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Phytochemical evaluation and *in vitro* antioxidant studies of *Piper nigrum* (L.)

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Abstract
Piper nigrum L. is most extensively used spice all over the world. It consists of major bioactive compound piperine which is an alkaloid. It is widely used in various herbal cough syrups and it is also used in anti-inflammatory, anti-malarial, anti-leukemia treatment. Extraction and isolation of bioactive compound is the new challenge in the field of phytochemistry. The aim of the present study was to evaluate the extractive value of *P. nigrum* L. in different solvent fractions. Phytochemical analysis of fractions was conducted to detect the presence of various phytochemical constituents. The objective of the study was investigated by studying their total phenol and flavonoid content (TPC and TFC), *In vitro* antioxidant parameters (DPPH, FRAP, ABTS, DMPD, and TAA). The plant part used for the medicinal purposes have been investigated and compared with synthetic antioxidants, BHA, BHT and ascorbic acid. The qualitative analysis of crude extract and solvent fractions revealed the presence of various phytochemicals of pharmacological significance. The total phenols and total flavonoids in *Piper* were found to be 178.42±3.29mg/g of GAE and 157.22±5.24mg/g of QE respectively. The highest DPPH scavenging assay showed IC_{50} value of 21.21±0.297µg. There has been a direct relation between Phenol, flavonoid and DPPH scavenging assay. ABTS assay showed IC_{50} value as 8.32±0.164µg and DMPD assay showed IC_{50} value as 59.47±0.470µg.

Keywords: *Piper*, phytochemical, antioxidant, DPPH, ABTS, DMPD, etc.

Introduction
India is indigenous source of many medicinal plants. The information regarding the medicinal plants was even found in the oldest books such as Rigveda [1]. *Piper nigrum* L. (family-Piperaceae) commonly known as Black pepper or black gold is most extensively consumed spice over worldwide, is also known as king of spices and exported all over the world from India [2]. It is widely used in different traditional systems of medicine like Ayurvedic and Unani System of medicines [3, 4]. Besides playing an agent of flavour boosting herbs and spices they are also known for their preservative and medicinal value [5]. In recent times, worldwide interest in the traditional medicine has significantly amplified phytochemical and biological studies at home and abroad due to these medicines being safer and cheaper than the synthetic drugs [6, 7].

Energy demands of the body are met by the utilization of oxygen which also leads to free radical production, resulting in cellular damage [8]. This accumulation may lead to facilitated aging and certain neurological disorders like, cellular damage, mitochondrial cell death and damaged RNA-repair system. Another consequence includes the development oxidative stress in the brain due to some environmental chemical or toxin, giving rise to reactive oxygen species (ROS) as a by-product [9]. Cancer and other pathophysiological conditions are attributed to the production of ROS [10]. Lipids, proteins and DNA are the most vulnerable targets of free radicals giving rise to malondialdehyde, 4-hydroxy nonemal, carbonyl moieties and DNA damage; disturbing the genetic stability [11]. According to numerous studies medicinal plants play an efficient role in chemo-prevention of ROS-related problems.

Secondary metabolites are substances manufactured by plants that make them competitive in their own environment. These small molecules exert a wide range of effects on the plant itself and on other living organisms. Earlier studies put forth a direct correlation between phenolics and antioxidant activities [12, 13].

The present study was undertaken to evaluate extractive value of different solvents depending upon their polarity from non-polar solvents to polar. Preliminary phytochemical analysis along with the quantification of phenol and flavonoid was also done. The study was conducted to find out *In vitro* antioxidant potential of *Piper nigrum* L.

Antioxidant parameters like DPPH, FRAP, ABTS, DMPD, and TAA have been investigated and compared with synthetic antioxidants, BHA, BHT and ascorbic acid.

Materials and Methods

Collection and drying

Fresh peppercorn of *Piper nigrum* L. was collected from Ayurvedic herbal medicinal shop in Nagpur district. The black pepper was washed thoroughly three times with sterile distilled water. The materials were dried under hot air oven at 45°C for 3 hours and powdered in a mechanical grinder. The powdered samples were sealed in separate polythene bags until the time of the extraction.

Extract preparation

Fifty gm of powdered black pepper was extracted successively with several organic solvents such as n-hexane, methanol, petroleum ether, acetone, ethyl acetate, and ethanol. All the solvents were used depending upon their polarity from non-polar solvent to semi polar to polar solvent. For this purpose, 250mL each of solvents were put in Soxhlet extractor for serial fraction until the extract was clear. The extracts were filtered through Whatman No.1 (40) filter paper and then for further use kept in a refrigerator.

Extractive value

The dry powdered plant material of *Piper nigrum* L. was extracted with methanol, ethanol, acetone, chloroform, ethyl acetate, and petroleum ether using a maceration process. 1gm of the coarsely powdered plant material was weighed in a weighing pan and transferred into a dry 250mL conical flask. Then the flask was filled with different solvents (15mL) separately. The flasks were covered with aluminium foil and kept aside for 24hrs at room temperature, shaking frequently. The mixtures were filtered through Whatmann No. 1 filter paper into a 50mL conical flask. After the filtrate has obtained, it was then transferred into a weighed petry plates. The obtained extracts were concentrated to dryness by keeping filtrate for complete evaporation of solvent [14].

The extractive value in percentage was calculated by using following formula and recorded.

Extractive value (%) = Weight of dried extract / Weight of plant material X 100

Preliminary Phytochemical Analysis

Preliminary phytochemical tests were performed as per the standardized procedures [15-17].

Test for Phenols

Ferric chloride test

Take 2mL of crude extract of plants. In that add 3-4 drops of 5% $FeCl_3$ solution. Bluish black colour confirms the presence of phenols.

Test for Flavonoids

Lead acetate test

In 1-2 mL of plant extract, add 1mL of 10% lead acetate solution. Blue colour confirms the presence of flavonoids.

Shinoda test

In aqueous extract of plants some pieces of magnesium metal ribbons were added. Then add few drops of concentrated HCl. After a minute or two appearance of pink, crimson or magenta colour confirms the presence of flavonoids.

Alkaline reagent test

Take 1-2mL of aqueous extract of plants. To this add 2mL of 2% NaOH, it will give intense yellow colour. To this solution add 3mL of 5% HCl, if it turns reaction mixture colourless indicates presence of flavonoids.

Test for Alkaloids

Hager's test

In 1-2mL of plant extract add freshly prepared Hager's reagent (1g picric acid in 100mL hot water). It gives yellow precipitate indicating presence of alkaloids.

Dragendorff's test

Few drops of Dragendorff's reagent were added to the plant extract which gives orange, red or creamy precipitate confirms presence of alkaloids.

Mayer's Test

In plant extract (2mL) when added Mayer's reagent (potassium mercuric iodide), it gives yellow, whitish or creamy precipitate that confirms the presence of alkaloids.

Wagner's test

Take 2mL of plant extract and in this add1mL of Wagner's reagent. Reddish brown precipitate confirms presence of alkaloids.

Test for Steroids

Salkowski test

In 2mL of plant extract, add 2mL chloroform and 1-2mL concentrated sulphuric acid, the reddish-brown colour at the junction of aqueous and chloroform layer indicates presence of steroids.

Test for Tannins

Bramer's Test

In plant extract (diluted) add 2-3 drops of 5% $FeCl_3$ solution. Green or bluish black precipitate indicates presence of tannins.

Lead acetate test

In the 2mL of plant extract add 10% lead acetate solution. Appearance of white precipitation confirms the presence of tannins.

Potassium dichromate test

After addition of 10% potassium dichromate solution in 2mL of plant extract, tannins gives the red or dark colour precipitate.

Gelatin Test

1mL of 1% gelatin solution in 10% NaCl was prepared and added to 2mL of extract. Formation of white precipitate indicates presence of tannins.

Test for Saponins

Foam test

5mL of aqueous extract or 500mg of dry extract was heated and shaken with 5mL distilled water. Foam produced persisted for 10 minutes indicates presence of saponins.

Olive oil test

In 5mL of extract a few drops of olive oil was added and the solution was shaken vigorously. Formation of emulsion confirms presence of saponins

Test for Glycosides

Keller-Kiliani test

To the 2mL of plant extract 1mL of glacial acetic acid was added followed by addition of a few drops of $FeCl_3$ and at the end 1mL of H_2SO_4 added slowly and the solution allowed settling. A reddish-brown colour ring appears at the junction of two layers and the upper layer turns bluish green. These results suggest the presence of cardiac steroidal glycosides (aglycon).

Legal's test

2mL of concentrated extract mixed with 2mL of pyridine, few drops of 2% freshly prepared sodium nitroprusside solution and few drops of 20% NaOH. Blue or pink coloration indicates presence of aglycon moiety.

Liebermann's test

2mL of extract was mixed with 2mL of acetic anhydride. Solution was heated then after cooling a few drops of concentrated H_2SO_4 was added from the sides of the test tube. Appearance of the blue or green colour precipitate indicates presence of glycosides.

Test for Terpenoids

Acetic anhydride test

2mL of acetic anhydride was added to 2mL of extract followed by addition of 2-3 drops of concentrated H_2SO_4. The deep red coloration indicated the presence of terpenoids.

Chloroform test

In this test, to the 2mL of plant extract 2mL chloroform was added and the solution was evaporated in a water bath to make its concentrate. Later 3mL H_2SO_4 was added and the solution was boiled. The grey colour will appear when the terpenoids are present.

Total Phenol content (TPC)

Folin-Ciocalteu method was used to determine the total phenolic contents of methanolic extract by UV spectrophotometer [18]. In brief, 2.5mL of 10% Folin-ciocalteu reagent and 2mL of 7.5% sodium carbonate were added to 500µg of extract. The reaction mixture was incubated at 45 °C for 45 minutes and the blue coloured phosphomolybdic /phosphotungstic acid complex was measured at 760nm. The TPC value was calculated using gallic acid standard and presented as mg GAE/g of extract. The analysis was performed in triplicates.

Total Flavonoid Content (TFC)

Aluminium chloride colorimetric method of was used to analyse the flavonoid content of methanolic extract of *P. nigrum* L. fruits [19] with slight modification. 200µL of 5% sodium nitrite was added to 200µg of extract and allowed to react for 5min. 300µL of 10% aluminium chloride was added to the mixture and after 5min, 2mL of 1M NaOH was added and the absorbance of the orange-red aluminium complex was taken at 510nm. Quercetin (standard flavonoid compound) was used to construct a standard calibration curve. The TFC value was calculated using the quercetin standard and presented as mg QE/g of extract. All tests were carried out in triplicates.

***In vitro* antioxidant assays**

2, 2-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging assay

The antioxidant activity of crude extracts and various purified compounds from the plants can be ascertained using DPPH assay. The assay was conducted using the procedure prescribed by Tuba and Gulcin (2008), [20] with required alterations according to Kedare & Singh (2011) [21]. Purple coloured $DPPH^{\bullet}$ solution was prepared in methanol till the absorbance was achieved to 0.950±0.025 at 517nm. 3mL methanol was added to 4, 8, 12, 16 and 20µg of plant extract followed by addition of 1mL $DPPH^{\bullet}$ solution. The reaction mixture was vortexed and incubated at RT for 30minutes in the dark. Absorbance of the pale-yellow hydrazine product was measured at 517nm with blank containing only methanol. IC_{50} values of samples were calculated along with the ascorbic acid, BHA and BHT standards.

2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid radical ($ABTS^{\bullet+}$) scavenging assay

$ABTS^{\bullet+}$ scavenging activity of the plant extracts was determined by first generating ABTS radical cation ($ABTS^{\bullet+}$) by mixing 7mM ABTS and 2.45mM potassium persulfate in deionized water and kept at room temperature overnight (12-16hrs) and finally the absorbance of $ABTS^{\bullet+}$ was adjusted to 0.750±0.025 at 734nm. Later, 3mL methanol and 1mL $ABTS^{\bullet+}$ solution was added to 2, 4, 6, 8 and 10µg of plant extract. After 10minutes of incubation at RT, the absorbance of decolorized/ scavenged $ABTS^{\bullet+}$ was measured at 734nm with blank containing only methanol [22]. IC_{50} values of samples were calculated along with the ascorbic acid, BHA and BHT standards.

N, N-dimethyl-p-phenylenediamine dihydrochloride radicle ($DMPD^{\bullet+}$) scavenging assay

DMPD cation radical ($DMPD^{\bullet+}$) was produced by the reaction of DMPD with ferric chloride in acetate buffer. For this, 500µL of 100mM DMPD was added to 50mL of 0.1M acetate buffer (pH 5.3) and then 100µL of ferric chloride was added to produce $DMPD^{\bullet+}$. Finally, the absorbance of this solution was adjusted by using acetate buffer or ferric chloride to 0.900±0.100 at 505nm. Then, 2mL of the $DMPD^{\bullet+}$ solution was added to 10, 20, 30, 40 and 50µL of extract and incubated at RT for 10 minutes and the discoloration is noted at 505nm by using acetate buffer as blank [23]. IC_{50} values of samples were calculated and then the results were compared along with the standards like ascorbic acid, BHA and BHT.

Ferric ion reducing ($Fe^{3+} \rightarrow Fe^{2+}$) antioxidant power (FRAP) assay

The FRAP assay for formation of intense Perl's Prussian blue complex of the Fe^{2+} - ferricyanide complexes from yellow coloured Fe^{3+} - ferricyanide complexes by the reducing power of plant extract was also performed [20]. Briefly, different concentrations of plant extracts (5, 10, 20, 30, 40 and 60µg) was taken and reacted with 2.5mL of 1% potassium ferricyanide in 2.5mL sodium phosphate buffer (0.2M; pH 6.6) and incubated at 50 °C for 20minutes. Then 2.5mL of 10% trichloroacetic acid was added. 2.5mL of this reaction mixture was taken then diluted with 2.5mL distilled water and 0.5mL of 0.1% ferric chloride was added. The absorption of the complex was measured at 700nm.

Phosphomolybdenum method for total antioxidant activity (TAA)

In this method different concentrations of plant extract (20, 40, 60, 80 and 100µg) were reacted with 5.4mL phosphomolybdenum reagent, made up of 28mM sodium phosphate, 4mM ammonium molybdate and 0.6M sulfuric

acid. The reaction mixture was then incubated at high temperature of 95 °C for 90min, cooled at room temperature and subsequently the absorbance of green phosphate/Mo(V) complex formed was noted at 695nm [24].

Statistical Analysis

All the analyses were performed in triplicate experiments (N=3). The results of TPC, TFC, TAA and FRAP were calculated as mean of observations ± SD. Whereas for DPPH, ABTS and DMPD scavenging activities, the means of IC_{50}±SD was calculated.

Results and Discussion

Extractive value

Every plant has hundreds of thousands of compounds, and because of these compounds every plant has medicinal values. Extractive value determines the number of active constituents in a particular medicinal plant when extracted with a solvent. It is used for evaluation of crude drugs when they cannot be estimated by any other method. Any medicinal plant or plant part with medicinal and nutraceutical benefits cannot be consumed whole. The crude drugs can be evaluated using the extractive value procedure. It ascertains the quality as well as purity of the drug. The extractive value of extracts of *Piper nigrum* L. was investigated and represented in Table 1 & Fig. 1. From the present study, it was found that among all the six solvents used methanol has the highest potential (12.07±0.351) to extract maximum secondary metabolites from the *Piper nigrum* L. whereas the lowest yield was obtained from the petroleum ether extract (3.23±0.379). According to recent studies, extracts from *Piper nigrum* L. may have therapeutic benefits by increasing the bioavailability of nutrients and drugs. This has prompted more research in pharmacology to find better ways to treat patients [25]. It has been discovered that *Piper nigrum* L. extracts contain antibacterial, anti-inflammatory, and hypolipidemic qualities, indicating possible uses in the treatment of chronic illnesses [26]. Evidence of Piper nigrum's medicinal qualities supports its extraction value in the pharmaceutical and nutraceutical industries.

Table 1: Percent extractive value of *Piper nigrum* L.

Solvent	% EV Mean ± SD
P. Ether	3.23±0.379
Acetone	6.47±0.208
Ethyl acetate	6.70±0.200
Chloroform	6.90±0.173
Ethanol	8.57±0.252
Methanol	12.07±0.351

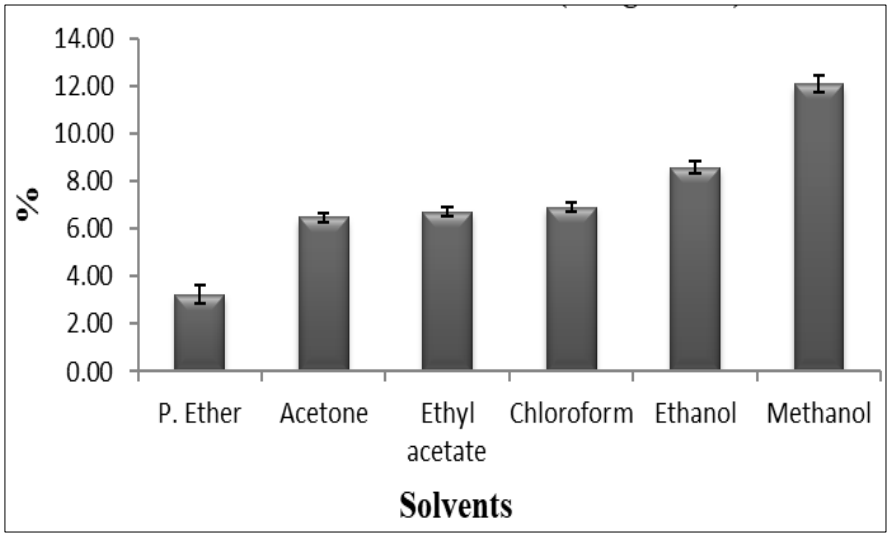

Fig 1: Percent extractive value of *P. nigrum* L.

Preliminary phytochemical screening

Piper nigrum L. extracts from plants are rich in secondary metabolites with therapeutic effects suggested by Indian tradition. These therapeutic effects are displayed due to the presence of numerous phytochemicals in it. Presence of these phytochemicals manifested as the result of preliminary qualitative phytochemical screening. We have tested the availability of phenols, flavonoids, glycosides, alkaloids, terpenoids, tannins, steroids and saponins. Excluding terpenoids, all the other phytochemicals were present in the extracts. Among all the solvents Methanol has potentially the best solvent for the presence of phytochemicals as the extractive value data also suggested. The results of phytochemical analysis are presented in Table 2.

Alkaloids, flavonoids, terpenes, and essential oils are all present in *Piper nigrum* L of these, piperine has been studied the most [27]. The pungent taste of black pepper is due to Piperine. Piperine in black pepper has anti-inflammatory, antioxidant, anticancer effects [28]. *Piper nigrum* L. and its phytochemicals have health benefits supported by research. Ticktin *et al*. (2006) [29] found *Piper nigrum* L. extracts have strong antioxidant properties, helping fight diseases like cancer and heart disease. The presence of flavonoids and other phenolic compounds may contribute to these antioxidant properties. Tannins have the tendency of binding to proteins and blocking the protein synthesis. Therefore, the current preliminary phytochemicals screening might prove valuable in the detection and further quantitative analysis of these therapeutically important compounds [30]. *Piper nigrum* L. is essential for culinary use and contains bioactive compounds with health potential. Further research can deepen its applications in food, medicine, and health.

Table 2: Phytochemical screening of *Piper nigrum* L.

	Chemical tests	Methanol	Ethanol	Acetone	Ethyl acetate	Petroleum ether	Chloroform
Flavonoids							
1)	Lead Acetate Test	+	+	-	-	-	-
2)	Alkaline Reagent Test	-	-		-	-	-
3)	Shinoda Test	+	+	-	+	+	-
Phenol							
1)	Ferric Chloride Test	+	+	+	+	-	-
Tannin							
1)	Bramer's Test	-	+	+	-	-	-
2)	Gelatin Test	+	+	-	+	-	-
3)	Potassium dichromate test	+	-	+	+	-	+
4)	Lead acetate test	+	+	+	+	+	+
Glycosides							
1)	Keller-killiani's test	+	-	+	+	+	+
2)	Legal Test	+	-	-	-	+	-
3)	Liebermann's Test	-	+	-	-	-	-
Saponins							
1)	Foam Test	-	-	-	-	-	-
2)	Olive oil Test	+	+	+	+	+	-
Alkaloids							
1)	Hager's test	+	+	+	+	+	+
2)	Dragendorff's test	+	+	-	+	-	-
3)	Mayer's Test	+	+	-	+	-	-
4)	Wagner's test	+	+	+	+	+	-
Steroids							
1)	Salkowski's Test	+	+	+	+	+	-
Terpenoids							
1)	Chloroform Test	-	-	-	-	-	-
2)	Acetic anhydride test	-	-	-	-	-	-

Total Phenolic Content

Piper nigrum L., commonly known as black pepper, is not only valued for its flavour profile but also for its rich phytochemical composition, particularly in terms of phenolic compounds and flavonoids. The analysis of total phenolic and flavonoid content in *Piper nigrum* L. is significant as these compounds play crucial roles in its antioxidant properties and potential health benefits.

The total phenolic contents of different extracts of *Piper nigrum* L. were determined by UV spectrophotometric method. Table 3, Figure 2 showed the total phenolic content in *Piper*. The highest total content of phenolic compounds was found in ethyl acetate extract (178.42±3.287 mg/g) of gallic acid equivalent followed by methanol (149.74±1.823 mg/g) whereas petroleum ether extract had the least phenolic content i.e. 90.35±2.662 mg/g of gallic acid equivalent. The given values are mean ± SD of three different determinations.

Total phenolic content (TPC) serves as an important indicator of the antioxidant potential of plant-based foods. Phenolic compounds are known for their ability to scavenge free radicals and mitigate oxidative stress, which is implicated in various chronic diseases [31]. Studies have consistently demonstrated that *Piper nigrum* L. contains a high concentration of phenolic compounds, primarily due to its diverse array of biochemical constituents including flavonoids, tannins, and phenolic acids.

Phenolic and flavonoid compounds possess antimicrobial, anti-allergic, anticancer, and anti-inflammatory properties. They are crucial for reproduction and growth, serving as a defence against pathogens [30] making their quantitative analysis essential for drug quality assessment.

Total Flavonoid Content

The content of total flavonoids from *Piper nigrum* L. varied from 18.95±4.660 (petroleum ether extract) to 157.22±5.238 mg/g (ethyl acetate extract) of quercetin equivalents/g (Table 4, Figure 3). The phenolic and flavonoid compounds in different extracts vary due to the polarities of compounds in the raw material, which are crucial secondary metabolites consisting of cyclized diphenylpropane structure [32] and are secreted in plants in the form of pigments in flowers, fruits, seeds, and leaves, for recruiting pollinators and seed dispersers, in defence as feeding deterrent and antimicrobial agents, and in UV protection. Flavonoids show antioxidant activity mostly by chelating free radical forming metal ions like Fe^{2+} by formation of coordinate bonds with them by its –C=O and –OH groups [33].

Flavonoids, a group of polyphenolic compounds found in plants, possess diverse chemical and biological activities, including radical scavenging properties, essential for normal growth and defence against infection and injury. Various studies show *Piper nigrum* L. has high flavonoid content, especially quercetin and kaempferol derivatives [34].

Various factors affect the TFC in *Piper nigrum* L. It includes extraction method, plant variety and harvest time. Ethanol and methanol have shown to effectively extract flavonoids, resulting in higher yields compared to other such solvents. By gaining an understanding of these variables, extraction procedures can be optimized, enhancing the effectiveness and quality of *Piper nigrum* L. extracts used in therapeutic and dietary applications.

Table 3: Total Phenol in *P. nigrum* L. (mg/g of GAE)

Solvents	Mean± SD
P. Ether	90.35±2.662
E. acetate	178.42±3.287
Acetone	108.68±2.089
Methanol	149.74±1.823

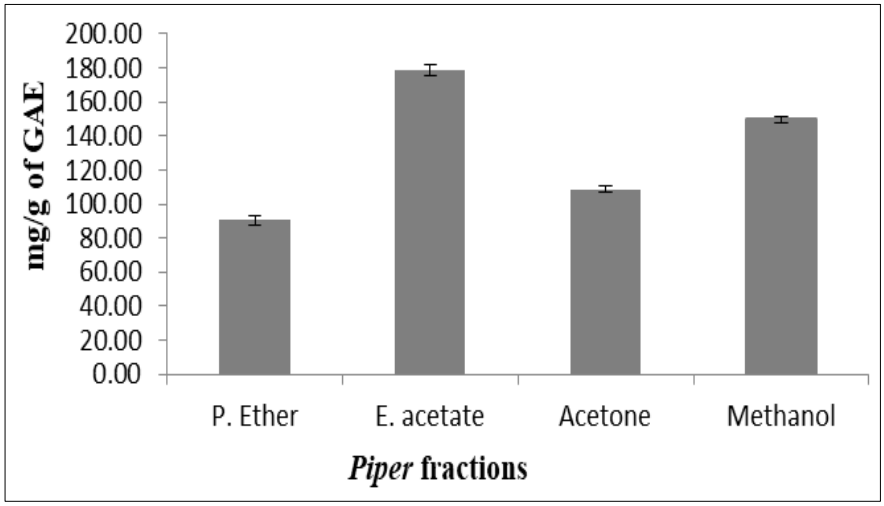

Fig 2: Total Phenol in *Piper nigrum* L. (mg/g of GAE)

Table 4: Total Flavonoid in *Piper nigrum* L. (mg/g of QE)

Solvents	Mean ± SD
P. Ether	18.95±4.660
E. acetate	157.22±5.238
Acetone	44.88±2.138
Methanol	72.65±7.710

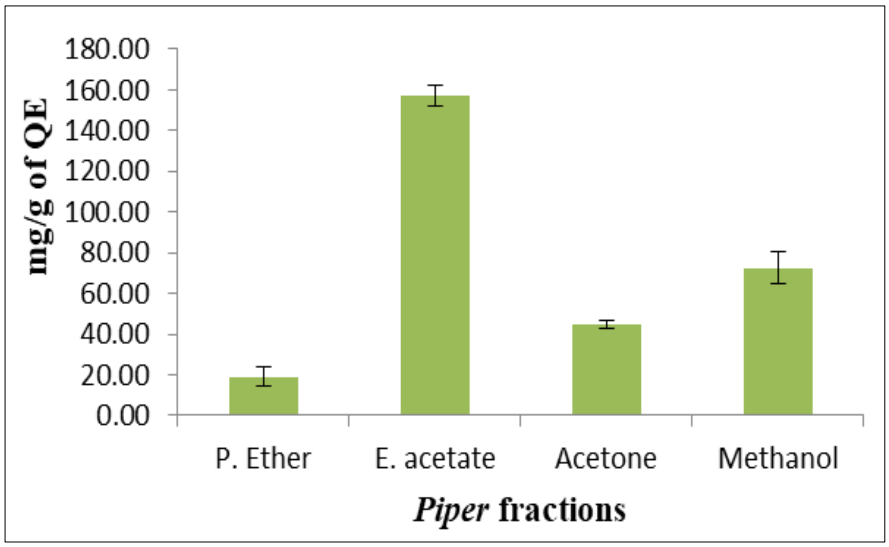

Fig 3: Total Flavonoid in *Piper nigrum* L. (mg/g of QE)

***In vitro* antioxidant capacity**

Several *In vitro* tests were adopted to evaluate the antioxidant activity of solvent extracts at different concentrations. High antioxidant values in *Piper nigrum* L. can be related to the high concentrations of phenols and flavonoids. Antioxidants are a major primary defence system against ROS and free radicals [35]. To check this hypothesis, we studied the antioxidant properties of *Piper* by DPPH, ABTS and DMPD free radical scavenging assay, FRAP and phospho-molybdenum antioxidant (TAA) assay. The results obtained were compared with artificial antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and ascorbic acid.

2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH˙) scavenging assay

The DPPH radical scavenging activity is based on the reduction of purple coloured DPPH˙ to its yellow hydrazine product ($DPPH^{-H}$) by hydrogen or electron donating capacity of the plant compounds [36]. The study revealed that ethyl acetate fraction has the best scavenging activity with IC_{50} value 21.21±0.296µg (Table 5, Figure 4) among the other fractions studied. Other workers also studied the DPPH scavenging activity in *Piper*. Gulcin (2005) found 48±5.18% activity in ethanol extract of black pepper [37]. Nahak and Sahu (2011) found 53.07±0.04% activity in methanol extract at 50 µg/ml [38].

Table 5: IC_{50} value of *Piper nigrum* L. Fractions

Std/Fractions	IC_{50}±SD
BHA	4.30±0.125
Ascorbic Acid	5.24±0.29
BHT	13.7±0.307
E. Acetate	21.21±0.296
Methanol	29.79±0.973
Acetone	47.62±2.980
P. Ether	88.71±2.537

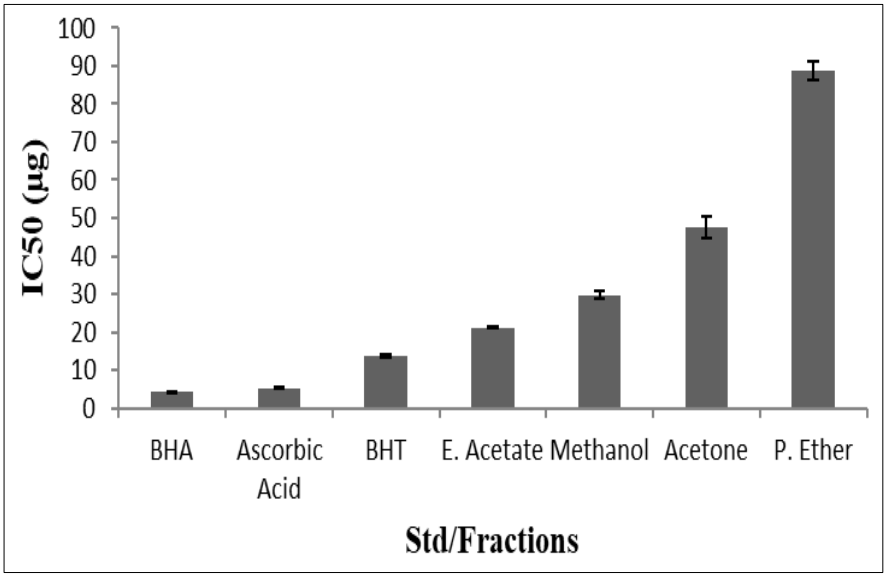

Fig 4: IC_{50} value of *Piper nigrum* L. Fractions

Correlation between Phenol, flavonoid and DPPH Antioxidant activity

Polyphenols are important plant compounds that show anti-oxidant activity. Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids and flavonoids [39]. It is reported that the phenolics are responsible for the variation in the anti-oxidant activity of the plant [40]. They exhibit anti-oxidant activity by inactivating lipid free radicals or preventing decomposition of hydro peroxides into free radicals [41, 42]. From the above studies, it can be concluded that there is a correlation between phenol, flavonoid and DPPH antioxidant. As the phenol and flavonoid content was found to be maximum in ethyl acetate fraction of *Piper*, the IC_{50} value also showed the maximum scavenging potential of ethyl acetate fraction in DPPH assay (Figure 5).

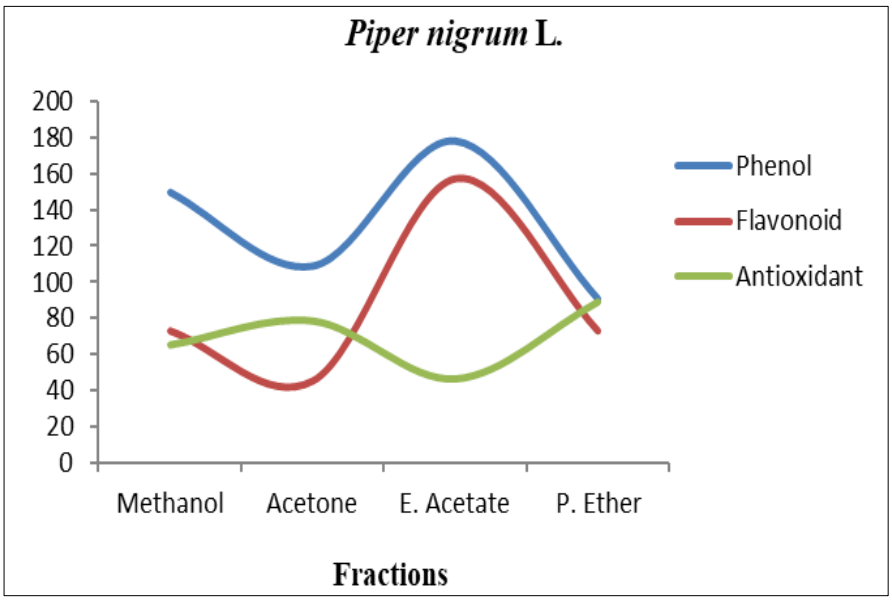

Fig 5: Correlation between Phenol, flavonoid and Antioxidant activity

2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid radical ($ABTS^{\bullet+}$) scavenging assay

ABTS radical cation decolorization assay is also another significant total antioxidant activity screening method. Phenols and flavonoids contribute to quality and nutritional value in terms of modifying colour, taste, aroma and flavour. The phenolic compounds act as antioxidant agents. $ABTS^{\bullet+}$ scavenging activity demonstrates the capacity of the phytochemicals to neutralize ROS by hydrogen atom transfer (HAT) or single electron transfer (SET) mechanism. The methanolic extract of *Piper* did not have the best HAT or SET capacity against synthetic standards. It shows $ABTS^{\bullet+}$ scavenging with IC_{50} value 8.32±0.164µg (Table no. 6, Fig. 6).

Table 6: ABTS Radical cation decolorization assay (IC_{50})

Std/Plants	Mean IC50±SD
Ascorbic acid	2.51±0.125
BHA	2.14±0.066
BHT	3.10±0.833
Piper	8.32±0.164

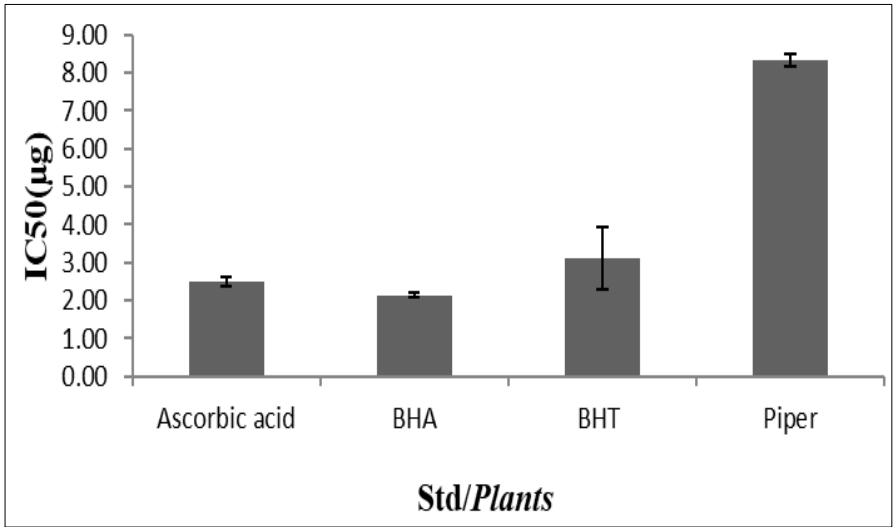

Fig 6: ABTS Radical cation decolorization Assay (IC_{50})

N, N-dimethyl-p-phenylenediamine dihydrochloride radicle ($DMPD^{\bullet+}$) scavenging assay

When the compound N,N-dimethyl-ρ-phenylenediamine (DMPD) is in the presence of a suitable oxidant solution, a coloured radical cation is formed (DMPD•+). Antioxidant compounds, which can transfer a hydrogen atom to DMPD•+ cause a discoloration of the solution. In our study, the obtained highest $DMPD^{\bullet+}$ scavenging activity of *Piper* methanolic extract indicates that it contains hydrophilic antioxidants in abundance. It showed the IC_{50} value of 59.47±0.470µg.

Table 7: N, N-dimethyl-ρ-phenylenediamine (DMPD) assay

Std/Plants	IC50±SD
Ascorbic acid	14.9±1.683
BHA	46.92±1.280
Piper	59.47±0.470

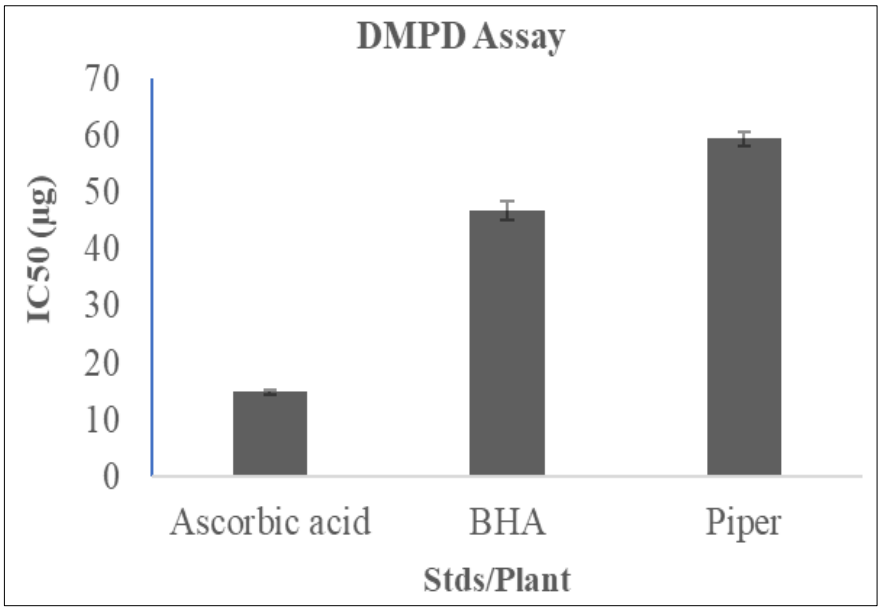

Fig 7: N, N-dimethyl-ρ-phenylenediamine (DMPD) assay

Ferric ion reducing ($Fe^{3+} \rightarrow Fe^{2+}$) antioxidant power (FRAP) assay

The reducing capacity of the plant extracts was determined by FRAP assay which is based on the single electron transfer mechanism. High reducing capacity of plant extract is indicative of its potential antioxidant capacity. The reducing power assay of *Piper* may serve as a significant index of its potential antioxidant activity. In fact, the presence of reductants (antioxidant) in samples causes the reduction of Fe^{3+}/ferracyanide complex to ferrous form [43]. Therefore Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm [44]. Table no. 8, Fig. 8 revealed the reducing power activity of *Piper* at various concentrations. It can be observed that their reducing power increased with increasing of the concentration of each extract (5–60µg/ml).

Table 8: FRAP values for Std/Plant

Conc. (µg)	Ascorbic acid	BHA	BHT	*Piper*
5	0.104±0.011	0.045±0.005	0.037±0.003	0.035±0.001
10	0.201±0.004	0.088±0.005	0.080±0.001	0.082±0.001
20	0.400±0.005	0.155±0.019	0.156±0.001	0.163±0.001
40	0.807±0.013	0.333±0.003	0.315±0.008	0.275±0.001
60	1.148±0.025	0.487±0.004	0.450±0.006	0.365±0.003

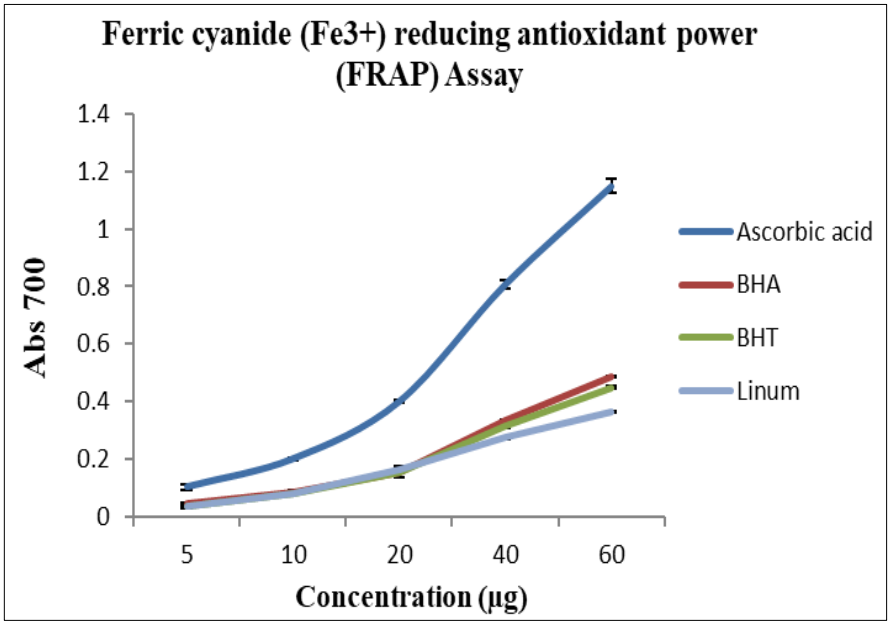

Fig 8: FRAP values for Std/Plant

Phospho-molybdenum method for total antioxidant activity (TAA)

The Phospho-molybdenum assay is a quantitative method to evaluate the antioxidant capacity. This method assesses both water-soluble and fat-soluble antioxidants from the plant extract. Figure 9 reveals the total antioxidant activity of *Piper* at various concentrations.

It can be observed that their reducing power increased with the increasing of the concentration of each extract (20–100µg/ml), (Prieto *et al*., 1999) [24].

Table 9: TAA for Std/Plant

Conc. (µg)	Ascorbic Acid	BHA	BHT	*Piper*
20	0.10±0.007	0.09±0.007	0.07±0.002	0.04±0.002
40	0.23±0.006	0.20±0.010	0.14±0.003	0.10±0.003
60	0.36±0.008	0.28±0.015	0.20±0.006	0.18±0.008
80	0.49±0.010	0.43±0.004	0.25±0.004	0.24±0.002
100	0.64±0.005	0.57±0.021	0.32±0.006	0.27±0.011

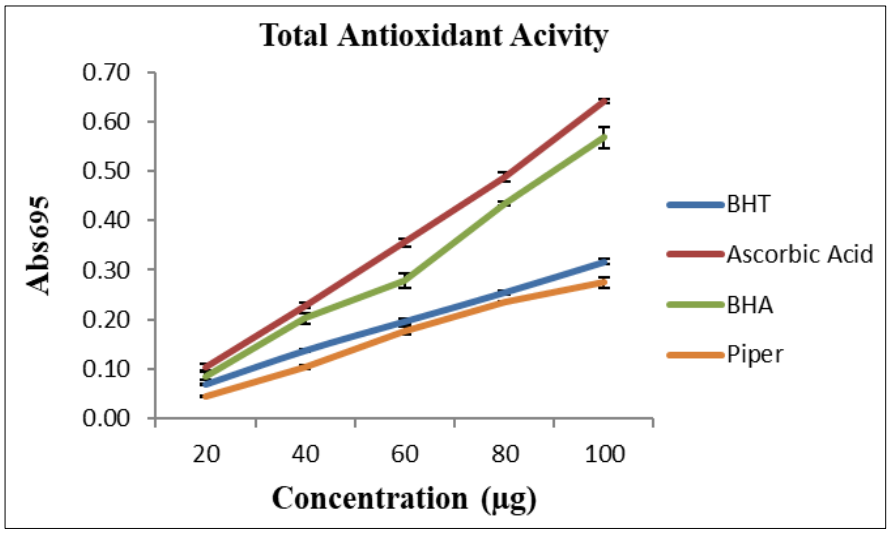

Fig 9: TAA for Std/Plants

Conclusion

Phytochemical analysis of *Piper nigrum* L. reveals a variety of bioactive compounds. The result showed good source of phytochemical constituents. To standardize extraction techniques and comprehend their mechanisms and possible interactions, more research is required. Piper nigrum's phenolic and flavonoid content suggests its potential as an antioxidant food ingredient. Further research is needed to understand its health benefits and standardize extraction methods. The findings suggest its application in functional foods and nutraceuticals, highlighting its possible role in disease prevention and management. Flavonoids act as the scavengers of free radicals and these are the phyto-constituents to be focused on for investigation of many biological activities. Antioxidant properties can be used as easily accessible source of natural antioxidants and as a possible food supplement as well as in the pharmaceutical industry.

Conflict of Interest

Authors declare that they do not have any Conflict of Interest.

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