Exploration of antibacterial and antioxidant potential of a few members of the family Piperaceae

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Abstract

Piperaceae is a highly diverse and large family composed of five genera of which Piper and Peperomia are the most abundant. The current study endeavours five Piper species (Piper betle, Piper nigrum, Piper longum, Piper chaba and Piper retrofractum) and one species of Peperomia (Peperomia pellucida) concerning their phytochemical contents, antioxidant and antibacterial properties. Besides their economic uses, these plants also possess curative properties that have been exploited ethnomedicinally since the primaeval days. The methanolic extracts of both fresh (FL) and shade dried (SDL) leaves of these plants showed the presence of various phytochemicals. Among the studied plants, polyphenols like total phenolics (TPC), total flavonoids (TFC) and total tannins (TTC) contents were maximum in P. betle FL extract (TPC:39.50±0.99 mg GAE/ g extract, TFC: 19.40±0.57 mg QE/ g extract and TTC 11.08±0.11 mg GAE/g extract) and significantly higher than the others. Antioxidant efficacies of the extracts by total antioxidant activity (TAA), ferric reducing antioxidant power (FRAP) and ability to scavenge different radicals (DPPH, ABTS, NO and SO), were also highest in P. betle.

The study also highlighted the strong antibacterial activities of the extracts against both Gram-positive and Gram-negative bacteria. P. betle FL extract showed the highest activity representing the maximum zone of inhibition (24.65±0.21 mm) and lowest MIC/MBC values (0.58±0.04/0.65±0.07 mg/ml) against E. coli. These findings exhibit the potential of these plant extracts, especially P. betle, in the prospective exploration of plant-derived antioxidants and therapeutic uses of these plants for developing novel antibacterial drugs.

Keywords: Piperaceae, *Piper*, *Peperomia*, Polyphenolics, Antibacterial, Antioxidant.

Introduction

The beneficial roles of plants were marked from primeval periods and the conventional acquaintance has been disseminated over the eras till modern days. A large variety of plant-derived secondary metabolites like phenolics, flavonoids, alkaloids, tannins, terpenoids and saponins are known as effective antimicrobics and are of pharmacological significance.[12] The medicinal properties of such compounds have been explored due to their compelling pharmacological behaviour, minimal toxicity and cost effectivity.[16] Plants remained important bio-resources for traditional medicine systems over the centuries and the bioprospecting of plant resources led to the development of folk medicines, nutraceuticals, food supplements, pharmaceutics and modern medicines.[12]

Plants produce a wide range of natural antioxidant molecules that can scavenge reactive oxidative species (ROS) responsible for oxidative damage during oxidative stress. Antioxidants from plants like flavonoids, tannins, coumarins, anthocyanins, chromones, lignans, stilbenes, carotenoids and vitamins exhibit varied properties including anti-inflammatory, anti-analgesic, antiviral, antibacterial, anticancer, anti-ageing etc.[19] The increase in multi-drug resistance in pathogenic microorganisms poses a grave threat to mankind due to the random use of synthetic antimicrobial drugs. To contend with this situation, new antimicrobials are required and plants with medicinal principles are of great choice for novel antimicrobial agents.[6] Considering these facts, the study intended to explore two diverse bioactivities namely antioxidant and antibacterial efficacies in a few members of Piperaceae.

Piperaceae is a large family of angiosperm, generally known as the pepper family consisting of 5 genera and about 3600 species. However, most of the species are clustered within the two main genera, which are *Piper* and *Peperomia*.[37] *Piper nigrum* L., a source plant of black and white pepper, is a well-known member of the family. The *Piper betle* L. leaves, consumed in various ways in South Asian countries, are considered a trifling stimulant and are used by different communities during religious occasions as well. Betel leaves are also known for their vast ethnomedicinal properties since ancient times. In addition, other *Piper* species like *P. longum* L., *P. chaba* Trel. & Yunck. and *P. retrofractum* Vahl. are reported for their distinct bioactivities.[37] *Peperomia pellucida* (L) Kunth, another representative of the family Piperaceae has also been reported for its ethnobotanical uses and pharmacological activities.[31]

Although the fruits of *P. nigrum* and *P. longum* have been known significantly for their pharmacological and economical values[37], studies on their leaves are gaining importance in recent days due to the presence of a wide spectrum of phytochemicals. [6,17] Along with fresh leaves, dried leaves are also beneficial in various ways if the phytoconstituents remain unaltered. However, contradictory reports exist regarding the loss of bioactivities during drying

methods.[33] Keeping this view, we have used two types of leaf samples- fresh leaf (FL) and shade dried leaf (SDL) in the study.

The present study emphasises a comparative investigation of six members of Piperaceae namely: *P. betle, P. nigrum, P. longum, P. chaba, P. retrofractum* and *Peperomia pellucida* about their phytoconstituents, varied antioxidant activities and antibacterial properties using their leaf methanolic extracts. This endeavour aims to designate effective antioxidants and potent antibacterials from these important members of the family Piperaceae, which can be useful in the forthcoming period for the development of natural antioxidants and plant-derived antimicrobials.

Material and Methods

Plant materials: *Piper betle* (paan, betel) leaves were procured from a 'paan boroj' of Shimurali, Nadia, West Bengal (23.044364º N, 88.512731º E, 12m above sea level). Leaves of *Piper nigrum* (golmorich, black pepper), *Piper longum* (pipul, Indian long pepper, Pippali), *Piper retrofractum* (Javanese long pepper), *Piper chaba* (chui jhal or piper chilli) were acquired from the Spice Garden, Bidhan Chandra Krishi Viswavidyalaya, Kalyani (22.989893º N, 88.449395º E, 11m above sea level). We also collected the leaves of *Peperomia pellucida* (luchi pata, shining bush plant) from the University of Kalyani campus, Kalyani, West Bengal (22.989133º N and 88.447411º E, 11m above the sea level). The plants were identified with the help of a taxonomic manual[29] and the voucher specimens were preserved at the departmental repository at the University of Kalyani.

Methanolic extract preparation: Mature leaves of different plant species were collected, cleaned and air dried to remove surface moisture. Leaf samples (20g) were trimmed into small pieces and extracted using 90% methanol (v/v) by maintaining the sample to solvent ratio at 1:20 (w/v), kept for 48h at room temperature (RT; 30±2º C) with agitation (45 rpm) and eventually were filtered to obtain fresh leaf filtrates. Meanwhile, fresh leaves (20g) were shade dried for 15 days, pulverized to form a powdered sample and extracted similarly to obtain shade dried leaf filtrates. The fresh and shade dried leaf filtrates were then concentrated under reduced pressure in a rotary evaporator (Büchi, Switzerland) at 40º C to obtain the fresh leaf extract (FL) and shade dry leaf extract (SDL) respectively. These crude extracts were preserved at 4º C until further use.

Phytochemical analyses of the extracts

Qualitative tests: Qualitative detections of different phytochemical groups like sugars, phenolics, flavonoids, tannins, alkaloids, terpenes, iridoid glycosides and saponins were done following standard methods as described by Raj et al.[32]

Quantitative tests for polyphenolics: Determination of different polyphenolic components in the plant samples was done following similar methods described by Ojha et al.[23] Briefly, the extracts were mixed with Folin Ciocalteu (FC) reagent and sodium carbonate, kept for incubation at dark for 45 min and the absorbances were recorded at 765 nm. Phenolic contents were assessed from a calibration curve (r^2= 0.982) of gallic acid and represented as gallic acid equivalent (mg GAE/ g).

Total flavonoid contents (TFC) were performed by successive addition of 5% sodium nitrite and alkaline aluminium chloride (10%) in the extract samples and after incubating at dark for 10 min, the absorbances were taken at 510 nm. The quantifications were done with the use of a standard curve of quercetin (r^2= 0.984) and expressed as quercetin equivalent (mg QE/ g).

For total tannin content (TTC) estimation, extracts were mixed consecutively with 0.1M ferric chloride and 8mM potassium ferricyanide and incubated for 10 min at RT and measured at 720 nm. A standard curve of gallic acid (r^2= 0.993) was prepared to quantitate the tannin contents as gallic acid equivalent (mg GAE/ g).

Determination of antioxidant activities: Antioxidant activities were measured by total antioxidant activity (TAA), ferric reducing antioxidant power (FRAP) and radical scavenging assays like 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS), superoxide (SO) and nitric oxide (NO) following previously described methods.[32]

In brief, TAA was determined by the phospho-molybdenum method in which extracts were mixed with sulfuric acid, sodium phosphate and 4 mM ammonium molybdate sequentially and incubated for 90 min at 90±2º C. The reaction mixtures were measured at 695 nm and the quantitation was done using a standard curve (r^2= 0.983) of ascorbate and denoted as ascorbic acid equivalent (mg AAE/ g).

For FRAP assay, extract samples were mixed with FRAP reagent, incubated for 10 min at 37º C and measured at 594 nm. The estimates were calculated from a standard curve of ferrous sulphate (r^2= 0.994) and represented as mM Fe^{2+} equivalent/mg of extract.

The DPPH scavenging assay was performed by mixing extracts (in varying concentrations: 0.025-1.0 mg/ml) and DPPH solution (6 x 10^{-5} M). After incubation (15 min; RT; at dark), the absorbances were measured at 517 nm. In ABTS assay, extracts in different dilutions were mixed with appropriately diluted ABTS reagent (formulated by mixing 2.45 mM potassium persulfate and 7 mM ABTS in deionized water, set aside at dark condition for 16h which was then diluted to an optical density of 0.70±0.02) and recorded at 734 nm. The NO radical scavenging property was assessed by mixing the extracts with an equal volume of sodium nitroprusside under illumination for 2h.

The mixtures were then treated with Greiss reagent, kept for 10min at RT and the absorbances were taken at 542 nm. In SO assay, different concentrations of extracts were added in 0.05 M phosphate buffer (pH 7.5) which were mixed sequentially with 0.018 mM riboflavin, 0.32 mM ethylenediaminetetraacetic acid (EDTA) and 0.04 mM nitro blue tetrazolium (NBT). The mixtures were then subjected to illumination at RT for 1.5h and absorbances were taken at 590 nm.

In all the scavenging assays, the activity in percentage was calculated by:

$$\text{Scavenging activity (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

The 50% scavenging of the radicals (IC_{50} values) was calculated from the percent scavenging of a sample employing different concentrations.

Evaluation of antibacterial activity

Bacterial strains in the study: The antibacterial assays were performed using three gram-positive (*Bacillus subtilis* MTCC 121, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* MTCC 3086) and three gram-negative (*Escherichia coli* MTCC 443, *Vibrio cholerae* N16961, *Salmonella enterica* serovar Typhi C-6953) bacteria. They were cultured aerobically either in nutrient broth (NB; HiMedia) or in tryptone soy broth (TSB; HiMedia) at 37º C with agitation (45 rpm) and whenever required, agar plates of the said media were used. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) plates were used during the antibacterial assays with incubation at 37º C for 24 h.

Bacterial inhibitory assay: The inhibitory assay was performed following the agar-plate-based disc diffusion assay of Kirby-Bauer[3] with modifications.[32] Bacterial suspensions (100 µl, comprising around 2×10^8 colony forming units/ml) were swabbed over MHA plates to obtain uniform bacterial growth. Meanwhile, methanolic leaf extracts of different dilutions (3, 6 and 12 mg) were made from the stock solutions (200 mg/ml; in methanol) and applied on sterile filter paper discs (5 mm). Methanol (60 µl) and ampicillin (6 µg/ disc) were used as negative and positive control respectively. The dried paper discs were aseptically positioned over the bacterial smear, incubated overnight at 37º C and the bacterial inhibition zones were measured.

Estimation of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The MIC and MBC of the extracts against different bacteria were determined based on the micro-well dilution method[13] elaborated by Raj et al.[32] The extracts were diluted serially (0.25-15 mg/ml) in MHB and poured into the wells (190 µl) of a microtiter plate (Tarsons, India). Bacterial inoculums (10 µl, 1×10^7 CFU/ml) were applied to the wells to attain the resulting volume of 200 µl in each well. Appropriate controls like a positive control (ampicillin), extract control (extracts without inoculum) and inoculum control (growth medium only with inoculum, excluding extract) were also examined in parallel with the extract treatments.

Subsequently, the microwell plates were incubated overnight at 37°C with mild shaking (35 rpm) and the absorbances were recorded at 620 nm to monitor bacterial growth with the aid of a microtiter plate reader (BioTek, Switzerland). The MICs were determined by evaluating the minimum concentration of the sample where no growth of the bacteria was evident.

The MBC was estimated using a loopful of bacterial suspension (approximately 5 µl) from each well of the MIC plate and the suspensions were streaked on MHA plates. The plates were kept overnight at 37°C and the lowest concentration, at which no bacterial colonies appeared, was determined as the MBC of the sample.

Statistical analyses: The data were illustrated as mean ± standard deviation (SD) of three replications. One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test ($p<0.05$) was used to compare the results of phenolic estimates and antioxidant assays. In the disc diffusion assay, significant variations ($p<0.05$) between/among the different extract concentrations were determined by comparing critical differences (CD).

Results and Discussion

Phytochemical analyses: Our study revealed the presence of different phytochemicals in the six plant species detected by qualitative analyses as represented in table 1. The phytochemical groups like sugars, phenolics, flavonoids, tannins, alkaloids, terpenes, iridoids, saponins were detected at high to moderate levels in all the plants with higher amounts of phenolic components in *P. betle* and *P. nigrum* while higher terpene contents were detected in *P. nigrum*, *P. longum* and *Peperomia pellucida*. Iridoid glycosides and saponins were detected in *P. betle*, *P. retrofractum* and *Peperomia pellucida*. A host of studies by various researchers revealed the extraction of wide-ranging phytochemicals by methanol in different plants.[27,46]

Methanol is a widely used solvent for extraction as its polar nature helps to extract the polar compounds easily,[41] however, the extraction of many non-polar compounds is also facilitated by the solvent.[15,39] Methanolic extraction of different phytochemical classes in the leaves of different *Piper* spp.[9,44] and *Peperomia pellucida*[25] was also documented by others. Among the different plant parts, leaves are known to contain rich sources of phytochemicals, particularly phenolics,[21,47] presumably due to their higher photosynthetic efficacies.[47] As such distinctions between phytochemical contents in FL and SDL were mostly absent following qualitative analyses which may be due to the feeble sensitivity of the employed assays.

Table 1
Qualitative estimation of phytochemicals in methanolic extracts of a few members of Piperaceae.

Plant Species		Phytochemicals							
		Sugars	**Phenolics**	**Flavonoids**	**Tannins**	**Alkaloids**	**Terpenes**	**Iridoids**	**Saponins**
PB	FL	+	++	++	++	++	+	+	+
	SDL	+	++	++	++	++	+	+	+
PN	FL	+	++	++	++	+	++	-	-
	SDL	+	++	++	++	-	++	-	-
PL	FL	+	+	+	+	+	++	-	-
	SDL	-	++	+	+	+	++	-	-
PR	FL	+	++	+	+	+	+	++	+
	SDL	-	++	+	+	-	+	+	+
PC	FL	+	++	+	+	-	+	+	-
	SDL	-	++	+	-	-	+	-	-
PP	FL	++	+	++	-	-	++	++	+
	SDL	++	+	++	-	-	++	+	+

'++' High, '+' Moderate, '–' Absent; PB: *Piper betle*, PN: *Piper nigrum*, PL: *Piper longum*, PR: *Piper retrofractum*, PC: *Piper chaba*, PP: *Peperomia pellucida*; FL- Fresh leaf extract, SDL- Shade-dried leaf extract.

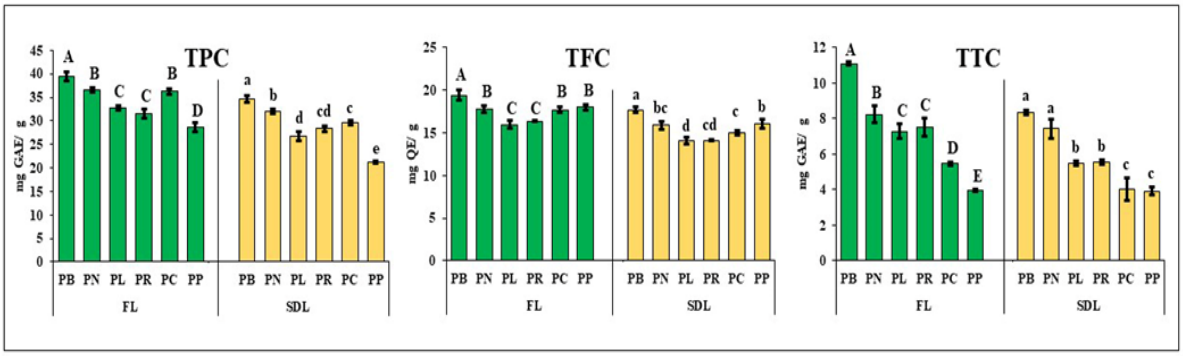

Figure 1: Quantitative estimation of Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Tannin Content (TTC) in different leaf extracts.
PB: *Piper betle*, PN: *Piper nigrum*, PL: *Piper longum*, PR: *Piper retrofractum*, PC: *Piper chaba*, PP: *Peperomia pellucida*; FL- Fresh leaf extract, SDL- Shade-dried leaf extract; GAE: Gallic acid equivalent, QE: Quercetin equivalent.
Different alphabets (uppercase for FL and lowercase for SDL) used in the figure represent significant differences ($p<0.05$) calculated by Tukey's Post-hoc test.

The quantitative estimates of polyphenolic components in the leaf extracts were depicted in figure 1. All the studied plants possessed substantial TPC, TFC and TTC contents and among them, *P. betle* represented the highest yield in every component. In comparison between the leaf samples, FL demonstrated a better yield than SDL in most cases. The highest TPC (mg GAE/g extract) was observed in *P. betle* (FL: 39.50±0.99; SDL: 34.70±0.71) significantly ($p<0.05$) differing from others while the lowest yield was noticed in *Peperomia pellucida* (FL: 28.70±0.99; SDL: 21.20±0.28). The total phenolics of other plants were placed in between these two plants. In flavonoid contents (TFC; mg QE/g extract), the superiority was attained by *P. betle* (FL: 19.40±0.57; SDL: 17.65±0.35) with significant ($p<0.05$) variations from other plants and among them, the lowest flavonoid yield was observed in *P. longum* (FL: 15.96±0.42; SDL: 14.05±0.36).

The flavonoid content in *Peperomia pellucida* was remarkably higher (FL: 17.98±0.32; SDL: 16.08±0.53) but fell short to reach the highest value. The TTC yields (mg GAE/g extract), however, followed a nearly similar trend to TPC with the highest in *P. betle* (FL: 11.08±0.11; SDL: 8.33±0.11) and lowest in *Peperomia pellucida* (FL: 3.93±0.07; SDL: 3.93±0.25) with significant ($p<0.05$) variations in most of the cases. The predominance of phenolic compounds in the leaf tissues was also documented by various studies[21,47] and also from our previous studies.[24] The better yield of phenolics using either FL or SDL remained conflicting as several reports indicated higher yields with FL[26,30] while superior phenolic yields with SDL were also reported by many researchers.[24,35]

However, in our study, the TPC, TFC and TTC contents were greater with FL than SDL pointing towards the inactivation or degradation of certain phytochemicals during drying. The proposition is strengthened by the earlier observations revealing the loss of various essential oils and other compounds during drying of *Arum palaestinum*[30] and *P. betle*[33] leaves. Various studies estimated different phenolic components in *Piper* spp.[3,40,43,45] or *Peperomia pellucida*[22] individually.

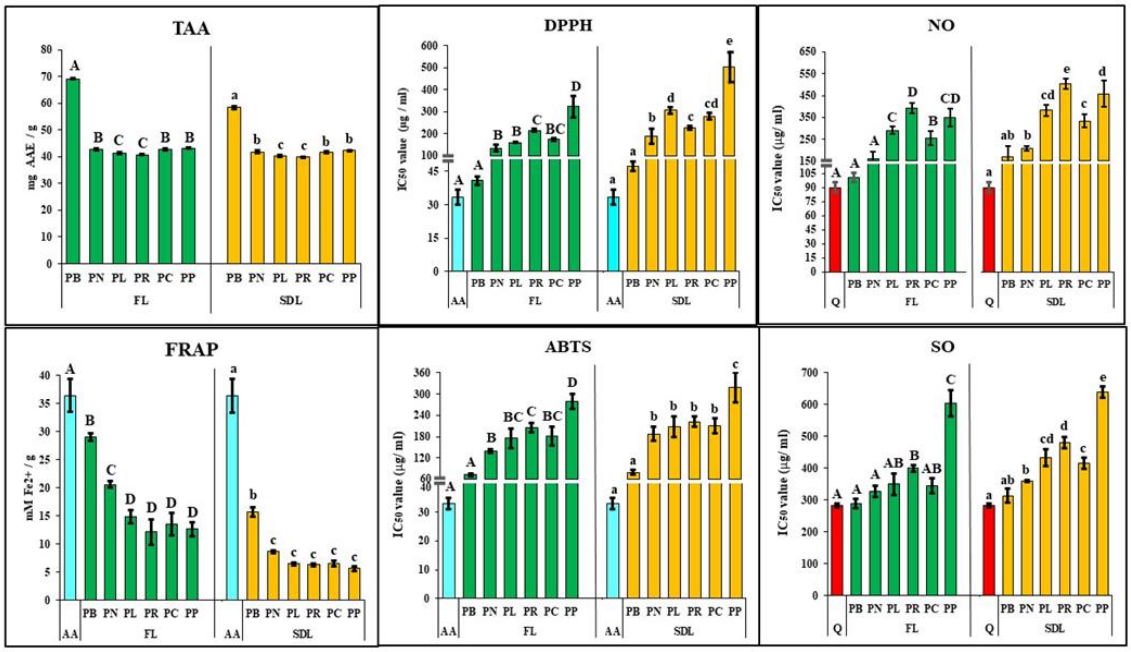

Figure 2: Antioxidant properties of fresh (FL) and shade dried (SDL) leaf extracts by total antioxidant activity (TAA), ferric reducing antioxidant potential (FRAP) and radical scavenging [DPPH, ABTS, Nitric oxide (NO) Superoxide (SO)] assays.

PB: *Piper betle*, PN: *Piper nigrum*, PL: *Piper longum*, PR: *Piper retrofractum*, PC: *Piper chaba*, PP: *Peperomia pellucida*; AA: Ascorbic acid, Q: Quercetin.

Different alphabets (uppercase for FL and lowercase for SDL) used in the figure represent significant differences ($p<0.05$) calculated by Tukey's Post-hoc test

However, this study presented a comparative investigation of phenolic components of six important members of the family Piperaceae. Among the six plants, *P. betle* leaf extracts exhibited the highest TPC, TFC and TTC values while in other members, the estimates varied substantially. Such variations among the plants may depend on various factors like plant genotype, tissue types, growth stages and environmental factors among others.[18]

Antioxidant activities: The antioxidant activities of different plant extracts employing several assays were illustrated in figure 2. The higher estimates of TAA and FRAP are positively correlated with their antioxidant activities while inversely proportional relationships exist between IC_{50} values in the radical scavenging assays (DPPH, ABTS, NO and SO) and their bioactivities.

The antioxidant activities of these plants on an individual basis were reported by various studies,[3,22,34,40,43,45] however, the study made a comparative evaluation among the plants in determining the most efficient one. Our study also encompasses a number of antioxidative assays of different principles as it has been evinced that the effectiveness of antioxidants is better arbitrated through diverse analyses.[28] In all the antioxidant assays, FL extracts showed higher antioxidant activities than SDL and the observation was also in agreement with several studies performed on different plants.[28,35] Such a decrease in activities was attributed to the degradation or modification of antioxidant phytochemicals during drying.[5]

The TAA activity (mg AAE/gm extract) was maximum in FL extract of *P. betle* (69.05±0.21) followed by *Peperomia pellucida* (43.10±0.14), *P. nigrum* (42.70±0.48), *P. chaba* (42.63±0.26), *P. longum* (41.28±0.30) and *P. retrofractum* (40.70±0.14). A similar trend was also observed in SDL extracts but at a lesser amount. In both FL and SDL, *P. betle* activities were significantly ($p<0.05$) higher than the others. In other reports, the TAA activities at varying degrees were also documented in *Piper* spp.[3,34,39] and in *Peperomia pellucida*.[22] The higher TAA activity in the studied plants was corroborated by their rich phenolic contents and that was also evidenced in previous reports.[3,34,42]

The FRAP assay also displayed maximum activity (mM Fe^{2+}/g) in *P. betle* (FL: 36.42±2.96, SDL: 15.77±0.86) with significant variations ($p<0.05$) than the other plants. However, in both FL and SDL extracts, the other plants showed near-identical activities with a nonsignificant ($p>0.05$) relationship excepting the FL extract of *P. nigrum*. Among the different plants, FRAP activity of *P. betle* FL extract was much closer to the reference (positive control) ascorbic acid, though differing significantly ($p<0.05$). The FRAP activity was deduced in *Piper* spp. using different solvent extracts and better activity was shown by many in hydroalcoholic fractions[3,34] while better activity with ethyl acetate was documented by few.[1]

The leading antioxidant activity of *P. betle* was again documented in the radical scavenging assays represented by

their lower IC_{50} values. Results also revealed greater antioxidant activities in FL than SDL. In the DPPH assay, the lowest IC_{50} values (mg/ml) were observed in *P. betle* (FL: 40.77±1.91; SDL: 47.29±2.08), almost approaching the activities of standard ascorbic acid (33.38±3.35) showing nonsignificant ($p>0.05$) variations. Such a strong activity of *P. betle* points toward its greater potential as a source of antioxidants having more antioxidant molecules in the extract. The other leaf extracts manifested weaker activities with much higher IC_{50} values showing significant variations ($p<0.05$) to *P. betle* as well as ascorbic acid.

Similar trends were observed in the ABTS assay with a minimum IC_{50} value in *P. betle* (FL: 72.38±4.50; SDL: 79.73±6.41) with non-significant relations to the positive control ascorbic acid. The other *Piper* spp. demonstrated higher IC_{50} values differing significantly ($p<0.05$) from *P. betle*, however, variations between themselves in SDL extracts were nonsignificant. The highest IC_{50} values were demonstrated by *Peperomia pellucida* (FL: 278.64±20.70; SDL: 318.29±42.45), significantly ($p<0.05$) differing from other samples.

In NO and SO assays, the lowest IC_{50} value among the plants was also observed in *P. betle* FL extract (NO: 100.80±4.98 and SO: 290.04±14.22) followed by *P. nigrum* FL extract (NO: 159.22±35.15 and SO: 327.96±18.64) and the estimates were not statistically significant with the standard quercetin (NO: 90.01±5.72 and SO: 282.51±6.37 respectively).

Assessment of all antioxidant assays revealed the superiority of *P. betle* over the other studied members of the family Piperaceae. The highest activities of *P. betle* in all the antioxidant assays can be interrelated with their higher polyphenolic contents as polyphenols are regarded as one of the major contributors of antioxidants in plants.[42]

Table 2
Growth inhibition zones (mm) showing antibacterial activity of the extracts against different bacterial strains

Plant Extract	Conc. (mg/ disc)	*B. subtilis* MTCC 121		*S. aureus* ATCC 25923		*S. epidermidis* MTCC 3086		*E. coli* MTCC 443		*V. cholerae* N16961		*S.* Typhi C-6953	
		FL	SDL	FL	SDL	FL	SDL	FL	SDL	FL	SDL	FL	SDL
PB	3	19.50± 1.70	11.5± 0.85	14.85± 0.07	8.95± 0.21	17.35± 1.63	13.20± 0.71	20.85± 1.48	11.95± 0.78	15.60± 2.40	8.65± 0.78	15.90± 1.41	11.00± 1.27
	6	23.20± 1.41	17.35± 0.64	16.90± 0.14	13.55± 0.35	19.35± 0.78	14.90± 1.41	22.85± 1.06	14.35± 0.49	17.35± 0.78	12.70± 1.41	17.90± 1.41	13.95± 1.20
	12	24.45± 0.92	19.80± 1.56	18.85± 1.34	14.70± 0.28	20.45± 0.64	15.80± 1.56	24.65± 0.21	16.60± 0.99	18.70± 1.56	16.25± 0.49	20.20± 0.42	15.20± 1.41
PN	3	8.30± 0.71	6.80± 0.08	8.05± 0.35	ND	7.45± 0.64	5.40± 0.57	8.35± 0.78	7.27± 0.75	7.45± 3.46	5.27± 0.46	6.85± 1.34	5.45± 0.07
	6	9.45± 0.35	7.85± 0.89	9.23± 1.07	ND	8.95± 1.20	7.40± 2.12	9.7± 0.28	9.02± 2.37	7.45± 3.46	6.10± 1.49	8.30± 1.84	7.25± 0.92
	12	10.55± 0.78	8.70± 0.92	9.47± 0.38	6.17± 0.55	10.1± 0.71	8.47± 1.42	10.80± 0.42	9.90± 3.37	9.90± 4.10	7.17± 1.52	9.40± 0.71	9.45± 0.78
PL	3	7.20± 0.99	6.58± 0.64	6.97± 0.47	ND	9.15± 3.18	ND	6.30± 0.71	6.15± 0.79	6.45± 2.05	6.40± 0.72	6.85± 1.48	5.87± 0.28
	6	7.85± 1.34	7.80± 1.36	7.90± 0.95	ND	10.55± 3.32	ND	7.15± 0.92	7.35± 0.30	7.70± 2.69	6.93± 0.49	7.35± 0.64	6.95± 1.34
	12	8.95± 1.34	8.93± 1.53	8.47± 1.07	5.53± 0.47	11.95± 1.48	6.30± 0.20	7.95± 0.92	8.85± 0.66	9.05± 2.47	7.37± 0.42	9.40± 0.71	7.25± 0.49
PR	3	6.26± 0.21	ND	ND	ND	ND	ND	6.53± 0.35	ND	ND	ND	ND	ND
	6	7.86± 0.21	ND	6.07± 0.38	6.37± 0.74	7.35± 0.78	6.40± 0.46	7.70± 0.56	6.52± 0.50	7.25± 0.42	ND	6.32± 0.33	ND
	12	8.23± 0.28	7.03± 0.71	8.13± 0.35	7.67± 0.67	8.25± 0.07	7.80± 0.60	8.30± 0.66	7.50± 0.45	8.11± 0.63	ND	7.49± 0.52	ND
PC	3	6.95± 0.64	5.93± 1.14	6.20± 1.44	ND	5.40± 0.57	ND	7.95± 2.76	7.07± 0.21	5.95± 1.35	5.70± 0.36	5.95± 0.21	5.67± 0.38
	6	8.55± 1.06	7.63± 1.14	6.70± 2.00	ND	7.35± 2.05	5.77± 0.35	9.80± 2.83	8.63± 1.42	6.85± 1.48	6.00± 0.95	6.65± 0.92	5.75± 0.21
	12	9.15± 0.07	8.90± 2.63	7.80± 1.56	6.36± 0.55	8.10± 1.70	7.37± 1.32	11.85± 2.19	11.23± 2.99	8.65± 2.33	7.00± 1.11	7.85± 2.76	7.55± 0.49
PP	3	5.85± 0.07	5.65± 0.90	5.90± 0.26	ND	5.85± 0.07	ND	5.95± 0.21	5.67± 0.86	ND	ND	5.50± 0.71	ND
	6	7.00± 0.14	6.25± 1.95	7.90± 0.61	ND	7.10± 0.57	ND	7.20± 0.14	6.42± 0.62	7.70± 0.14	6.70± 0.28	6.45± 2.05	ND
	12	9.35± 0.64	7.45± 2.53	9.57± 0.35	6.17± 0.55	7.45± 0.64	6.33± 0.47	8.60± 0.42	7.75± 1.91	9.55± 0.49	8.85± 1.48	6.95± 2.76	ND
CD value at 0.5% level		0.504	1.005	0.654	0.292	0.766	0.534	0.695	0.988	0.787	1.285	0.777	0.587
Amp	0.006	41.00± 1.10		41.80± 1.20		31.10± 0.60		22.00± 0.60		10.80± 0.20		21.80± 0.80	

PB: *Piper betle*, PN: *Piper nigrum*, PL: *Piper longum*, PR: *Piper retrofractum*, PC: *Piper chaba*, PP: *Peperomia pellucida*; FL- Fresh leaf extract, SDL- Shade-dried leaf extract; Amp: Ampicillin; ND = Not Detected

A positive correlation between the polyphenol contents and antioxidant activities was also established by several previous studies.[23,36] The noticeable activities of *P. nigrum* leaf extract in NO and SO scavenging assays also highlight its potential in combating oxidative damage and warrant further exploration.

Antibacterial activities: The inhibitory activities of bacterial growth by methanolic extracts (3, 6 and 12 mg) of the members of Piperaceae against different bacterial strains following disc diffusion assay, are presented in table 2. The different plant extracts manifested sufficient antibacterial activity in a dose-dependent manner showing maximum activities at the highest concentration (12 mg). The variations between the extract concentrations were significant ($p<0.05$) in most cases. It is well-known that concentration-dependent increments in activities are the hallmark of the specific mode of action by any bioactive compounds. The concentration-dependent increments in antibacterial activities in our study established the specificity of these extracts. Similar observations were noticed in various plant extracts showing distinct bioactivities. [14,32]

The FL extracts were much more efficacious than the SDL extract types in all the studied plants and *P. betle* FL extract (12mg) unveiled the highest inhibitory activity with the inhibition zones (mm) against *E. coli* (24.65±0.21) followed by *B. subtilis* (24.45±0.92), *S. epidermidis* (20.45±0.64), *S.* Typhi (20.20±0.42), *S. aureus* (18.85±1.34) and *V. cholerae* (18.70±1.56). Among the SDL extracts, *P. betle* exhibited the highest activities against the bacterial strains, though failed to reach their FL counterparts. There are divergent reports about the efficacy of FL and SDL in different plant types.[2,10]

In our study, FL extracts were superior to SDL and that was also evidenced by various studies.[8,10] It was demonstrated that several metabolites were either degraded or inactivated during drying[5,30] and in *P. betle*, the loss of essential oils was reported in dried leaves.[33] However, various other plant species showed better antimicrobial activity and phytochemical yield in SDL.[2] In both FL and SDL extracts of *P. betle*, the inhibitory effect was noticeable from the initial concentration (3 mg) and displayed a significant ($p<0.05$) upsurge in activity with increments in extract concentration (Figure 3, Table 2).

The antibacterial efficacy of *P. betle* was documented by several solvent extracts including methanol and the putative phytochemicals in such activities were postulated by various researchers.[2,44] Our study also affirmed the effectiveness of *P. betle* in inhibiting pathogenic bacteria of which few are multidrug-resistant. The greater efficiency of the species can be interrelated with its higher phytochemical contents, particularly polyphenols, as phenolics are implicated in various bioactivities.[44]

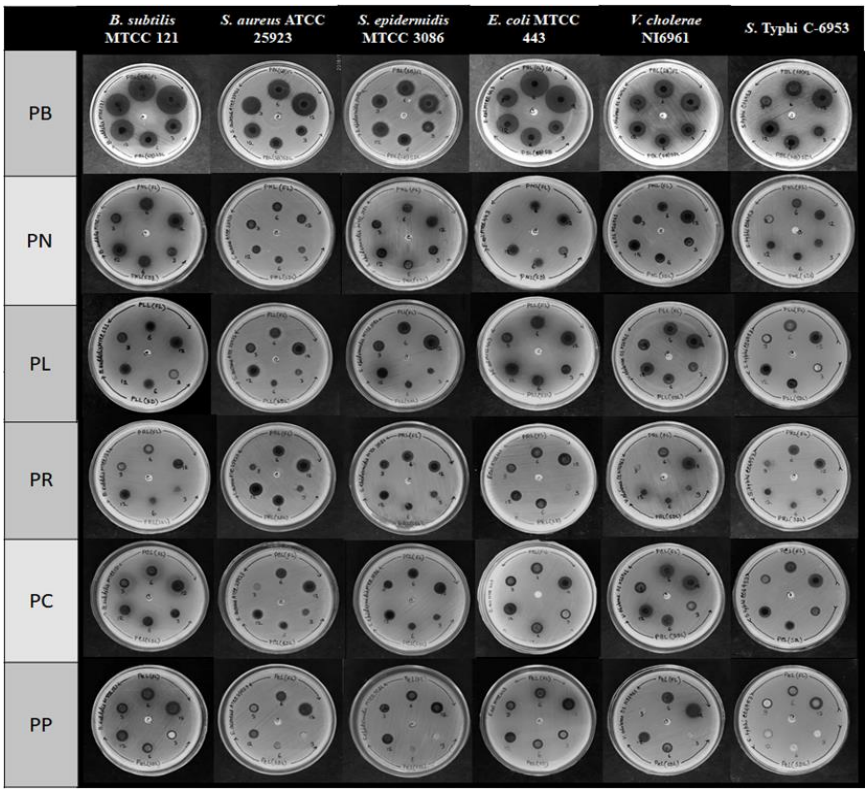

Figure 3: Disc diffusion assay showing antibacterial activities of different members of Piperaceae against different bacterial strains.

PB: *Piper betle*, PN: *Piper nigrum*, PL: *Piper longum*, PR: *Piper retrofractum*, PC: *Piper chaba*, PP: *Peperomia pellucida*; Each plate represents fresh leaf extracts (FL) at upper half and shade-dried leaf extracts (SDL) at the lower half; increments in extract concentrations (3, 6 and 12 mg/ disc) are shown in the clockwise direction; c: vehicle control

Although various solvent extracts showed antibacterial efficacy to different degrees, hydroalcoholic solvents were proven to be a better choice[3,34] and our study also corroborated with that. The remarkable inhibitory effects of *P. nigrum* (inhibition zones ranging from *B. subtilis*:10.55±0.78 to *S. aureus*:9.47±0.38 in FL extract and *E. coli*:9.90±3.37 to *S. aureus*:6.17±0.55 in SDL extract) are worth mentioning, albeit failed to reach the level of *P. betle*. The antibacterial efficacy of *P. nigrum* was evaluated by many workers and found promising activity in its leaves.[24,42,47]

The other three *Piper* species (*P. longum*, *P. retrofractum*, *P. chaba*) and *Peperomia pellucida* demonstrated relatively weaker inhibitory activities than *P. betle* and on many occasions, the lower extract concentrations of these plants, SDL, in particular, failed to produce any inhibition zones. The lowest activities among the six plants were observed in *Peperomia pellucida* in both FL and SDL. The antibacterial activities of the above *Piper* spp.[11,36,43] and *Peperomia pellucida*[25] were reported discretely following various solvent extracts.

The antibacterial efficacies of the plant extracts against different bacteria were determined by their MICs and MBCs (Table 3). Results revealed that FL extracts were with lower MIC and MBC estimates than SDL indicating greater antibacterial potency of the FL extracts.

Among the six plants, *P. betle* demonstrated highest activities against *E. coli* (MIC: 0.58±0.04; MBC: 0.65±0.07) followed by *B. subtilis* (MIC: 0.59±0.04; MBC: 0.67±0.00), *S. epidermidis* and *S. aureus* (MIC: 0.63±0.04; MBC: 0.70±0.00), *V. cholerae* (MIC: 0.73±0.04; MBC: 0.80±0.00) and *S.* Typhi (MIC: 0.90±0.00; MBC: 0.95±0.07) using FL extracts. The SDL extracts of the species were also effective but to a lesser degree than FL. The other studied members of Piperaceae documented much higher MICs and MBCs reflecting their weak antibacterial efficacies, significantly ($p<0.05$) differing from *P. betle*. Ampicillin, used as a positive control, demonstrated the lowest MICs and MBCs among all the treatments with significant ($p<0.05$) differences from all the plant extracts. The lower MIC and MBC values of *P. betle* corroborating its higher antibacterial efficacy were also evidenced by many reports.[3,44]

Table 3
Minimum Inhibitory Concentration (MIC; mg/ml) and Minimum Bactericidal Concentration (MBC; mg/ml) of fresh (FL) and shade dried (SDL) leaf extracts of different members of Piperaceae.

Plant Extracts	Gram-positive						Gram-negative					
	***B. subtilis* MTCC 121**		***S. aureus* ATCC 25923**		***S. epidermidis* MTCC 3086**		***E. coli* MTCC 443**		***V. cholerae* N16961**		***S.* Typhi C-6953**	
	FL	**SDL**	**FL**	**SDL**	**FL**	**SDL**	**FL**	**SDL**	**FL**	**SDL**	**FL**	**SDL**
PB	0.59± 0.04	0.85± 0.07	0.63± 0.04	1.75± 0.35	0.63± 0.04	1.75± 0.35	0.58± 0.04	1.50± 0.71	0.73± 0.04	1.50± 0.00	0.90± 0.00	3.00± 0.00
	0.67± 0.00	0.90± 0.07	0.70± 0.00	2.50± 0.71	0.65± 0.07	2.00± 0.00	0.65± 0.07	1.75± 0.35	0.80± 0.00	2.00± 0.00	0.95± 0.07	3.75± 0.35
PN	4.50± 0.71	7.25± 0.35	7.25± 0.35	9.50± 0.71	6.50± 0.35	9.25± 0.35	5.25± 0.35	7.25± 0.35	9.25± 0.35	12.25± 0.35	12.00± 1.41	12.50± 0.71
	5.75± 0.35	7.50± 0.71	8.00± 0.00	10.75± 0.35	7.25± 0.35	9.50± 0.71	6.00± 0.00	8.00± 0.71	10.00± 0.00	12.50± 0.71	13.00± 1.41	14.75± 0.35
PL	6.50± 0.71	10.25± 0.35	7.50± 0.71	11.25± 0.35	7.25± 0.35	9.25± 0.35	7.25± 0.35	11.00± 0.00	10.25± 0.35	12.25± 0.35	12.50± 0.71	13.75± 0.35
	7.25± 0.35	11.50± 0.71	8.00± 0.71	12.50± 0.35	8.25± 0.35	10.00± 0.00	8.50± 0.35	12.50± 0.71	11.00± 0.00	13.50± 0.71	14.75± 0.35	14.00± 0.71
PR	8.25± 0.35	11.25± 0.71	9.00± 0.71	ND	9.25± 0.35	ND	8.50± 0.71	11.00± 0.35	11.25± 0.35	ND	12.50± 0.71	ND
	9.25± 0.35	12.50± 0.71	10.75± 0.35	ND	9.50± 0.71	ND	9.25± 0.71	12.25± 0.35	12.50± 0.71	ND	14.00± 0.71	ND
PC	7.25± 0.35	10.25± 0.35	9.25± 0.35	14.25± 0.53	9.25± 0.35	13.25± 0.35	8.00± 0.00	10.00± 0.00	10.25± 0.35	12.75± 0.35	13.25± 0.35	13.75± 0.35
	8.25± 0.35	11.50± 0.71	9.75± 0.35	15.75± 0.35	9.50± 0.71	14.00± 0.00	8.75± 0.35	11.50± 0.71	11.50± 0.71	13.50± 0.71	14.75± 0.35	14.75± 0.35
PP	5.25± 0.35	8.25± 0.35	8.75± 0.35	ND	7.25± 0.35	ND	5.50± 0.71	8.00± 0.00	9.50± 0.71	13.00± 0.00	13.00± 0.71	ND
	6.00± 0.00	9.00± 0.00	9.50± 0.71	ND	8.25± 0.35	ND	6.25± 0.35	8.75± 0.35	10.50± 0.71	14.50± 0.71	14.25± 0.35	ND
Amp	0.012±0.01		0.013±0.03		0.011±0.01		0.013±0.01		0.021±0.02		0.028±0.01	
	0.015±0.00		0.014±0.01		0.013±0.03		0.013±0.01		0.023±0.03		0.040±0.00	

PB: *Piper betle*, PN: *Piper nigrum*, PL: *Piper longum*, PR: *Piper retrofractum*, PC: *Piper chaba*, PP: *Peperomia pellucida*; Amp: Ampicillin; ND= Not Detected; for each plant extract the upper and lower row represent MIC and MBC respectively.

To summarize, the study made a comparison between different members of Piperaceae to provide a broader perspective of the antibacterial and antioxidant efficacies of these members among which *P. betle* leaf methanolic extract showed its supremacy over other studied plants.

Conclusion

Plant-derived antioxidants and antimicrobial agents are occupying the centre stage day by day as they are safe and reliable. The study presented a comparative assessment of the polyphenol contents, antioxidative and antibacterial efficacies of six plants belonging to the family Piperaceae using leaf methanolic extracts. In addition, a comparison between fresh and shade dried samples was done to get an idea about their use. Findings from the study revealed significant antioxidant and antibacterial activities in all the plants at varying degrees and fresh leaves manifested better activities than shade dried leaves.

Among these plants, *Piper betle* fresh leaf extract showed the highest antioxidant and antibacterial activities that were corroborated by their rich polyphenolic contents. It is envisioned from the study that the potent antioxidant and antibacterial properties of *P. betle* leaves can be exploited for the isolation of phytochemicals to be used as dietary antioxidants and in therapeutics as antimicrobial(s) of plant origin.

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