate antioxidant and anti-inflammatory activities. The antioxidant potential of all 9 varieties of methanolic leaf extracts were evaluated by six methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, superoxide radical O2-scavenging activity by the riboflavin-light-nitroblue tetrazolium (NBT) system, Lipid peroxidation assay was done by a modified thiobarbituric acid reactive species (TBARS) assay and Nitric oxide (NO) scavenging activity . Total phenolic and total flavonoid content also determined by standard protocol. Anti-inflammatory effect of the extract evaluated on LPS induced RAW 264.7 cell line in dose dependent manner. RP-HPLC analyses of 9 varieties of methanolic crude extract, was done and picks were monitored at 254 nm. All of the nine extracts demonstrated highest antioxidant activities at concentration of 1 mg/ml. Among the nine varieties, five (C,E,F,G and I) demonstrated anti-inflammatory activity, one(H) is proinflammatory and three varieties of *P.betle* leaf (A,B and D) didn't show significant anti-inflammatory activity. the F variety was most effective at a concentration of 250 µg/ml to retained cell viability 99%, very close to control and positive control (Dexamethasone) but H variety of *P.betle* leaf extract showed pro-inflammatory activity and cell viability decreased by 29% than control. The results of the experiments suggest that selected variety of P.betle may be used as natural antioxidant as well as an alternative or supplementary herbal remedy for the treatment of inflammatory disease. Thus, the present study warrants further investigation involving components of *P.betle* for possible development of new class of anti-inflammatory drugs.

Introduction

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, like pathogens, damaged cells and irritants. It is a mechanism of innate immunity, and is characterized by pain, heat, redness, swelling and loss of function.

Betel vine (*Piper betle L.*), commonly called paan, and is the leaf of an evergreen, perennial vine of the piperaceae family. Betel leaves are consumed after heavy meals as a digestive stimulant and mouth freshener. Paan is said to have a number of medicinal properties, including anti-inflammatory and anti-oxidant activities.

Betel (*Piper betle*) belongs to the genus Piper of the family Piperaceae. The plant originated in central and eastern peninsular Malaysia, where it is locally called sirih [1,2]. It is distributed throughout east Africa and the tropical regions of Asia. It is a commercial crop that is widely cultivated in many parts of India and Sri Lanka [3]. The use of betel leaf can be traced as far back as two thousand years [4]. Fresh leaves are chewed with betel nut, *Areca catechu* (Arecaceae), and other adjuvants (betel quid) in most parts of India and the habit of chewing betel is so widely prevalent that it ranks next to consumption of alcohol, coffee and smoking [5,6]. Due to their strong pungent aromatic flavour, betel leaves are used as a masticatory in Asia. Its common names are betel (in English), paan (in Indian), phlu (in Thai) and sirih (in Bahasa Indonesian) [7]. Betel leaves are reported to contain an aromatic oil [8], minerals [9], glycosides [10], enzymes, vitamins, essential amino acids [11] and tannins [12].

It has been reported that betel leaf has a wide spectrum of therapeutic properties. The extract of betel leaves possesses antimutagenic, anticarcinogenic, antiplaque, antidiabetic, anti-inflammatory and antibacterial bioactivities [13-18]. Generally plants that have significant therapeutic properties have been found to be rich in phenolics, with high antioxidant properties [19]. This correlation has been confirmed with the antioxidant activity being detected in the extract of betel leaf [20]. The consumption of antioxidant-rich foods helps neutralize the free radicals in the body, thus preventing or delaying the oxidative damage of lipids, proteins and nucleic acids [21]. It has been shown that the antioxidants could reduce mortality rate of cardiovascular disease [22,23], and protect against cancer and other chronic diseases [24]. The aqueous extract of the inflorescence of *Piper betle* extract was effective in scavenging H_2O_2, superoxide radical and hydroxyl radical [25,26]. The extract also prevented hydroxyl radical-induced DNA strand breaks in the PUC18 plasmid [26]. Recently, Manigauha et

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al, [27] observed that methanolic extracts of the betel leaves possess reducing power, DPPH radical, superoxide anion scavenging and deoxyribose degradation activities [27,28]. Studies have also shown that the hydroalcoholic extract of the betel leaf possesses nitrogen oxide scavenging effect in vitro [26,28].

The betel leaf has long been used as a household remedy for the inflammation of the oral cavity [29]. Ethanolic extracts of betel leaf has been reported to possess anti- inflammatory activities at non- toxic concentrations, in the complete Freund's adjuvant-induced model of arthritis in rats. Eugenol, one of the principal constituents of betel leaf, has been shown to possess anti-inflammatory effects in various animal models of studies with various inflammogens [30]. Betel leaf constituents, eugenol, hydroxychavicol and alpha-tocopherol,have also been shown to enhance the levels of GSH in mouse skin and liver [31,32].

Different species of betel leaves, for instance, from female (Bengaluru local) type, male (Madras) type and Meetha (sweet) type have been analysed for their anti-oxidant activity by FRAP (Ferric Reducing Anti-Oxidant Potential) Assay [1], their radical scavenging activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay [1], their anti-cancer activity by inhibiting proliferation of cells [2], their apoptosis-inducing activity by inhibiting proliferation of cells and Sub G1 flow cytometry [2], and their cryoprotective actuighngnivity towards human fibroblast cells by MTT Assay [3]. The total phenol content and the flavonoid content of the betel leaves have been estimated, using Folin-Ciocalteau method and NaNo2-AlCl3-NaOH respectively. Two of the constituent phenolic compounds have been identified as chevibetol and allyl pyrocatechol [4].

The anti-oxidant and anti-inflammatory properties of betel leaves have been attributed to various components of the leaves. Leaves with higher phenol and flavonoid content have been found to have higher anti-oxidant, radical scavenging and anti-cancer activities [1,2]. Quinic acid present in the betel quid may also play a role in health protection. [6].

Hydroxychavicol (HC) is a novel component of betel, which has been found to inhibit platelet aggregation. HC is a potent COX-1/COX-2 inhibitor, ROS scavenger and inhibits platelet calcium signalling. HC could be used as a therapeutic agent for cardiovascular diseases through its anti-inflammatory effects, without any effect on haemostatic functions [7].

The anti-ulcerogenic activity has also been attributed to the high flavonoid content of betel leaves. It has been seen that betel extracts have the ability to heal gastric ulcers [8] and peptic ulcers [9].

The ethanolic extract of betel has been tested for radioprotective activity, and it has been found that it can prevent DNA-strand breaks induced by radiation. This property has been attributed to its radical-scavenging and lympho-proliferative activities [4].

The aim of our study was to first detect potential anti-inflammatory and antioxidant activities of 9 varieties of *Piper betle* leaf extracts which are traditionally known as anti-geriatric compounds. High content of various phenolic and non-phenolic compounds and other uncharacterized moieties may contribute to its use, not only as a highly nutritive, edible plant part, but also as a nutraceutical substance, to be used prophylacticallyas well as therapeutically in oxidative inflammatory diseases.

Materials and Methods

Reagents

Chemicals, such as ethylenediamine tetra acetic acid (EDTA), trichloroacetic acid (TCA), butanol, ammonium molybdate, and sodium dodecyl sulphate, benzoic acid, sodium phosphate, and DMSO were purchased from E. Merck (India) Limited. 1,1 Diphenyl-2-picrylhydrazyl (DPPH), malondialdehyde, potassium ferricy anite, thiobarbituric acid (TBA) were procured from Sigma, USA. N-butanol, ferrous sulphate, ferric chloride, Folins reagent, Riboflavin, naphthylethylenediamine dihydrochloride (NED), and sulphanilamide in phosphoric acid, sodium bicarbonate, sodium hydroxide, and potassium hydroxide were purchased from Sisco Research Laboratories PVT Ltd India. Nitroblue tetrazolium (NBT), MTT reagent [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and DMEM media were purchased from Himedia, India. Fetal bovine serum (FBS) was purchased from Gibco. DCFH-DA and DHR 123 were purchased from Invitrogen. All other reagents were of analytical grade.

Preparation of plant extract

Fresh leaves were washed under distilled water and shade dried for 7 days and powdered. Then extraction was carried out by slightly modified method of Swati Dhote et al. [5]. Briefly, powered leaves were extracted with 80% methanol (1 g: 20 ml) with the help of cold maceration, at room temperature for about 24 hrs, shaking frequently. The process of extraction was repeated for three times. The solution was filtered using Whatman's filter paper no1 and the solvent, allowed to evaporate completely to obtain the extract. The extract was stored in sterile glass vials at 4°C until use.

DPPH radical-scavenging activity

The antioxidant activity of the crude methanolic *P.betle* extracts was evaluated against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals [33]. Various concentration of the methanolic extract was added to a 0.004% methanolic solution of DPPH on a 96 well ELISA plate. After 30 min incubation, Absorbance was determined at 517 nm and the percent inhibition activity was calculated.

Assay of superoxide radical O_2^- scavenging activity

Riboflavin-light-nitrobluetetrazolium (NBT) system [34] was used to determine the superoxide dismutase activity of the extract according to Martinez et al. [35]. Each 0.1 ml of reaction mixture contained 13 mM methionine, 50 mM phosphate buffer (pH 7.8), 100 μM EDTA, 2 μM riboflavin, NBT (75 μM) and various doses of sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 15 min of illumination from a fluorescent lamp.

Lipid peroxidation assay

Egg yolk homogenates as lipid-rich media [36] was used as lipid peroxide which was measured by modified thiobarbituric acid reactive species (TBARS) assay [36]. MDA which was produced by lipid peroxidation of polyunsaturated fatty acids by $FeSO_4$, reacts with two molecules of thiobarbituric acid (TBA) and pinkish red chromogen with an absorbance maximum at 532 nm, was measured using a 96 well ELISA plate reader. Percentage of inhibition of lipid peroxidation was calculated at various concentration of the crude methanolic *P.betle* extract [37].

Nitric oxide (NO) scavenging activity

Spontaneous decomposition of sodium nitroprusside (20 mM) in phosphate buffer (pH 7.4) generates nitric oxide which reacts with oxygen molecules to produce nitrite ions. Griess reaction was used to measure Nitrite ions. Slight modified method of Shirwaikar et al. [38] was used to determine the nitric oxide scavenging activity of the extracts 0.2 ml of sodium nitroprusside (20 mM) in PBS (pH 7.4) and 1.8 ml of PBS solution was added and incubated at 37°C for 3 h. 1 ml of different concentration of extract was taken and diluted with 1 ml of Griess reagent [1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine]. Similarly a blank was prepared containing the equivalent amount of reagents (only the sodium nitroprusside and PBS), but without the extract. The absorbance of these solutions was recorded at 540 nm against the corresponding blank solution. Ascorbic acid (100 µg/ml) was used as the reference. The percentage inhibition of nitric oxide was calculated as follows:

The NO scavenging activity(%) of methanolic extract of *P.betle* leaf $=[(A_b-A_t)-A_b]$ X 100, where A_b is the absorbance of the blank and A_t is the absorbance in the presence of *P.betle* leaf extract or positive control.

Determination of total flavonoid content

Aluminium chloride ($AlCl_3$) was used to determine total flavonoid content of the extras Fisetin was used as a standard 0.1 ml methanolic extracts were added to 0.3 ml distilled water, followed by 0.03 ml, $NaNO_2$ (5 %) and after 5 min 0.03 ml $AlCl_3$ (10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. Fisetin was used as positive control.

Determination of total phenolic content

Folin-Ciocalteau reagent was used to determine total phenolic content of the *P.betle* leaf extract [39]. The reaction mixture contained 200 µl of diluted extract, 800 µl of freshly prepared diluted Folin Ciocalteau reagent and 2 ml of 7.5 % sodium carbonate. The final mixture was diluted to 7 ml with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance was recorded at 765 nm. Gallic acid was used as standard and the results were expressed as mg Gallic acid (GAE)/10 g of the extract.

Cell culture

HEK 293 cell line and RAW 267.4 murine macrophage cell line was obtained from NCCS, Pune, India. The cells were grown in DMEM medium containing 5% inactivated fetal bovine serum, streptomycin (20 µg/mL) ,penicillin (100 U/mL), and kept at 37°C in T-25 tissue culture flasks at CO_2 incubator. Cell was grown to confluence in a humidified atmosphere containing 5% CO_2.

Cell viability using MTT assay

Cytotoxicity of betel leaf extract was measured according to Das et al. [40-42] by seeding , 5×10^4 cells/well in a 96-well plate and incubated for 24 h with different concentrations of the betel leaf extract . Each well was filled with 100 µL of medium and 10 µL of a tetrazolium salt, MTT after washing the cell properly. The plate was incubated for various time periods and then absorbance was measured at 540. The percentage of viable cells of control (without extract) considered as 100%. The assay was performed in triplicate twice.

HPLC analysis of the *P.betle* extracts:

Crude methanolic extract of *P.betle* leaf was filtered by 0.22 µm syringe filter (Milipore, Germany) and 100 µl of filtered was administered for HPLC analysis. The HPLC analysis was carried out using Waters 2695 System Controller coupled with 2487 dual absorbance detector and Waters 515 pump reversed-phase C18 columns was used as stationary phase. Acetonitrile/Water (1:1, flow rate 0.5 mL/min) was used as and peaks were detected at 254 nm [41].

Results

IC_{50} (half maximal inhibitory concentration) is a quantitative measure of how much of a drug is required to inhibit a biological process or its component by half. Lower the IC_{50}, the more efficient is the drug.

DPPH radical scavenging activity gives an idea of the anti- oxidant activity of the compound, and is expressed as the concentration of sample needed for 50% reduction of DPPH colour intensity. IC_{50} of lipid peroxidation assay, NO assay and superoxide radical assay are the concentrations needed to prevent lipid peroxidation, NO production and superoxide production, respectively, by 50%.

The lowest IC_{50} values are of varieties C and A, for DPPH radical scavenging activity, which are found to be close (1.02 fold higher and 1.09 fold lower, respectively) to the IC_{50} of ascorbic acid, a standard anti- oxidant, whereas that of variety D is very high (7.30 fold). The IC_{50} of varieties D, G and H are lower (1.13 fold, 1.16 fold and 1.15 fold respectively) than ascorbic acid, for lipid peroxidation assay. The NO scavenging activity of variety D is lowest among all the compounds (8.45 µg/ml), but it is 2.16 fold higher than ascorbic acid. Superoxide radical scavenging activity of varieties H and G are highest (IC_{50} 0.27 and 0.77, respectively), which are 11.56 and 4.05 folds lower than ascorbic acid.

Inhibition of DPPH radical, or DPPH radical scavenging activity, is seen in all 9 varieties of betel leaves, at a concentration of 1 mg/ml. Maximum inhibition is seen in variety C (81.03%), whereas minimum inhibition is seen in variety D (10%). The inhibitory activity of the compounds decrease in the order C>A>B>F>G>I>E>H>D.

The ability to prevent lipid peroxidation is seen in all 9 varieties of betel leaves, at a concentration of 1 mg/ml. Maximum inhibition is seen in variety H (77.5 %), whereas minimum inhibition is seen in variety C (22.2 %). The inhibitory activity of the compounds decrease in the order H>G>F>E>D>I>A>B>C.

Nitric oxide scavenging activity is seen in all 9 varieties of betel leaves, at a concentration of 1 mg/ml. Maximum inhibition is seen in variety D (41.8%), whereas minimum inhibition is seen in variety G (18.7%). The inhibitory activity of the compounds decrease in the order D>C>A>E>I>F>B>H>G.

Total phenol content of the *P.betle* leaves was assessed using the Folin-Ciocalteau method, using gallic acid as a standard. Results are expressed as mg gallic acid per 10 gms of extract. It is seen that variety A has the highest phenolic content (1.12 mg GAE/10 gm extract), whereas variety E has the lowest (0.76 mg GAE/10 gm extract). The phenol content of the samples decrease in the order A>B>C>H>I>D>G>F>E.

Total flavonoid content of the *P.betle* leaves was assessed using the aluminium chloride ($AlCl_3$) method, using fisetin as a standard. Results are expressed as mg fisetin per gm of extract. It is seen that variety B has the highest phenolic content (499.15 mg fisetin/gm extract), whereas

variety D has the lowest (46.01 mg fisetin/gm extract). The flavonoid content of the samples decrease in the order B>F>H>E>A>C>I>G>D.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. However, the viability of cells treated with sample A has not increased, but decreased from LPS. The decrease is seen at all the concentrations, with the maximum decrease at 10 µg/ml. This shows that sample A is not very effective in combating the inflammation caused by LPS.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. With addition of extract B, the viability has increased 1.12 fold with 250 µg/ml, and has increased very slightly with 100 µg/ml. However, the viability of cells has decreased with 50 µg/ml and 10 µg/ml. This shows that sample B has an anti-inflammaotyr effect only at high concentrations.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. With sample C, the viability has increased 1.36 fold with 250 µg/ml, and has increased very slightly with 100 µg/ml. However, the viability of cells has decreased with 50 µg/ml. This shows that sample C has an anti-inflammatory effect at higher concentrations.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. With addition of sample D, the viability has increased 1.10 fold with 250 µg/ml.It has increased very slightly with 100 µg/ml (1.01 fold) and with 50 µg/ml (1.01 fold). However, the viability of cells has decreased with 10 µg/ml (1.51 fold). This indicates that sample D has an anti-inflammatory effect at comparatively high concentrations, since the viability does not increase till 250 µg/ml.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. With treatment with sample E, the viability has increased 1.10 fold with 250 µg/ml, 1.11 fold with 100 µg/ml, 1.29 fold with 50 µg/ml and 1.42 fold with 10 µg/ml. This indicates that sample E has an anti-inflammatory effect at a concentration as low as 10 µg/ml. It is effective even at high concentrations, without being detrimental.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. The viability has increased 1.52 fold with 250 µg/ml, 1.36 fold with 100 µg/ml, and 1.33 fold with 50 µg/ml, with sample F, but has decreased 1.11 fold with 10 µg/ml. This indicates that sample F has an anti-inflammatory effect at concentrationsof 50 µg/ml and above, but not at 10 µg/ml.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. With addition of sample G, the viability has increased 1.21 fold with 100 µg/ml, 1.35 fold with 50 µg/ml, and 1.14 fold with 10 µg/ml, but has decreased 1.03 fold with 250 µg/ml. This indicates that sample G has an anti-inflammatory effect at lower concentrations, but may be toxic at higher concentrations.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. It is seen that with compound H, the viability has not increased even at low concentrations, indicating that sample H has a pro-inflammatory activity, rather than anti- inflammatory activity.

Cell viability of RAW 264.7 cells was assessed using MTT assay. Viability of untreated control cells was taken as 100%, and the viabilities of cells treated with extracts were calculated accordingly. Dexamethasone (dexa) was taken as positive control, and cells treated with only LPS as negative control. With LPS treatment, there is a 1.54 fold decrease in viability, which is restored by dexa treatment. With treatment with sample I, the viability has increased 1.35 fold with 250 µg/ml, 1.26 fold with 100 µg/ml, 1.16 fold with 50 µg/ml and 1.10 fold with 10 µg/ml. This indicates that sample I has an anti-inflammatory effect at concentrations as low as 10 µg/ml, and even at high concentrations without being toxic to the cells.

Discussion

Natural products are in great demand owing to their extensive biological properties and bioactive components which have proved to be useful against a large number of diseases. It has been proved that present extracts of *Piper betle*leaves show a wide array of activities like anti-inflammatory, antioxidant and also pro-inflammatory.

Various reactive oxygen species (ROS) can be formed in cells by transition metal {especially Fe (II)} mediated reactions along with oxygen metabolism [42], and radiation exposure [43], leading to deleterious effects on membrane lipids and DNA. For the present work, the antioxidant activity of the methanolic extracts of nine varieties of *P. betle* leaves were screened by the DPPH radical scavenging and reducing power assays. Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralising its free radical character [44,45]. The colour changes from purple to yellow and its absorbance at wavelength 517 decreases. Methanolic extract of *P. betle* leaf extract quenched DPPH free radical (Table 1). DPPH assay shows that, in this system, the radical-scavenging activities of the

Varieties Of *P.betle* Leaves	Concentration (µg/ml)	% Of Inhibition ± SD, n=3	Regression Equation
A	10	8.83 ± 1.322	y=13.42x-15.62 r2=0.602
	50	12.87 ± 0.000	
	100	9.87 ± 1.027	
	250	18.57 ± 0.740	
	1000	73.11 ± 0.848	
B	10	2.59 ± 0.385	y=10.83x-16.34, r2=0.625
	50	6.49 ± 0.555	
	100	6.23 ± 0.623	
	250	10.90 ± 0.286	
	1000	54.54 ± 1.210	
C	10	8.31 ± 0.734	y=16.11x-20.53 r2=0.699
	50	8.96 ± 1.314	
	100	16.10 ± 0.787	
	250	24.67 ± 1.438	
	1000	81.03 ± 0.634	
D	10	4.54 ± 0.287	y=1.352x+2.824, r2=0.972
	50	5.32 ± 0.522	
	100	6.62 ± 1.510	
	250	7.92 ± 0.156	
	1000	10.00 ± 0.257	
E	10	3.76 ± 0.244	y=4.89x-3.654, r2=0.864
	50	5.97 ± 0.124	
	100	7.40 ± 0.249	
	250	13.63 ± 0.216	
	1000	24.41 ± 0.616	
F	10	9.48 ± 0.990	y=8.985x-8.569, r2=0.560
	50	8.44 ± 0.620	
	100	8.96 ± 1.070	
	250	12.85 ± 0.739	
	1000	52.20 ± 0.662	
G	10	0	y=9.817x-16.44, r2=0.778
	50	2.46 ± 0.353	
	100	4.67 ± 0.317	
	250	15.19 ± 1.039	
	1000	42.72 ± 0.714	
H	10	5.06 ± 0.283	y=3.519x-0.145, r2=0.743
	50	7.14 ± 0.388	
	100	8.96 ± 0.808	
	250	9.35 ± 0.560	
	1000	21.55 ± 0.670	
I	10	4.93 ± 0.194	y=6.61x-4.978, r2=0.823
	50	7.53 ± 0.400	
	100	12.46 ± 0.389	
	250	15.19 ± 0.883	
	1000	34.15 ± 0.845	

Table 1: DPPH Scavenging Activities of *P.betle* leaf.

various *Piper* leaf extracts are in this order: C>A>B>F>G>I>E>H>D (Table 1). Methanolic extract of C variety *P.betle* leaf exhibited the highest antioxidant activity by strongly inhibiting 50% of DPPH radicals at a lower concentration (4.38 µg/ml) where ascorbic acid showed 50% inhibition at 4.78 µg/ml (Figure 1). Among 9 varieties of *P.betle* leaf, D variety was very less active in scavenging free radicals. IC_{50} value of D variety of *P.betle* leaf was 34.9 µg/ml (Figure 1).

All of the extracts demonstrated highest inhibition of lipid peroxidation at concentration of 1mg/ml (Table 2). The percentage of lipid peroxidation inhibition, IC_{50} values (concentration of sample required to scavenge 50% free radical or to prevent lipid peroxidation by 50%) was calculated from regression equations. IC_{50} values of the nine varieties of *P. betle* leaves were compared with the IC_{50} value of ASA in each system to assess the antioxidant property of *P. betle* leaves (Figure 1). IC_{50} value is inversely related to the activity. The percentage of lipid peroxide inhibition can be described in the following order: G>H> D>E>F>I>A>C>B. Among the nine varieties of betle leaf extract, G,H, D and E varieties showed more inhibition of lipid peroxidation than ascorbic acid. G variety of betle leaf has the highest activity which was about 1.16 times more than that of ASA.IC_{50} value of lipid peroxidation of F variety *P.betle* leaf extract was same as ASA, i.e. 5.57 µg/ml (Figure 1). But A,B,C& I varieties of *P.betle* leaf extract were less effective than ASA in inhibiting lipid peroxidation. The results suggest that consumption of G,H,D, and E varieties of *P. betle* leaf may afford a cytoprotective effect.

Nitric oxide (NO) is a potent pleiotropic mediator in physiological processes and a diffusible free radical too in the pathological conditions. It reacts with superoxide anion and form a potentially cytotoxic molecule, the 'peroxynitrite (ONOO-)'. Its protonated form, peroxynitrous acid (ONOOH), is a very strong oxidant [46,47] . The main route of damage is the nitration or hydroxylation of aromatic compounds, particularly tyrosine. Under physiological conditions, peroxynitrite also forms an adduct with carbon dioxide dissolved in body fluid and responsible for oxidative damage of proteins in living systems [48,49]. Methanolic extract of 9 varieties of *P. betle* leaf were examined for their possible regulatory effect on nitric oxide (NO) levels using sodium nitroprusside as an NO donor in vitro. All of the *P. betle* leaf extracts demonstrated direct scavenging of NO and exhibited significant activity. The potency of scavenging activity was in the following order: D>C>A>E>I>F>B>H>G (Table 3). IC_{50} value of the D variety of *P. betle* leaf extract was 8.45µg/ml, whereas IC_{50} value of ascorbic acid was 3.92 µg/ml (Figure 1) [50].

Phenols are the largest group of secondary metabolites of plant. Plant phenolic compounds have various biological effects, including

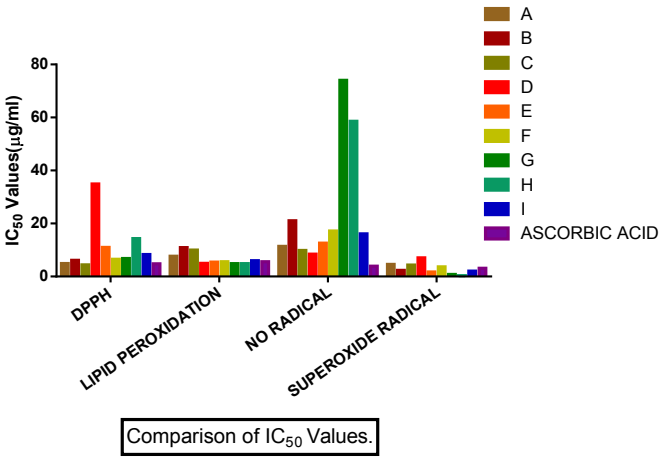

Figure 1: IC_{50} values were calculated from these regression equations. IC_{50} values of DPPH radical scavenging activity lipid peroxidation, NO radical scavenging activities and superoxide radical scavenging activity of nine different varieties of *P.betle* leaves (A,B,C,D,E,F,G,H & I) were compared with the IC_{50} value of Ascorbic Acid in each system to assess the antioxidant property of *P.betle* leaves.

Varieties Of *P.betle*Leaves	Concentration (µg/ml)	% Of Inhibition ± SD, n=3	Regression Equation
A	10	6.4 ± 0.883	y=7.01x+(-3.73) r^2=0.870
	50	9.1 ± 1.620	
	100	10.7 ± 0.679	
	250	28.6 ± 0.861	
	1000	31.7 ± 0.399	
B	10	4.7 ± 0.204	y=4.6x+(-0.08) r^2=0.982
	50	9.3 ± 0.571	
	100	13.9 ± 0.352	
	250	16.7 ± 0.183	
	1000	24.0 ± 0.667	
C	10	1.2 ± 0.174	y=5.43x+(-4.23) r^2=0.976
	50	5.2 ± 0.380	
	100	14.2 ± 0.620	
	250	17.5 ± 0.432	
	1000	22.2 ± 0.485	
D	10	11.6 ± 0.338	y=10.91x+(-3.93) r^2=0.873
	50	18.0 ± 0.545	
	100	22.9 ± 0.996	
	250	32.7 ± 0.797	
	1000	58.8 ± 2.900	
E	10	10.7 ± 0.892	y=10.5x+(-6.8) r^2=0.643
	50	15.8 ± 2.540	
	100	16.1 ± 0.108	
	250	19.6 ± 1.960	
	1000	61.3 ± 0.120	
F	10	1.2 ± 0.484	y=13.19x+(-23.29) r^2=.615
	50	1.6 ± 0.285	
	100	6.2 ± 0.470	
	250	8.9 ± 0.961	
	1000	63.5 ± 0.350	
G	10	8.0 ± 0.136	y=12.99x+(-12.49) r^2=0.716
	50	15.8 ± 0.529	
	100	15.5 ± 0.097	
	250	24.5 ± 0.338	
	1000	68.6 ± 0.097	
H	10	11.6 ± 1.120	y=13.34x+(-14.84) r^2=0.519
	50	11.6 ± 0.240	
	100	12.0 ± 0.272	
	250	13.2 ± 0.985	
	1000	77.5 ± 0.147	
I	10	2.4 ± 0.400	y=10.79x+(-14.07) r^2=0.841
	50	7.6 ± 0.253	
	100	9.9 ± 0.588	
	250	22.9 ± 0.588	
	1000	48.7 ± 0.596	

Table 2: Prevention of lipid peroxidation by *P.betle* leaf.

antioxidant (1,49,50,51,52).Plant phenolic compounds are highly effective free radical scavengers and antioxidants. Consequently, the antioxidant activities of plant/herb extracts are often explained by their total phenolic content with good correlation. We also observed similar correlations in the present study [51].

Figure 2 demonstrated the total phenolic content (TPC) of nine varieties of methanolic extracts of *P.betle* leaf. It was found that the A variety of *P.betle* leaf extract has the highest TPC, i.e. 1.121 ± 0.019 mg gallic acid/10 gm of *P.betle* leaf extract [52].

Anti-inflammatory versus pro- inflammatory effect

The effect of methanolic extract of 9 varieties of *P.betle* leaf on the viability of RAW 264.7 cells was evaluated therapeutically in a dose dependent manner (Figure 3). RAW 264.7 cells were treated with 1µg/ml of E.coli LPS and then various concentrations of methanolic *Piper* leaf extracts (250 µg/ml, 100 µg/ml, 50 µg/ml and 100 µg/ml) were added into it therapeutically and incubated for 24 h. Sample A did not exhibit any anti-inflammatory effect on LPS induced inflamed

Varieties Of *P.betle*Leaves	Concentration (µg/ml)	% Of Inhibition ± SD, n=3	Regression Equation
A	10	15.8 ± 0.044	y=3.54x+9.82, r^2=0.748
	50	16.3 ± 0.161	
	100	18.4 ± 0.216	
	250	20.1 ± 0.194	
	1000	31.6	
B	10	14	y=1.78x+12.62 r^2=0.972
	50	16.8	
	100	18.1	
	250	19.2	
	1000	21.7	
C	10	15.8 ± 0.088	y=4.30x+7.66 r^2=0.584
	50	16.2 ± 0.095	
	100	16.4 ± 0.161	
	250	18.0 ± 0.044	
	1000	36.4 ± 0.264	
D	10	17.1 ± 0.078	y=5.01x+7.65 r^2=0.548
	50	17.8 ± 0.040	
	100	18.2 ± 0.066	
	250	18.5 ± 0.044	
	1000	41.8 ± 0.529	
E	10	16.0 ± 0.060	y=3.18x+9.98 r^2=0.570
	50	16.3 ± 0.095	
	100	16.6 ± 0.108	
	250	17.3 ± 0.078	
	1000	31.4 ± 0.120	
F	10	16.9	y=2.15x+13.13 r^2=0.812
	50	16.6	
	100	18.3	
	250	20.3	
	1000	25.8	
G	10	16.7	y=0.45x+16.69 r^2=0.806
	50	18	
	100	18.3	
	250	18.5	
	1000	18.7	
H	10	18.3 ± 0.044	y=0.55x+17.79 r^2=0.978
	50	18.8 ± 0.222	
	100	19.6 ± 0.033	
	250	20.1 ± 0.197	
	1000	20.4 ± 0.108	
I	10	19.1 ± 0.120	y=2.17x+15.01 r^2=0.573
	50	19.3 ± 0.040	
	100	19.6 ± 0.133	
	250	20.0 ± 0.116	
	1000	29.6 ± 0.851	

Table 3: NO Radical Scavenging Activities of *P.betle* leaves.

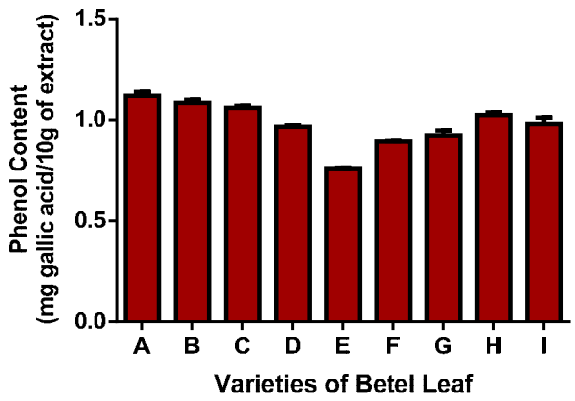

Figure 2: Bar graph showing comparative phenol content of 9 varieties of *P.betle* leaves. Results expressed as gallic acid equivalent (mg gallic acid/10gm) ± SEM of *P. betle* leaf extract, in duplicate.

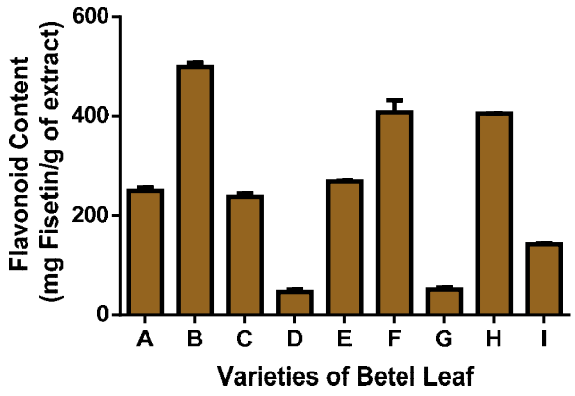

Figure 3: Bar graph showing comparative flavonoid content of 9 varieties of *P.betle* leaves. Results expressed as fisetin equivalent (mg fisetin/gm) ± SEM of *P.betle* leaf extract, in duplicate.

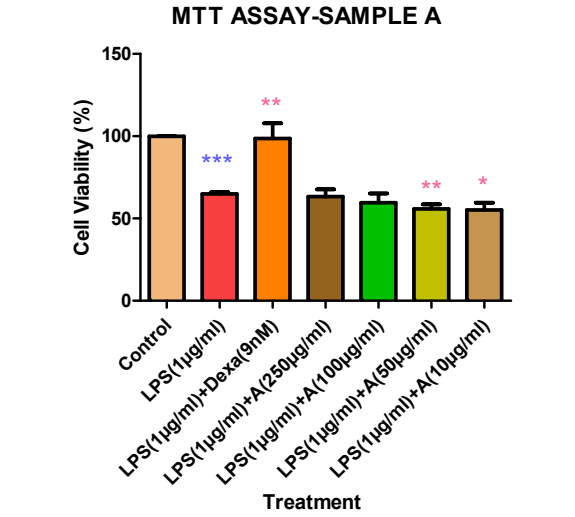

Figure 4: Bar graph showing effect of various concentrations of *P.betle* leaf extract (A) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control, to determine anti-inflammatory property *P.betle* leaf extract of sample A by MTT assay.

Raw 264.7 cells (Figure 4). Sample B (250 µg/ml) showed very less anti-inflammatory activity and it retained 72.69% viability, only 7.69% more than LPS induced RAW264.7 cells, which showed 65% viability (Figure 5). 250 µg/ml of sample C showed significant anti-inflammatory effect and it retained 88.4%viability, but the other three concentrations did not show effective viability on LPS treated RAW264.7 cells (Figure 6). Sample D retained 71.79% viability at a concentration of 250 µg/ml, and it is 6.79% more than negative control (Figure 7). In sample E, we found significant anti- inflammatory effect at every concentration and cell viability was retained 91.22%, 71.98 %, 84.01% and 92.09% at 250 µg/ml, 100 µg/ml, 50 µg/ml and 10 µg/m respectively on LPS treated RAW cells. Maximum anti- inflammatory activity was seen at a concentration of 250 µg/ml and 10 µg/ml, which is close to positive

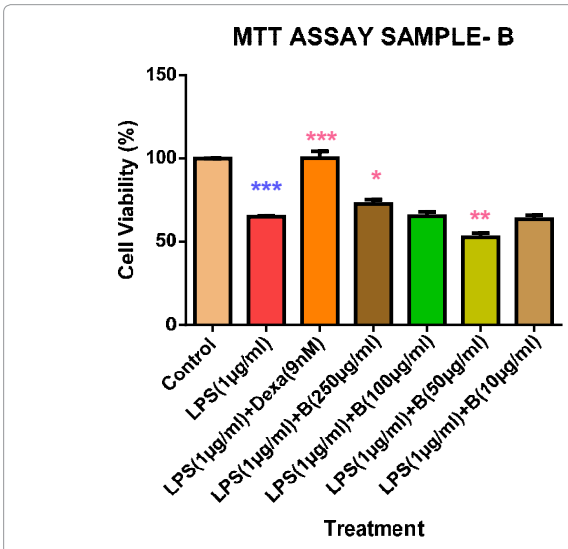

Figure 5: Bar graph showing effect of various concentrations of *P.betle* leaf extract (B) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

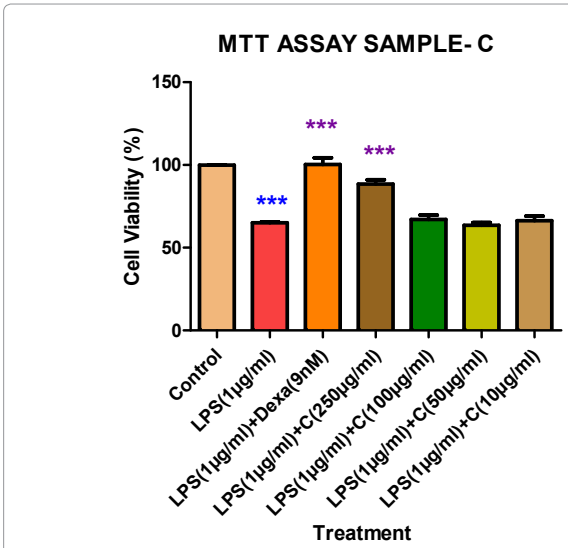

Figure 6: Bar graph showing effect of various concentrations of *P.betle* leaf extract (C) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

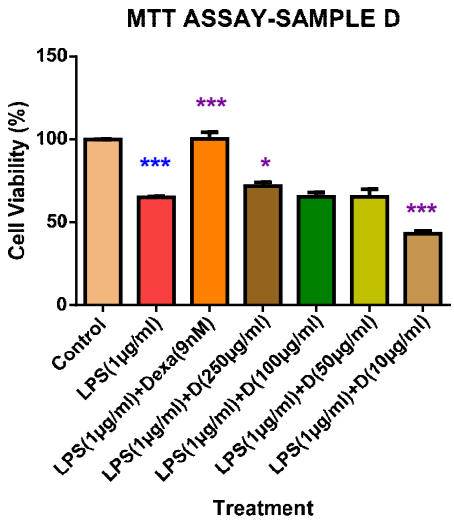

Figure 7: Bar graph showing effect of various concentrations of *P.betle* leaf extract (D) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

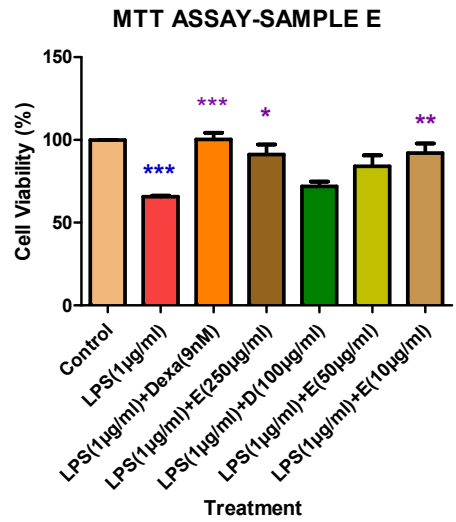

Figure 8: Bar graph showing effect of various concentrations of *P.betle* leaf extract (E) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

control i.e. Dexamethasone (Figure 8). Among the 9 varieties of *P.betle* leaf, the F variety was most effective concentration of 250 µg/ml and it retained cell viability 99%, very close to control and positive control. 2other concentrations of sample F, 100 µg/ml and 50 µg/ml, also showed significant viability, 88.48% and 86.49% respectively, under same experimental conditions. However, at a concentration of 10 µg/ml, there is no anti-inflammatory activity (Figure 9). Methanolic extract of sample G showed significant anti-inflammatory activity at a concentration of 50 µg/ml with respect to cell viability. At this concentration, it retained cell viability by 88.06 %, which is 23.06 % more than cell viability of negative control, i.e. 65% (Figure 10). 2 other concentrations of this sample, 100 µg/ml and 10 µg/ml, also showed anti-inflammatory activity, and retained cell viability by 78.86% and 73.9% respectively (Figure 10). But at the highest concentration (250 µg/ml), this variety of *P.betle* leaf showed lowest activity, but the other 7 varieties of *P.betle* leaf (A,B,C,D,E,F, and I) showed highest activity at concentration of 250 µg/ml. Variety I of *P.betle* leaf showed anti-inflammatory activity at 4 different concentrations. At a concentration of 250 µg/ml, it showed maximum viability of 87.74%, 22.74% more than negative control (Figure 11). Interestingly, sample H showed pro-inflammatory activity, whereas the other 8 varieties of *P.betle* leaf showed anti- inflammatory activity. Cell viability was retained 36.35%,39.61%,39.13% and 39.35% at concentrations of 250 µg/ml, 100 µg/ml, 50 µg/ml and 10 µg/ml of methanolic extract of sample H respectively,where LPS treated RAW cells showed 65% cell viability (Figure 12).

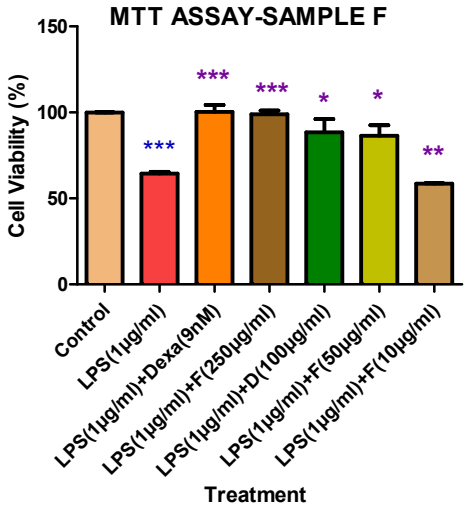

Figure 9: Bar graph showing effect of various concentrations of *P.betle* leaf extract (F) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

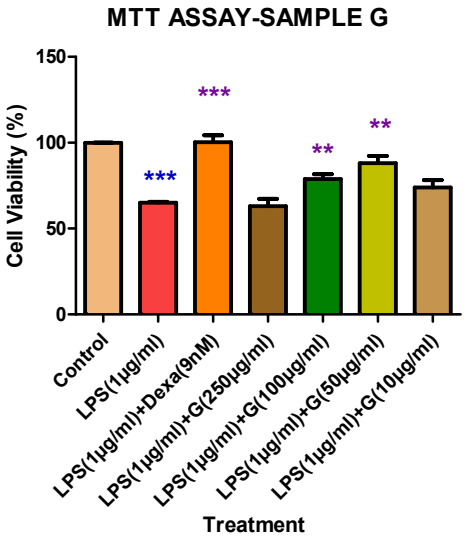

Figure 10: Bar graph showing effect of various concentrations of *P.betle* leaf extract (G) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

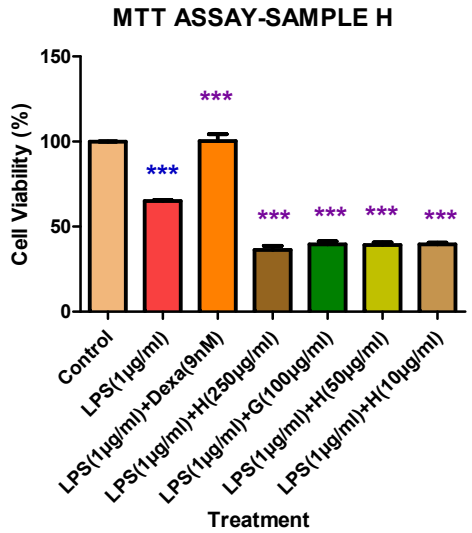

Figure 11: Bar graph showing effect of various concentrations of *P.betle* leaf extract (H) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

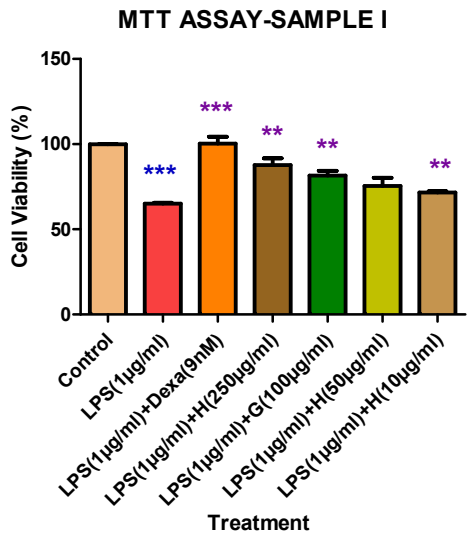

Figure 12: Bar graph showing effect of various concentrations of *P.betle* leaf extract (I) on viability of LPS induced inflamed RAW 264.7 cell line. Viability of Control (untreated) is considered as 100%, +Dexa (Dexamethasone) is used as positive control and +LPS as negative control.

HPLC profile

Figures 13-21 demonstrated the HPLC fingerprint of 9 crude extract of methanolic *P. betle* leaf. Among 9 varieties, B,D and F varieties of the *P.betle* leaf extracts showed very sharp and single pick at 254 nm (Figures 14,16 & 18) and F varieties showed highest anti-inflammatory activity .But Other six varieties of *P. betle* leaf extract showed multiple picks. Further analysis like MS, NMR etc. has to be done for identification of the pure bioactive compounds which have potent biological antioxidant and anti-inflammatory activities**.**

Conclusion

Piper betle L. (locally known as Paan) is a widely distributed plant in the tropical and subtropical regions, its leaves being largely consumed as a masticator and mouth freshener. It is valued as a mild stimulant and also has its use in Ayurvedic medicines.

The present study investigates on the free radical scavenging potential as well as total phenolic and aflavanoid contents of methanolic extract of 9 different varieties of *Piper betle* leaves (A,B,C,D,E,F,G,H & I). These extracts from diverse localities and varying on treatment, maturation at plucking etc. were used to evaluate antioxidant and anti-inflammatory activities. The antioxidant potential of all 9 varieties of methanolic leaf extracts were evaluated by six methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, superoxide radical O_2^- scavenging activity by the riboflavin-light-nitroblue tetrazolium (NBT) system, Lipid peroxidation assay was done by A modified thiobarbituric acid reactive species (TBARS) assay, Nitric oxide (NO) scavenging activity. By the Griess reaction and determination of total phenolic content was done by the Folin-Ciocalteau reagent. Gallic acid was used as standard to determine total phenolic content. Total flavonoid content was determined using aluminium chloride ($AlCl_3$) and the results were expressed as mg Fiseting *P. betle* leaf extract. *P. betle* leaves. MTT assay was performed to evaluate the anti-inflammatory effect of the extract on RAW 264.7 cell line in dose dependent manner. RP-HPLC analyses of 9 varieties of methanolic crude extract, was done in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase and pick were monitored at 254 nm. All of the 9 extracts demonstrated highest antioxidant activities at concentration of 1mg/ml. C variety *P.betle* leaf exhibited the highest DPPH radicals scavenging activity (IC_{50}-4.38 µg/ml) which was more than Ascorbic acid (IC_{50}-4.78 µg/ml) where D variety was less effective(IC_{50} :34.9 µg/ml). G variety of betle leaf extract was most effective to inhibit peroxidation of lipid, about 1.16 times more effective than Ascorbic acid. In superoxide and nitric oxide radical scavenging assays, among 9 varieties, D showed highest NO nitric oxide radical scavenging activity (IC_{50} 8 µg/ml) and H variety demonstrated highest superoxide radical O_2^- scavenging (IC_{50}- 0.77 µg/ml) when compared to the reference compounds. . It was found that B variety showed the highest phenolic content (499.15 mg fisetin/ gm extract), whereas variety D had the lowest (46.01 mg fisetin/ gm extract). Among the 9 varieties of *P. betle* leaf, the F variety was most effective at a concentration of 250 µg/ml and it retained cell viability 99%, very close to control and positive control but H variety of *P.betle* leaf extract showed pro-inflammatory activity and cell viability decreased by 29% than LPS induced RAW 264.7 cell. The results of the experiments suggest that selected variety of *P. betle* may be used as natural antioxidant as well as an alternative or supplementary herbal remedy for the treatment of inflammatory disease [53]. This is the first communication that betel leaves cannot be generalized in terms of their potential medicinal properties or toxic traits. It is a very fine environmental issue. The betel leaves used in the study have been collected from various geographical localities; have different farming profiles including various environmental traits and anthropogenic factors that lead to variations in their functional profiles. The details of the environmental and anthropogenic factors require detailed analyses and are beyond the scope of this paper. But this is a unique finding and needs to be brought to the attention of food scientists, drug hunters, ecologists, economists and social scientists because of the far reaching consequences of taking on leaf versus another and their long term health consequences as functional food. While some varieties show powerful anti-oxidant and anti-inflammatory activities, others show antagonistic functions which are potentially dangerous and long term use may lead to serious health conditions. These need to be avoided but

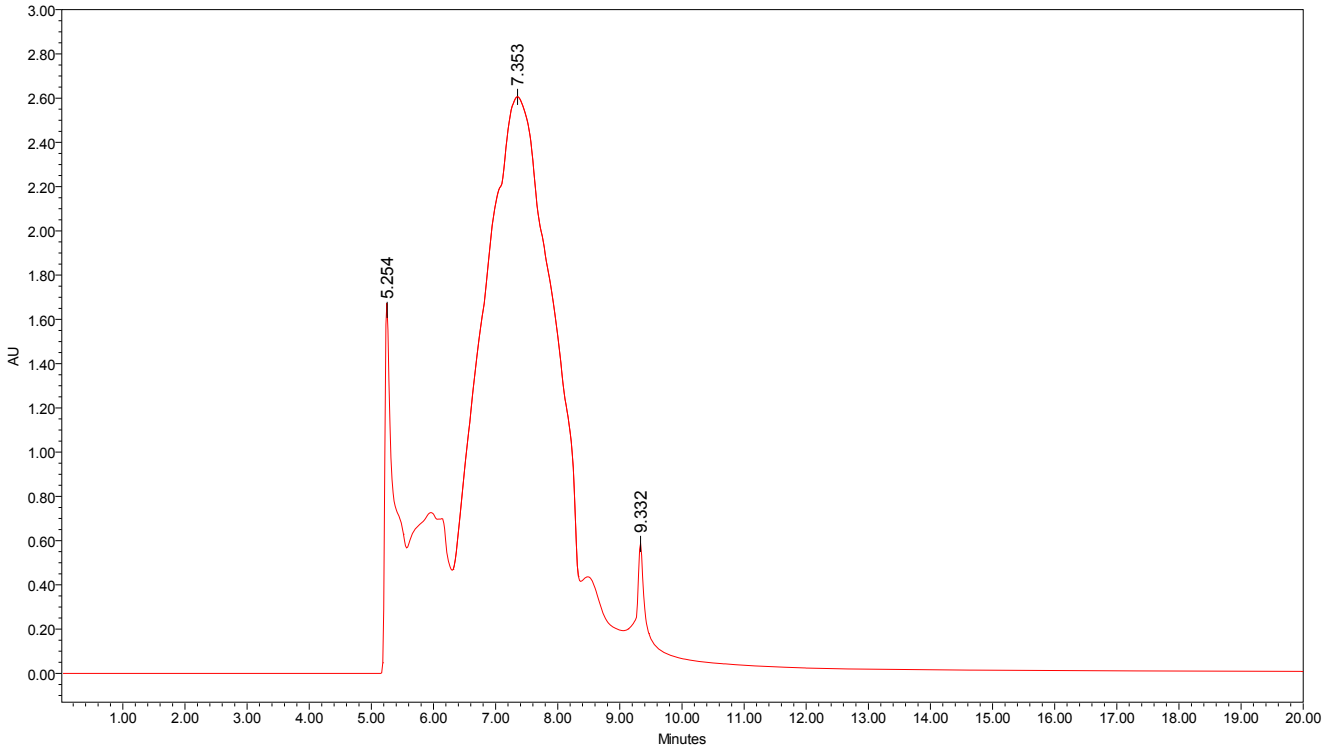

Figure 13: HPLC profile of a methanolic extract of variety A of *Piper betle*. Chromatogram of crude Sample A prepared from methanolic extract of *P.betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Three major peaks were identified at 254 nm and their retention times were 5.25, 7.36 and 9.33 min respectively.

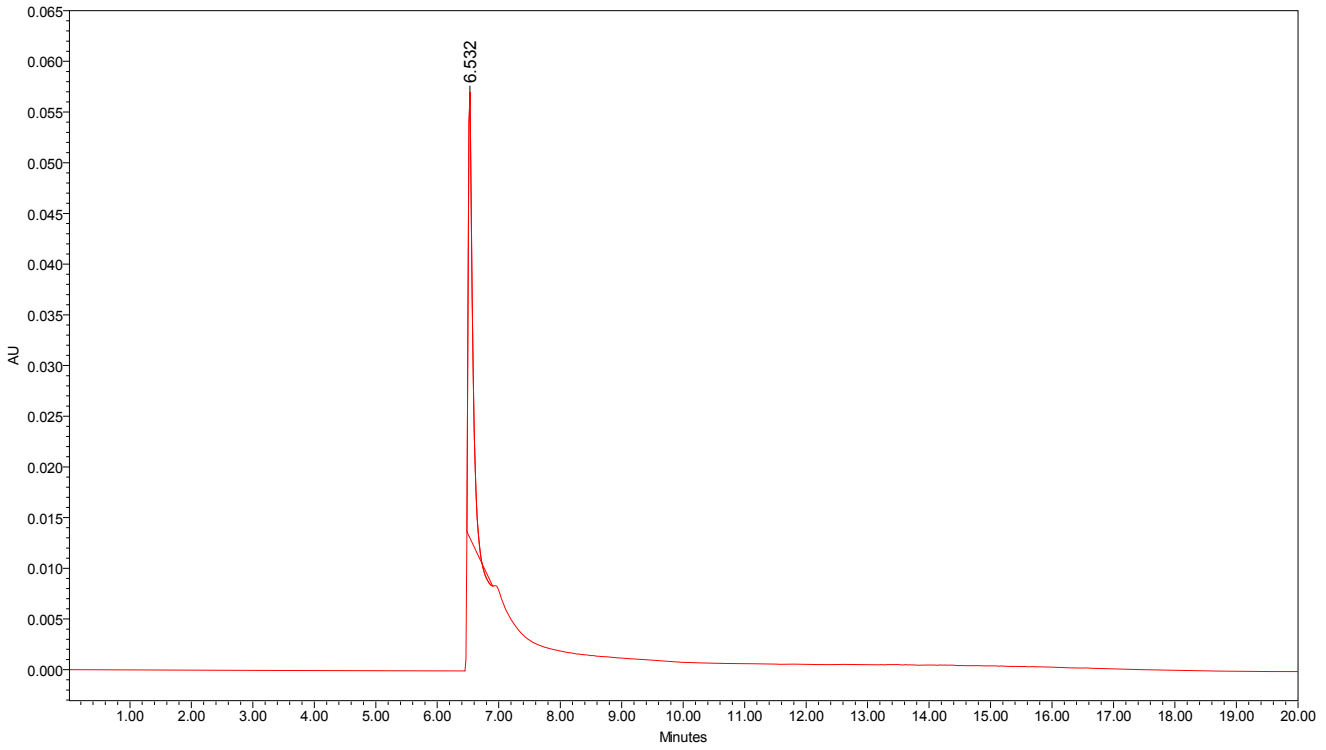

Figure 14: HPLC profile of a methanolic extract of variety B of *Piper betle*. Chromatogram of crude Sample B prepared from methanolic extract of *P. betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Only one peak was found at 254 nm and its retention time was 6.53 min.

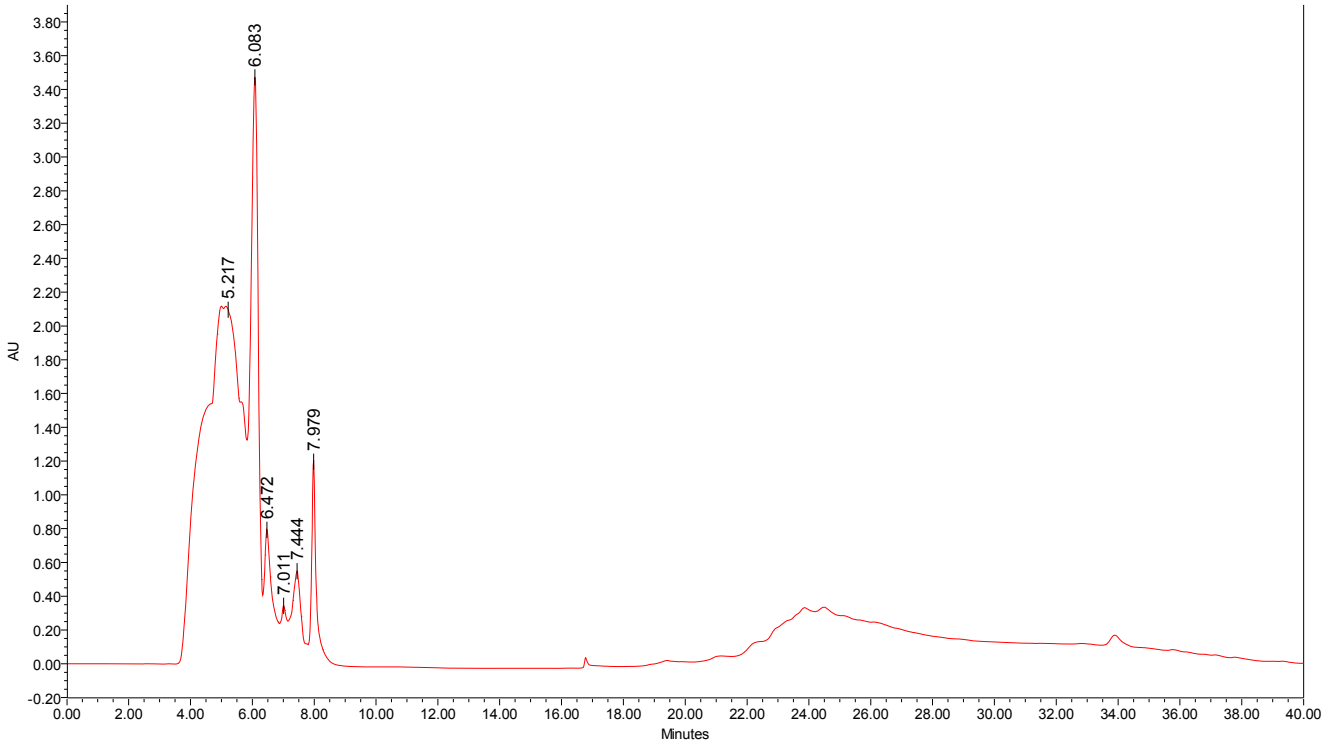

Figure 15: HPLC profile of a methanolic extract of variety C of *Piper betle*. Chromatogram of crude Sample C prepared from methanolic extract of *P. betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5ml/min. Six peaks were identified at 254nm and their retention times were 5.21, 6.08, 6.42, 7.01, 7.44 and 7.97 min respectively.

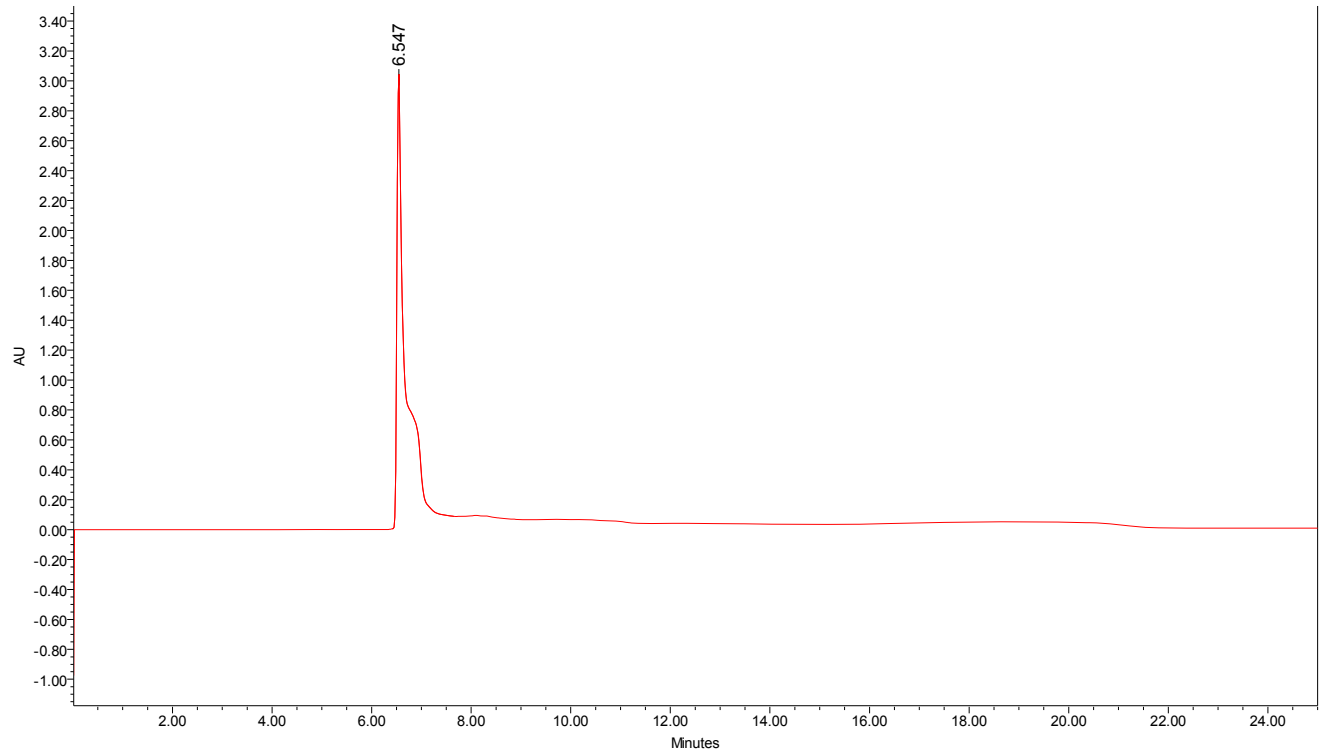

Figure 16: HPLC profile of a methanolic extract of variety D of *Piper betle*. Chromatogram of crude Sample D prepared from methanolic extract of *P.betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Only one peak was found at 254 nm and its retention time was 6.54 min.

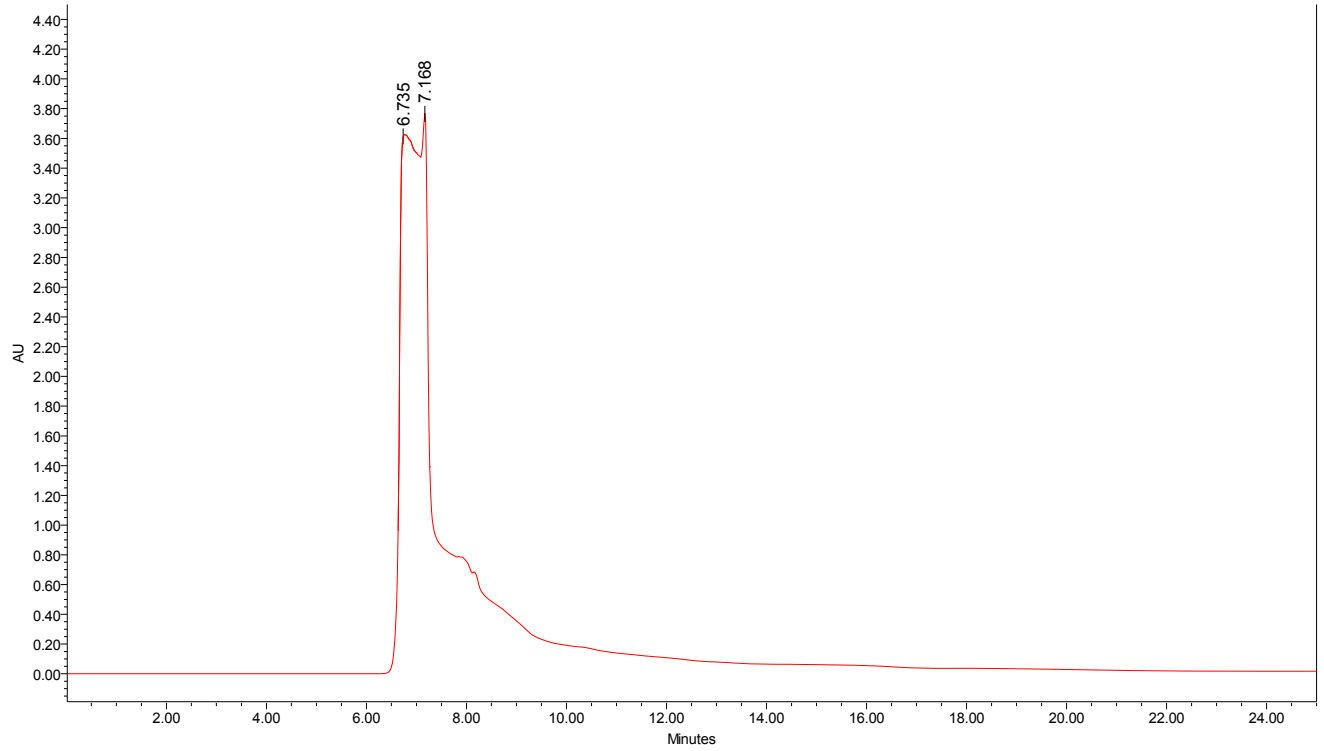

Figure 17: HPLC profile of a methanolic extract of variety E of *Piper betle*. Chromatogram of crude Sample E prepared from methanolic extract of *P. betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Only two peaks were found at 254 nm and their retention times were 6.73 and 7.16 min respectively.

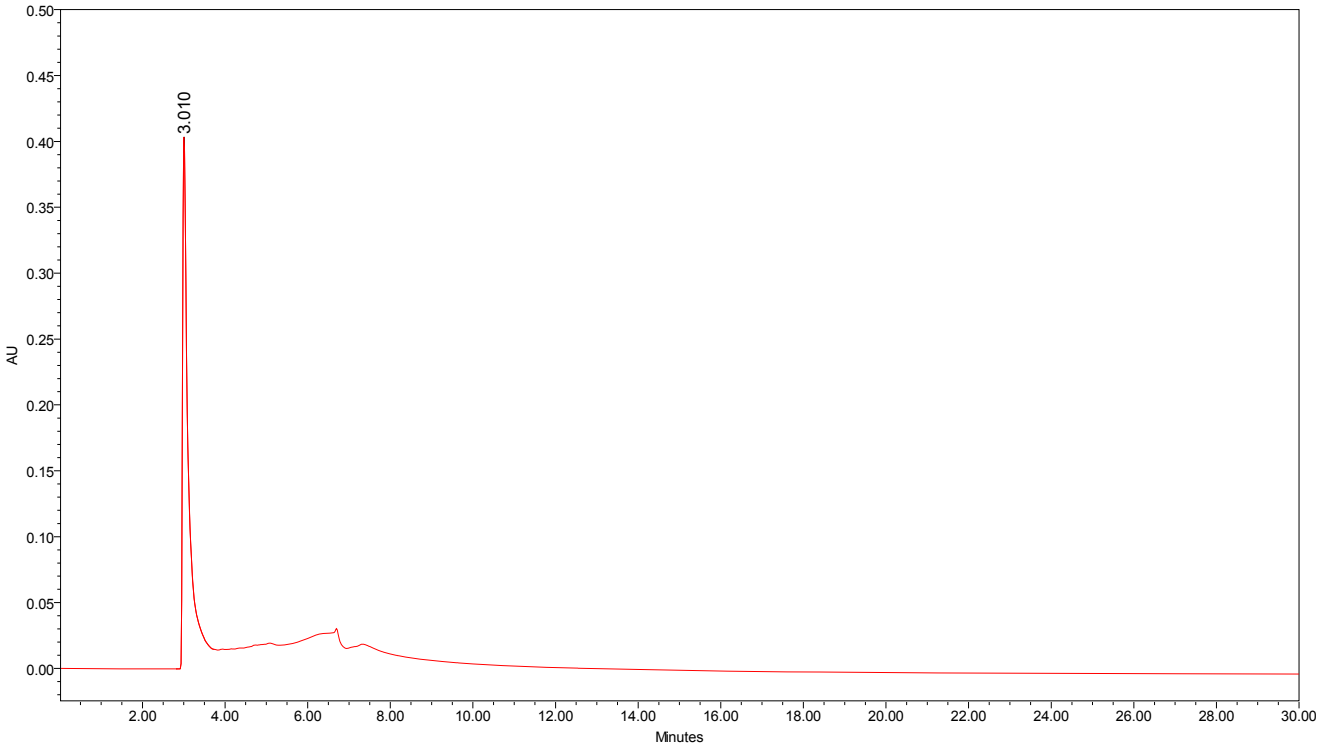

Figure 18: HPLC profile of a methanolic extract of variety F of *Piper betle*. Chromatogram of crude Sample F prepared from methanolic extract of *P. betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Only one peak was found at 254 nm and its retention time was 3.01 min.

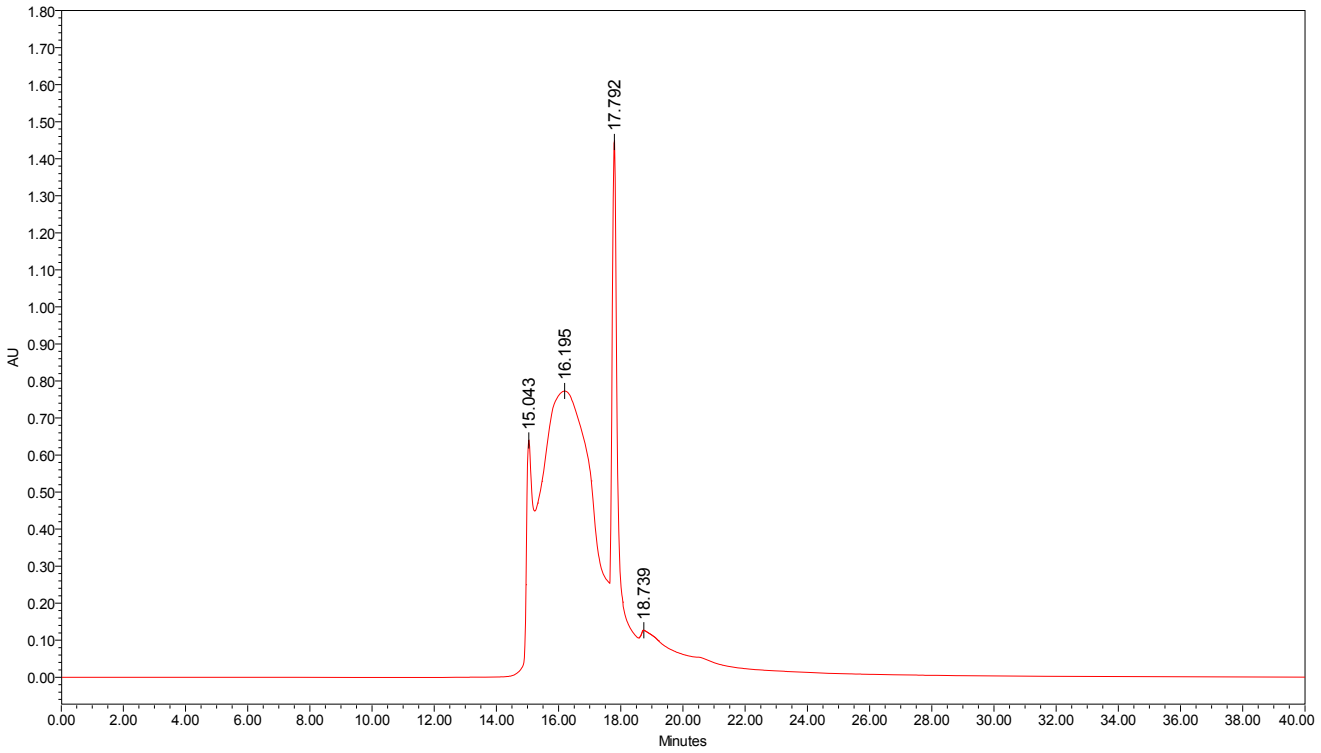

Figure 19: HPLC profile of a methanolic extract of variety G of *Piper betle*. Chromatogram of crude Sample G prepared from methanolic extract of *P. betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Four peaks were identified at 254 nm and their retention times were 15.04, 16.19, 17.79 and 18.79 min respectively.

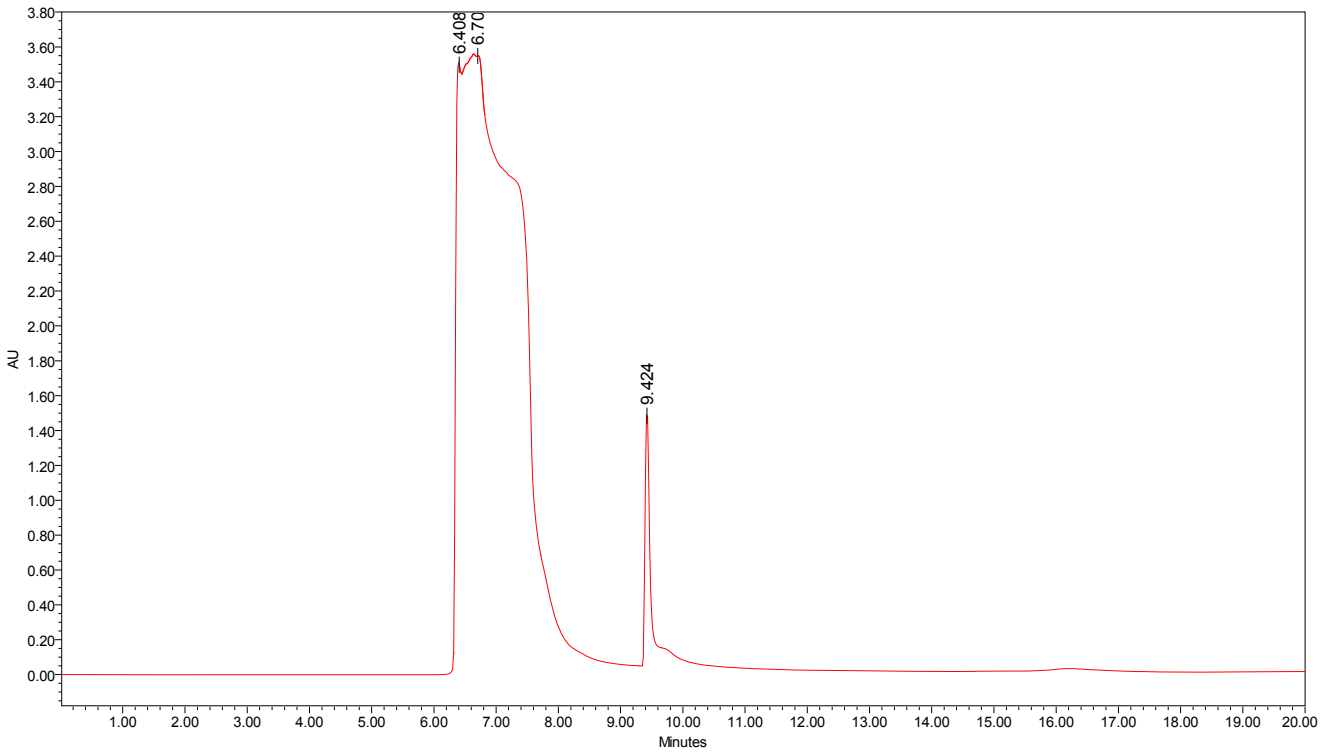

Figure 20: HPLC profile of a methanolic extract of variety H of *Piper betle*. Chromatogram of crude Sample H prepared from methanolic extract of *P. betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Three peaks were identified at 254 nm and their retention times were 6.40, 6.70 and 9.42 min respectively.

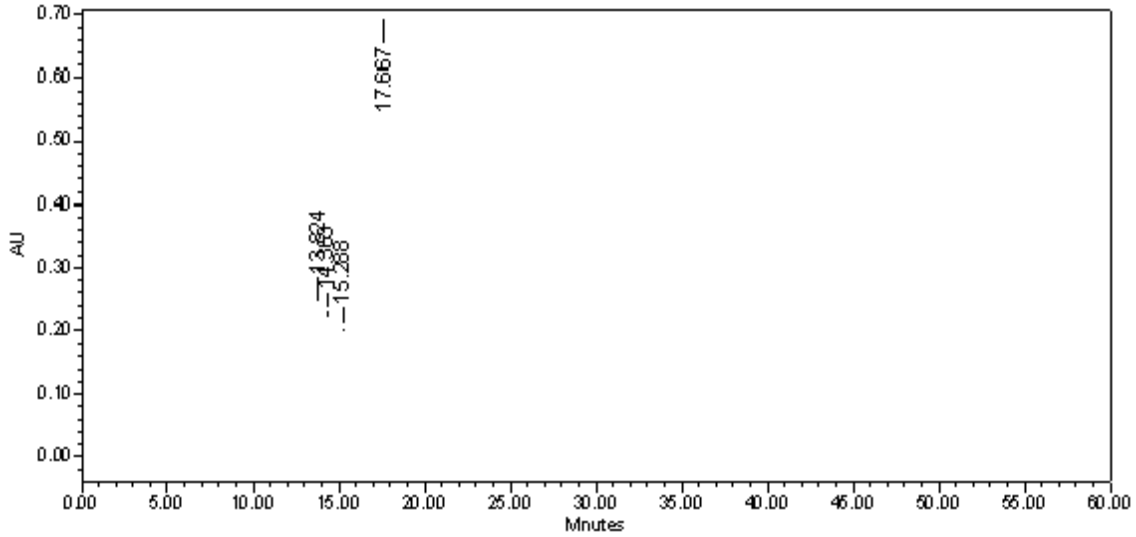

Figure 21: HPLC profile of a methanolic extract of variety I of *Piper betle*. Chromatogram of crude Sample I prepared from methanolic extract of *P. betle* L. leaf, on analytical RP-HPLC in isocratic mode using acetonitrile and water (1:1) as mobile phase and C18 column as stationary phase, at a flow rate of 0.5 ml/min. Four peaks were identified at 254 nm and their retention times were 13.82, 14.36, 15.28 and 17.66 min respectively.

common people generally believe paan to be of only one variety and do not consider the micro-nutrients aspects that must be factored in. Additionally biodiversity conservation also should be a major thrust area for the conservation /preservation of each variety which are by no means uniform and farming techniques and human interference and handling also must be thoroughly studies to understand the implication of each and every factor contributing to its ultimate functional phenotype and implications in terms of its transcriptomic components that in turn influence the metabolomics of the human being taking them as recreational food. Thus, the present study warrants further investigation involving components of **P. betle** for possible development of new class of anti-inflammatory drugs as well as focussed biodiversity conservation policies.

Contribution of Authors

RD has performed all assays, analysed data and drafted this manuscript. SM has analysed data and made significant intellectual contribution to this work. KM had performed some preliminary assays. UPS has mentored all the proteomic analyses pertaining to this project and helped in the acquiring of data and all final analyses. ERB has conceptualized the project, planned all experiments, analysed all data and wrote this manuscript.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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