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ORIGINAL ARTICLE

Antioxidant Properties of *Piper Betel* (L) Leaf Extracts from Six Different Geographical Domain of India

S.G. Jaiswal, M. Patel, D.K. Saxena and S.N. Naik*

Center for Rural Development and Technology, Indian Institute of Technology Delhi, HauzKhas, New Delhi-110016, India.

*Corresponding Author:

S. N. Naik

E-mail: naiksn@gmail.com

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Abstract

Six variety of betel leaf from different domain of India were extracted using five solvents in order to determine the effect of solvent and changing variety on total phenol content and antioxidant activity. Antioxidant activity was determined using DPPH, ABTS, FRAP, PCL (Photochemluminiscence) assay. However 80% methanol for TPC, 80% ethanol followed by 80% methanol for DPPH and 80% ethyl acetate for all other antioxidant assays proved to be the convenient extraction medium. Variety wise TPC of Desi bagla, DPPH of Sofia, ABTS and FRAP values of Banarasi safeda and PCL value of Calcutta were found to be more efficient. Weak to strong relationship were observed in different extracts of each solvent with highest r value of 0.95 ($p<0.001$) between TPC and ABTS. Wide range of correlation coefficient was derived within four different antioxidant assays of each betel leaf extracts with consistently high relationship observed between TPC and FRAP values.

Keywords: *Piper betel*, Phenols, Extraction, Photochemluminiscence, Antioxidant activity.

1. Introduction

Antioxidants is any substance that directly scavenges Reactive Oxygen Species (ROS) or indirectly acts to up-regulate antioxidant defences or inhibit ROS production (Khlebnikov *et al.*, 2007). It protects human being from deadly diseases such as cardiovascular disease, cancerous disease (Manch *et al.*, 2004), neurodegenerative disease, Parkinsons and Alzimers disease (Di Matteo and Esposito, 2003). Antioxidant supplements or antioxidant-rich food can help in reducing the oxidative damage from free radicals and active oxygen species (Zhao *et al.*, 1989). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and trolox are widely used as antioxidants in the pharmaceutical and food industry. However, they have been showing toxic or mutagenic effects (Halliwell *et al.*, 1992). Due to the toxicity of synthetic antioxidant, emphasis is given on development and isolation of natural antioxidants (polyphenols, tannins and saponins) from plant species (Juntachote and Berghofer, 2005). Out of these, phenolics are the major contributor of antioxidant activity in plant extracts due to their higher value in total content (Hodzic *et al.*, 2009), synergistic effectiveness as hydrogen donating capacity, reducing agent and free radical scavengers. Wong *et al.*, 2006, have optimized the extraction conditions for the maximum recovery of total phenolics which is an important aspect in the field of natural antioxidant for preservation of food.

Piper betel L. is the perennial dioecious vine which belongs to *piperaceae* family. It is originated from Malaysia and cultivated in tropical and subtropical parts of India. Due to ethno-medicinal properties, the plant is widely used in south-east Asian countries. Piper betel is the most favourite herb used for chewing purpose according to native people. Traditionally piper betel has socio-economic importance and gained valuable attention at ceremonial events. In *Ayurveda* (Indian medicinal system) betel leaf is known by its Vedic name Saptasira and used as adjuvant with different medicines for voice, purifying blood, laxative and appetizer. The essential oil contains high safrol along with eugenol, allyldiacetoxybenzene and chavibitol acetate as major constituents in Sri Lankan betel variety leaves (Kumartunga, 2003). Antioxidant action of betel leaf is very high, due to the presence of phenolic compound hydroxy-chavicol (4-allyl pyrocatechol) which have been proved to be preservative for vegetable oils up to the concentration of 0.03% without imparting their taste and odour

(Santhakumari *et al.,* 2003; The Wealth of India, 1989). Use of betel leaf extract as an antioxidant in butter cake retarded its oxidation and extends its shelf life. It was found to be a better source of antioxidant as compared to BHT and BHA (Lean and Mohamed, 1999). Hydroxy-chavicol shows anti-carcinogenic activity (Bhide *et al.,* 1991). The biological activity of major bioactive constituents such as allylpyrocatechol, hydroxychavicol, chavibetol of betel leaf were described (Lei *et al.,* 2003; Mula *et al.,* 2008; Rathee *et al.,* 2006; Razak and Rahim, 2003; Sharma *et al.,* 2009).

In the present study six variety of betel leaves were collected from different regions of India and extracted with five different solvents. Selection of solvents for extraction was made on the basis of good extraction efficacy for plant phenolics (Naczk and Shahidi, 2004). Extraction efficacies of phenolics are mainly affected by time, temperature, solubility, sample to solvent ratio, physical state of the sample and polarity of solvents (Naczk and Shahidi, 2006). Extraction of betel leaf was done by keeping all the above parameters constant and changing polarity of solvents. To our knowledge enormous study has been carried out on the antioxidant activity of different betel leaves variety (Rathee *et al.,* 2006; Dasgupta and De, 2004; Tamuly *et al.,* 2013). However, there is no literature available on the photochemiluminescence study of betel leaf. The present research is focused on two objectives (1) To investigate the photochemiluminescence activity of betel leaf extract of each variety and its comparison with other antioxidant evaluation methodology namely (DPPH), (ABTS) and (FRAP). (2) To determine the effect of solvents in extraction of polyphenols and antioxidants as well as study the correlation between different antioxidant activity assays with total phenol content.

2. Materials and Method

2.1 Chemicals

Standard compounds for the determination of radical scavenging activity viz., DPPH (2, 2-diphenyl-1-picrylhydrazyl), $ABTS^{+}$ (2, 2-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid), gallic acid and catechin were purchased from Sigma-Aldrich Fine Chemicals (St. Lous. MO). TPTZ (2,4,6-tripyridyl-s-triazine) was procured from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India), Folin-Ciocalteu reagent, methanol, ethanol, ethyl acetate, acetone, acetic acid, hydrochloric acid were obtained from Merck (Darmstadt, Germany). PHOTOCHEM antioxidant (ACW) kit used was obtained from Analytikjena (Konrad-Zuse-Strasse 1, Germany). Potassium per sulphate, acetate buffer, anhydrous sodium acetate, hydrochloric acid, ferric chloride, ferrous sulphate was purchased from Central Drug House (Pvt.) Ltd. (New Delhi, India).

2.2 Plant Materials and Extractions

Based on the shape, colour, taste and aroma, many verities of betel leaves are found in India. Out of these six, namely Banarasisafeda (PA), Calcutta (PB), Cuttack (PC), Desibagla (PD), Maharashtra (PE) and Sofia (PF) were collected from different provinces of India namely Uttar Pradesh, West Bengal, Odisha, Maharashtra and Bihar.

The leaves were stored at -40^{o}C for 12 hours, lyophilized in freeze dryer (Labconco, Kansas City, USA) and stored in dark at 4^{o}C before extraction. One gram freeze dried powder of each betel leaf variety was dissolved in 25 mL of solvent (80% methanol, 80% ethanol, 80% acetone, 80% ethyl acetate and distilled water) and extractions were carried out in shaking incubator (Lab Tech, LSI-2005RL, Hyderabad, India) for 2 hours. The extracts were filtered through muslin cloth and centrifuged at 4000 rpm for 15 min. The supernatant was filtered through 0.45µm Nylon-66 membrane syringe filters (mdi Membrane Technologies LLC, California, USA) and stored at 4^{o}C for further analysis.

2.3 Determination of Total Phenolic Contents

Total phenol content (TPC) of different variety of betel leaves was determined according to the Folin-Ciocalteu method with slight modification (Singleton and Rossi, 1965). 200 µL sample of leaf extract was transferred into a test tube containing, 1 mL of freshly diluted (10 fold) Folin-Ciolateu reagent. The mixture was allowed to stand at room temperature. After 8 min, 3 mL of 7.5% (w/v) sodium carbonate was added to the mixture and shaken manually. Then the mixture was incubated at room temperature for 60 min. The absorbance was recorded at 765 nm using UV – visible spectrophotometer (Shimadzu, UV-2600 Kyoto, Japan). Acidified methanol was used as blank. The calibration curve was plotted against gallic acid and expressed in terms of mg GA equivalents per g dry weight basis (mg GAE/g dw). The linearity of the range for TPC was measured as 0.05 – 0.5 mg GA/mL (R^2=0.9929).

2.4 Antioxidant Activity

2.4.1 DPPH Radical Scavenging Activity

The free radical scavenging activity of the leaf extract was measured by using $DPPH^{\bullet}$ radical with some modifications (Bermudez-Soto and Tomas-Barberan, 2004; Surveswaran *et al.,* 2007). According

to Payet*et al.*(2005), 100 µL extracts was reacted with 3.9 mL of 0.004% (80% methanol) DPPH solution. The reaction mixture was allowed to incubate in dark for 60 min. Then absorbance was measured against methanol at 515 nm using spectrophotometer (Shimadzu, UV-2600 Kyoto, Japan). Catechin was also used as a reference in this assay. A standard curve was obtained using catechin standard solution at concentration 0-1 mg/mL. The absorbance of the extract was compared to that of catechin standard curve and all results were expressed as mg Catechin equivalent per g dry weight basis (mg CE/g dw).

2.4.2 ABTS Radical Scavenging Assay

For the $ABTS^{\cdot+}$ assay the method previously described was followed (Re *et al.*, 1999). The working stock solution for $ABTS^{\cdot+}$ was prepared by mixing the equal quantities of 7.0 mM $ABTS^{\cdot+}$ solution and 2.45 mMpottasiumpersulphate and allowing them to react for 16 hours in dark. At the time of measurement, prepared working solution was diluted with 80% ethanol to the absorbance level of 0.70 ± 0.02 at 734 nm. A 100 µL of sample leaf was allowed to react with adjusted $ABTS^{\cdot+}$ working solution for 6 min. Catechin was used as standard and ABTS radical scavenging activity of all the extracts were expressed as mg Catechin equivalent per g dry weight basis (mg CE/g dw).

2.4.3 Ferric Reducing Antioxidant Potential (FRAP) Assay

Ferric reducing antioxidant potential of extract was analysed using the method proposed by Firuzi *et al.*(2005). FRAP solution was prepared by adding 200 mL of 300 mM acetate buffer (which was adjusted to pH of 3.6 by the addition of acetic acid) to 20 ml of 20 mM ferric chloride hexahydrate (dissolved in distilled water) and 20 mL of 10 mM 2,4,6-tri-(2-pyridyl)-s-triozine (TPTZ) (dissolved in 40 mMHCl). Ferrous sulphate standard graph was prepared by taking different concentration of ferrous sulphate (0.1-1 mM). 120 µL distilled water/standard/sample was added to 4 mL of the FRAP solution and absorbance was taken at 593 nm after 4 min. Ferrous sulphate equivalent concentration in mM was calculated from the standard graph and expressed as mmol ferrous sulphate equivalent/g on dry weight basis (mmol Fe^{+2}/g dw) .

2.4.4 Photochemiluminescence (PCL) Assay

In the history of different antioxidant analyses methods,Popov and Lewin (1994) described the mechanism of photochemiluminescence assay and after that Analytik Jena AG (Germany) commercialized this mechanism by the name of photochem. In this method chemiluminescent detection reagent luminol is used for photochemical generation of superoxide radical anion. This luminol act as photosensitizer as well as oxygen radical detection reagent.

$$S + h\upsilon + O_2 \longrightarrow [S^*O_2] \longrightarrow S^{\cdot+} + O^{\cdot-}$$

The composite reaction mixtures for the antioxidant capacities of water soluble (ACW) measurements were prepared as per the protocol given by Analytik Jena: A volume of 490 µL of reagent 1 and 10 µL conc. H_2SO_4 (95-97%) was added to the vial containing reagent 4 and vortexed for 20-30 seconds to get a standard concentration (10 mmol/L) of ascorbic acid. Reagent 4 working solution (0.1nmol/µL) was prepared by diluting the stock solution in 1:100 with reagent 1. Calibration curve was prepared by taking 0.5,1,2 and 3 nmol of ascorbic acid corresponding to 5-30 µL reagents 4 working solution. Appropriately diluted samples (10 µL) were added with 1490 µL of reagent 1, 1000 µL of reagent 2 and 25 µL of reagent 3. It was vortexed for 10-20 seconds and subjected for analysis. All the readings were taken in triplicates. The analysis parameters for antioxidant capacity are the difference between lag time of blank (L_0) and lag time of sample (L_1).

$$L = L_0 - L_1$$

Antioxidant capacities of water solubles are expressed in terms of mmol ascorbic acid equivalents per gram on dry weight basis (mmol AA/g dw).

2.5 Statistical Analyses

Statistical analyses were conducted using SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) version 16.0 for windows. One way ANOVA with Tukey's B test was performed to analyse the results. All the reported data were recorded in triplicates and results were expressed as a means ± standard deviation and means were accepted as significantly different at 95% confidence interval ($p<0.05$).

3. Results and Discussion

3.1 Total Phenol Content

Phenolics (secondary metabolites) of plant origin are the symbol of antioxidant properties which protect the human health from several diseases and have played the vital role in reducing free radicals produced by oxidation reactions (Podsedek, 2007). It has been noted that chavicol, allylprotocatechol, chavibetol and eugenol are the major pungent component of betel leaf responsible for antioxidant activity (The Wealth of India, 1989). This findings are

in relation with previous one where chavibetol, allylprotocatechol were fractionated through column chromatography (Rathee *et al.,* 2006). In earlier study, lesser value of total phenol content in betel leaf was found due to the use of high polar solvent (Maisuthisakul, 2008). The effect of solvent polarity on the extraction yield of total phenolics was elucidated by some researchers (Zhao *et al.,* 2006). In the present study we have tried to extract as much polyphenols from the betel leaves by using five different solvent systems. The results obtained for TPC have been depicted in Table 1. According to the polarity of extraction solvent, a wide variability was observed among the each betel leaf variety. The TPC for six variety betel leaf extract in five different solvents were found in the range of 0.29 to 2.62 mg GAE/g dw for PA, 0.08 to 2.59 mg GAE/g dw for PB, 0.09 to 2.38 mg GAE/g dw for PC, 0.07 to 2.87 mg GAE/g dw for PD, 0.16 to 2.07 mg GAE/g dw for PE and 0.04-1.16 mg GAE/g dw for PF. The observed descending order of contributed extraction solvent for TPC can be arranged as 80% methanol > 80% acetone > 80% ethanol >80% ethyl acetate > water. Highest TPC was observed in 80% methanol extract while lower TPC was observed in water extract. It is reported that, water: methanol (1:1) extract of *P.betel, P.betleoides* and *P.wallichii* was found to be the best solvent with significantly higher ($P<0.01$) phenolic content as compared to the other solvent (Rathee *et al.,* 2006).

On the other hand lower yield of TPC in aqueous betel leaf extract was also reported (Abrahim *et al.,* 2012). Our finding matches with the reported results. PD variety from West Bengal showed highest TPC among the all varieties. Our findings are in agreement with the previous results where betel variety of West Bengal had more phenol content compared to the Sweet and Mysore variety (Rathee *et al*., 2006).

3.2 Antioxidant Activity

The highest level of DPPH scavenging activity (Table 2) was observed for 80% ethanol extract of PF (133 mg CE/g dw) variety followed by PE (127.5 mg CE/g dw), while the lowest activity was observed for water extract of PA (2.48 mg CE/g dw) variety. Overall 80% ethanol was found to be the more suitable solvent over 80% methanol. The trend of antioxidant capacity of betel leaf extracts provided by DPPH assay after extraction with 80% ethanol was like PF>PE>PD>PC>PB>PA. However these results appeared to contradict a previous TPC report where 80% methanol exhibited more suitable solvent over 80% ethanol. There was significant difference ($P<0.05$) in the DPPH scavenging activity of different solvent extract for a particular variety except for PA and PC, where the result was not significantly different for solvent 80% acetone and 80% ethyl acetate. In another study, concentration dependent scavenging of DPPH radical was observed by three varieties of betel (Bangla, Sweet and Mysore) (Rathee *et al*., 2006). Highest DPPH scavenging activity was observed by the Bangla (IC_{50} 52.43 µg/mL) variety followed by Sweet and Mysore variety. The DPPH study of three betel leaf varieties using seven different solvents shows that there is no significant difference in the activity using the solvent methanol and ethanol. The lowest IC_{50}value obtained for methanol: water (1:1) extract was 36.5,45.5 and 50.4 µg/mL respectively for the *P.betle*, *P.betleoides*and *P.wallichii*variety respectively (Tamuly *et al.,* 2013).

$ABTS^{\cdot+}$ assay is used for both hydrophobic and lipophilic antioxidant system on the other hand DPPH is used in case of lipophilic antioxidant system (Kim *et al.,* 2002). Betel leaf extract values for $ABTS^{\cdot+}$ assay are shown in Table 3. Most of the values shows slightly more $ABTS^{\cdot+}$ scavenging capacity as compared to the DPPH method when catechin was used as standard in both the cases. Our findings are in agreement with the previous results reported (Khanam *et al.,* 2012), which shows lower antioxidant capacities for most of leafy vegetables equivalent to trolox, quercetin and ascorbic acid after comparison of DPPH assay to the ABTS assay.

Highest ABTS scavenging activity was obtained for 80% ethyl acetate extract of PA (79 mg CE/g dw) variety and the lowest value was for water extract of PF (6.45 mg CE/g dw) variety followed by PB (6.53 mg CE/g dw). 80% ethyl acetate was found to be the best extraction solvent for ABTS study of four (PA, PB, PC and PD) varieties out of six varieties. For PE (35.52 mg CE/g dw) and PF (26.82 mg CE/g dw) variety, 80% ethanol was found to be the best extraction solvent with significant difference.

The highest and lowest FRAP value was reported for 80% ethyl acetate extract of PA (1.35 mmol Fe^{+2}/g dw) variety and water extract of PF (0.04 mmol Fe^{+2}/g dw) variety (Table 4). 80% ethyl acetate was found to be the best extraction solvent for FRAP study of PA, PB, PC and PD variety. 80% Methanol was found to be most effective for PE (0.80 mmol Fe^{+2}/g dw) variety and 80% acetone was the best for FRAP study of PF (0.41 mmol Fe^{+2}/g dw) variety. In a similar study, methanol: water (1:1) reported to be the best solvent for ABTS and FRAP study of different betel leaf variety (Tamuly *et al.,* 2013).

PCL assay was chosen for antioxidant study of betel leaf extract because the superoxide radical (O_2 one of the dangerous reactive oxygen species) is directly linked with health issues and give antioxidant activities in nano molar range. This study is the first report on antioxidant activity of betel leaf extract by

Table 1: Total phenol content of six variety betel leaf extracted with five differentsolvents

Sample	TPC (mg GAE/g dw basis)				
	80% Methanol	80% Ethanol	80% Acetone	80%Ethyl Acetate	Water
PA	2.62±0.036^{c}	2.04±0.87^{b}	2.73±0.30^{c}	1.94±0.27^{b}	0.29±0.02^{a}
PB	2.59±0.020^{c}	1.85±0.066^{b}	1.89±0.25^{b}	2.06±0.22^{b}	0.08±0.02^{a}
PC	2.38±0.02^{c}	1.74±0.10^{b}	2.37±0.31^{c}	2.13±0.13^{c}	0.09±0.02^{a}
PD	2.87±0.12^{c}	1.8±0.03^{b}	1.72±0.13^{b}	1.84±0.05^{b}	0.07±0.01^{a}
PE	2.07±0.14^{c}	0.84±0.06^{c}	0.93±0.11^{c}	0.37±0.01^{b}	0.16±0.02^{a}
PF	1.16±0.02^{c}	1.1±0.03^{c}	1.45±0.20^{d}	0.52±0.02^{b}	0.04±0.01^{a}

Values are mean ± standard deviation of triplicate analyses. Results of each solvent extraction were analyzed separately. Different letters in the same row are significantly different ($p<0.05$) as measured by Tukey's B test.

Table 2: DPPH radical scavenging activityof six variety betel leaf extracted with five differentsolvents

Sample	DPPH (mg CE/g dw basis)				
	80% Methanol	80% Ethanol	80% Acetone	80%Ethyl Acetate	Water
PA	63.56±0.89^{d}	54.35±2.15^{c}	21.36±0.06^{b}	23.25±0.02^{b}	2.48±0.005^{a}
PB	58.95±0.18^{e}	55.37±1.25^{d}	19.04±1.6^{b}	22.71±0.09^{c}	4.46±1.04^{a}
PC	38.90±1.8^{c}	74.96±1.8^{d}	20.94±1.47^{b}	23.35±0.07^{b}	4.92±0.57^{a}
PD	51.78±1.46^{d}	77.71±1.62^{e}	16.52±0.41^{b}	22.42±0.13^{c}	2.96±0.94^{a}
PE	25.40±1.10^{d}	127.5±0.25^{e}	9.32±0.80^{b}	18.22±1.10^{c}	3.56±0.36^{a}
PF	35.25±2.20^{e}	133.5±1.77^{d}	17.47±0.84^{c}	21.42±0.64^{b}	7.20±0.98^{a}

Values are mean ± standard deviation of triplicate analyses. Results of each solvent extraction were analyzed separately. Different letters in the same row are significantly different ($p<0.05$) as measured by Tukey's B test.

Table 3: Antioxidant capacity of six variety betel leaf extracted with five differentsolvents measured by ABTS method

Sample	$ABTS^{\cdot+}$ (mg CE/g dw basis)				
	80% Methanol	80% Ethanol	80% Acetone	80%Ethyl Acetate	Water
PA	68.25±1.67^{c}	32.94±0.86^{b}	71.43±1.30^{d}	79±1.70^{e}	12.41±1.33^{a}
PB	46.93±1.49^{b}	68.38±1.08^{d}	55.95±1.28^{c}	78.32±2.17^{e}	6.53±1.30^{a}
PC	36.04±1.83^{b}	58.03±0.86^{c}	58.92±1.28^{c}	63.71±1.09^{d}	7.44±1.08^{a}
PD	70.30±2.60^{d}	49.90±2.30^{c}	42.83±0.71^{b}	78.10±1.68^{e}	8.81±0.73^{a}
PE	32.50±1.98^{b}	35.52±0.85^{b}	32.16±1.53^{b}	12.40±2.87^{a}	15.85±0.64^{a}
PF	26.2±0.63^{c}	26.82±2.58^{c}	24.11±0.85^{c}	22.63±0.64^{b}	6.45±1.06^{a}

Values are mean ± standard deviation of triplicate analyses. Results of each solvent extraction were analyzed separately. Different letters in the same row are significantly different ($p<0.05$) as measured by Tukey's B test.

Table 4: Antioxidant capacity of six variety betel leaf extracted with five differentsolvents measured by FRAP method

Sample	FRAP (mmol Fe^{+2}Eq./g dw basis)				
	80% Methanol	80% Ethanol	80% Acetone	80%Ethyl Acetate	Water
PA	0.65±0.03^{b}	0.67±0.06^{b}	0.90±0.04^{c}	1.35±0.01^{d}	0.09±0.005^{a}
PB	0.74±0.05^{b}	0.70±0.07^{b}	0.68±0.04^{b}	0.95±0.05^{c}	0.05±0.005^{a}
PC	0.64±0.02$^{b\text{-}c}$	0.56±0.07^{b}	0.69±0.04^{c}	0.90±0.05^{d}	0.04±0.005^{a}
PD	0.52±0.04^{c}	0.24±0.03^{b}	0.39±0.02^{c}	0.21±0.01^{b}	0.07±0.005^{a}
PE	0.80±0.03^{c}	0.63±0.04^{b}	0.56±0.06^{b}	1.10±0.11^{d}	0.07±0.005^{a}
PF	0.21±0.01^{b}	0.30±0.03^{c}	0.41±0.005^{d}	0.38±0.03^{d}	0.04±0.005^{a}

Values are mean ± standard deviation of triplicate analyses. Results of each solvent extraction were analyzed separately. Different letters in the same row are significantly different ($p<0.05$) as measured by Tukey's B test.

PCL method. Controversial statement was found in the literature regarding this method but possible explanation for its usefulness, effect of sample dilution and accuracy was also found (Hic and Balk, 2012).

The highest and lowest PCL activity was observed in 80% ethyl acetate extract of PB (49.30 mmol AA/g dw) and water extract of PF (0.39 mmol AA/g dw) variety as shown in Table 5.

Like ABTS and FRAP study, 80% ethyl acetate was found to be the best extraction solvent for photochem study of PA, PB, PC, PD and PF variety. 80% methanol exerted more PCL values for PE (31.98 mmol AA/g dw) variety. After comparison of all the methods except DPPH assay, it was confirmed that 80% ethyl acetate contributed as a better solvent for the extraction of antioxidant compound. These results were supported by the previous results where ethyl acetate showed the best solvent property for betel leaf antioxidant study (Maisuthisakul, 2008). PA and PF variety showed both highest and lowest antioxidant activity in both 80% ethyl acetate and water extract.

3.3 Correlation between TPC and Antioxidant Activity

Quantitative determination of TPC, DPPH, ABTS, FRAP, PCL results are greatly affected by the two variables (i) different solvent system and (ii) varieties of extract. Hence, correlation analyses between the studied parameters were analyzed within the extracts of each variable. Five different solvent extractions of six variety betel leaf shows strong to weak correlation between studied parameters when analyzed separately. Fig 1 (a) displays the relationship between TPC and DPPH of five different solvents.

Correlation coefficient (r) obtained from linear regression analyses between these two parameters were in a range of - 0.95 to 0.90 indicating that 80% ethanol was liable for negative correlation and 80% acetone ($p<0.05$) gives strong positive correlation between TPC and DPPH. The correlation between TPC and ABTS are shown in Fig 1 (b). Significant difference in all five different solvents with moderate to strong ($r = 0.51$ to 0.95) positive correlation was observed. Highest and lowest correlation were indicated by ethyl acetate ($p<0.05$) and ethanol ($p<0.001$) with significant difference.

r values between TPC and FRAP confirmed that more polar solvent gives highest correlation as shown in Fig 1 (c). This result was supported with the findings, in which highest correlation was found between TPC and FRAP of polar extracts as compared to non-polar extracts and antioxidant assays (Thaipong *et al.,* 2006). Between TPC and FRAP correlation was observed in the range of 0.15 to 0.73 with significant difference $p<0.01$ in water, methanol, acetone and no significant difference was found in ethanol and ethyl acetate. Strong and weak correlation was observed for water ($r=0.73$) followed by acetone ($r=0.72$) and ethyl acetate ($r=0.15$) respectively.

TPC and PCL correlation range varied from -0.52 to 0.94 as shown in Fig 1 (d). Strong positive correlation was found for ethyl acetate ($r=0.94$) with significant difference ($p<0.001$) followed by water (0.79). Weak correlation was found for ethyl acetate (-0.52) with no significant difference. After the evaluation of all correlation coefficients between TPC and antioxidant activities, highest and lowest were obtained in 80% ethyl acetate and 80% acetone respectively.

When Pearson's correlation coefficient (r) analyses were carried out in combination between TPC and different antioxidant activities of six betel leaf extract, wide variation were observed. This variation was compiled in Table 6.

ABTS and FRAP assay exhibits highest correlation values with TPC as compared to DPPH and PCL. On the other hand inverse correlation was found in PF extract of PCL method. PF variety also gave poor performance in all the methods with no significant difference except FRAP values which gave highest ($r=0.654$, $p<0.01$) performance.

Table 5: Antioxidant capacity of six variety betel leaf extracted with five different solvents measured by PCL method

Sample	PCL(mmol Ascorbic acid Eq./g dw basis)				
	80% Methanol	80% Ethanol	80% Acetone	80%Ethyl Acetate	Water
PA	9.61 ± 0.06^{c}	18.50 ± 0.27^{d}	7.14 ± 0.05^{b}	35.73 ± 1.11^{e}	2.57 ± 0.17^{a}
PB	9.22 ± 0.04^{c}	3.48 ± 0.04^{b}	1.19 ± 0.04^{a}	49.30 ± 0.44^{d}	1.10 ± 0.01^{a}
PC	10.69 ± 1.25^{d}	6.24 ± 0.02^{c}	4.29 ± 0.07^{b}	35.33 ± 0.73^{e}	1.23 ± 0.01^{a}
PD	3.57 ± 0.05^{b}	3.95 ± 0.03^{b}	5.39 ± 0.05^{c}	34.65 ± 0.76^{d}	0.40 ± 0.005^{a}
PE	31.98 ± 0.16^{e}	4.07 ± 0.05^{b}	21.45 ± 0.33^{d}	11.69 ± 0.21^{c}	0.48 ± 0.10^{a}
PF	1.54 ± 0.04^{b}	2.69 ± 0.09^{c}	1.66 ± 0.05^{b}	11.34 ± 0.10^{d}	0.39 ± 0.01^{a}

Values are mean ± standard deviation of triplicate analyses. Results of each solvent extraction were analyzed separately. Different letters in the same row are significantly different ($p<0.05$) as measured by Tukey's B test.

Table 6: Pearson's correlation coefficient (r) of total phenol content with antioxidant activities (i) DPPH (ii) ABTS (iii) FRAP (iv) PCL

Sample	DPPH	ABTS	FRAP	PCL
PA	0.637*	0.789**	0.623*	0.207
PB	0.721**	0.766**	0.859**	0.32
PC	0.373	0.790**	0.896**	0.399
PD	0.608*	0.862**	0.769**	0.189
PE	0.096	0.672**	0.909**	0.860**
PF	0.35	0.361	0.654**	-0.199

*significant at p<0.05, **significant at p<0.01

The trend of correlation between TPC and DPPH is in the range from 0.361 to 0.862, in TPC and FRAP is from 0.623 to 0.909, TPC and PCL is from -0.199 to 0.860. Overall highest r values was found in TPC and FRAP of PE variety (r=0.909) with significant difference of p<0.01 followed by PC (r=0.896). r values of PB variety in all methods shows overall consistent correlation performance with TPC. PCL method shows all the r values lower than other methods except PE variety (r=0.860, p<0.01).

4. Conclusion

The total phenol content and antioxidant potential of betel leaves collected from the different province of India are highly influenced by the extraction solvent. In relation to effect of different extraction solvent on antioxidant assay, 80% ethyl acetate exhibited highest extraction efficacy except DPPH assay where 80% methanol showed more extraction capacity. While 80% methanol was proved to be the better extraction medium for total phenol content. Variety wise distribution of antioxidant potential shows that FRAP and ABTS assay of Banarasi safeda and PCL assay for Calcutta variety showed highest antioxidant activity. Desibagla (PD) variety had maximum total phenol content. From this result it can be concluded that varying polarities of extraction solvent proved to be an effective tool in extraction of bio-actives compounds. Addition of 20% water in methanol, ethanol, acetone, ethyl acetate can improve the performance of extracting solvent and extract more antioxidant compound from the betel leaves which could be the promising source of natural antioxidant for food and pharmaceutical industries.

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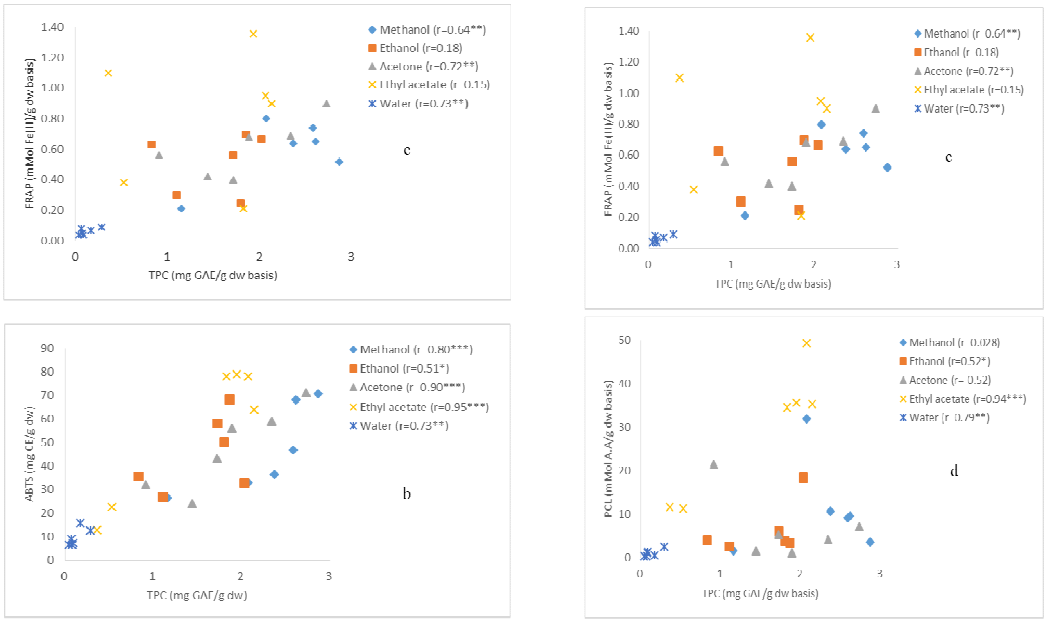

Fig 1: Effect of different solvent extractions on the correlation between (a) Total phenol content (TPC) and DPPH, (b) Total phenol content and ABTS, (c) Total phenol content and FRAP, (d) Total phenol content and phtocemluminiscence (PCL)

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