Evaluation of Antioxidant Assays in Hydroalcoholic Extract and Its Fractions With HPLC-DAD Screening and Quantification of Analytical Marker Compounds in *Piper longum* Fruit

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Abstract:

Piper longum fruit extracts and fractions were evaluated for their Phytochemical constituents and antioxidant Potency. Water extracts (PWA) showed higher activity in DPPH (IC_{50} 0.207±0.011 mg/ml), ABTS (IC_{50} 0.636±0.083 mg/ml), FRAP assay (20.806±0.658 µmol/10 mg AAE), than Hydro-alcoholic extracts (PHA). PHA showed higher activity in TRA assay (IC_{50}1.793±0.057 mg/ml) and TAC (1.279±0.244 µmol/mL AAE) assay with higher content of piperine (0.565±0.005%) and piperlongumine (0.061±0.001%), quantified simultaneously by HPLC: DAD. Among the hydroalcoholic fractionation of PHA, Chloroform fraction (PCL) showed higher yield (2.388±0.168%) with higher content of piperine (14.569±0.113%). Lowest yield was found in Hexane fraction (PH) (0.434±0.007%) while high piperlongumine (2.883±0.028) content. Ethyl acetate fraction (PEA) showed higher activity in DPPH (IC_{50} 0.058±0.001 mg/ml), ABTS (IC_{50} 0.413±0.117 mg/ml) and FRAP (24.234±4.052µmol/mL AAE). Chloroform fraction (PCL) showed higher activity in TRA (IC_{50} 0.375±0.037 mg/ml) and TAC (2.946±0.338 µmol AAE.) hence it appears that higher content of piperine and piperlongumine are not responsible for increased antioxidant potency in *Piper longum* fruits against DPPH, ABTS and FRAP assay.

Keywords*: Piper longum*, HPLC:DAD, Piperine, Piperlongumine, fractionations, Antioxidant.

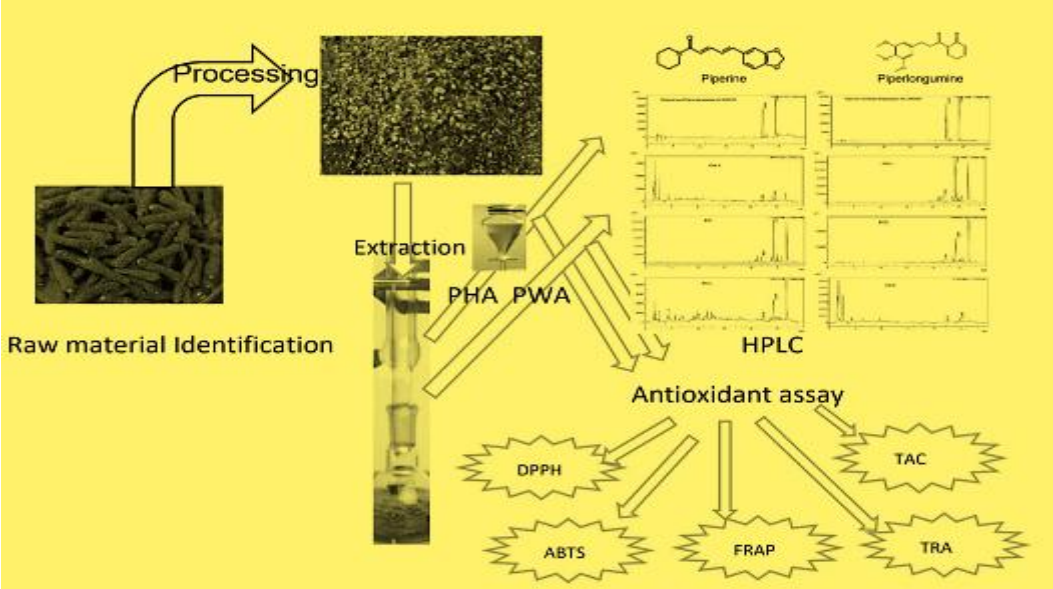

Figure 1: Graphical Representation of Abstract

1. Introduction.

Piperaceae family includes 4000 species. It is divided into five genera of which Piper and Peperomia are major two genera of the family[1]. Piper species are known for their commercial values, important of them are *Piper nigram, Piper longum* and *Piper betle* known for their high commercial value. *Piper longum* is one of the well-known species of Piperaceae family used traditionally in various countries viz. India, Malaysia, Singapore and other South Asian countries[1, 2] as an ingredient of food and spices[1, 2]. Hippocrates categorized it as a medicinal plant, as anti-tubercular, for respiratory tract infection, as antitumor, antidiabetic, cardio protective, hepatoprotective, antiapoptosis, antioxidant, anti-inflammatory, antiarthritic, myocardial protective, antifertility activity etc[3,4]. Some of compounds from the plant piperine, piperlongumine, pipermonaline, sesamin, diaeudesmin piperpongumine, piperdindecalidine are reported to treat chronic bronchitis, asthma, constipation, gonorrhea, tongue paralysis, diarrhea, cholera, malaria, hepatitis etc.[3]. Fruits of *Piper longum* act as analgesic for muscular pain and inflammation, leprosy, anti-dysenteric against fevers, jaundice, as an immunostimulant etc. The present work is focused on selection of extract and fractions of *Piper longum* fruit for potency against oxidation in a different assay based analysis and looking for the content of analytical compounds.

2. Results and discussion

2.1.Piper longum fruit extractions and fractionation.

On extraction of dried powdered material total extract yielded PWA and PHA about 14.157 ± 0.577% and 19.546 ± 0.161% respectively. PHA showed a higher yield as compared with PWA. PHA fractionation yield for different solvent was PH 0.434 ± 0.007%, PCL 2.388 ± 0.168%, PEA 1.956 ± 0.068% and for the material remaining PRE 12.448 ± 0.098%.

2.2.HPLC: DAD screening for quantification of Piperine and piperlongumine.

Screening and quantification of two analytical marker compounds in extracts and fractions was done by HPLC-DAD. Chromatogram of the standard, extracts and fractions are shown in Fig 1. The PWA and PHA showed presence of both analytical marker compounds piperine and piperlongumine as shown in Fig1. Content of piperine (0.565 ± 0.005) and piperlongumine (0.060 ± 0.001%) were found higher in PHA as compared to content of Piperine (0.051 ± 0.001%) and piperlongumine (0.008 ± 0.005) in PWA. Among the fractions both analytical markers compounds were present in all fractions as shown in Table 3. Piperine (14.569 ± 0.114%) content was found higher in PCL whereas piperlongumine (2.883 ± 0.021%) content was higher in PH. PEA showed moderate presence of piperlongumine (0.463 ± 0.021%) and piperine (3.804 ± 0.029%) as compared to PH and PCL. Piperine and piperlongumine were present in PRE in trace amount as shown in Table 1.

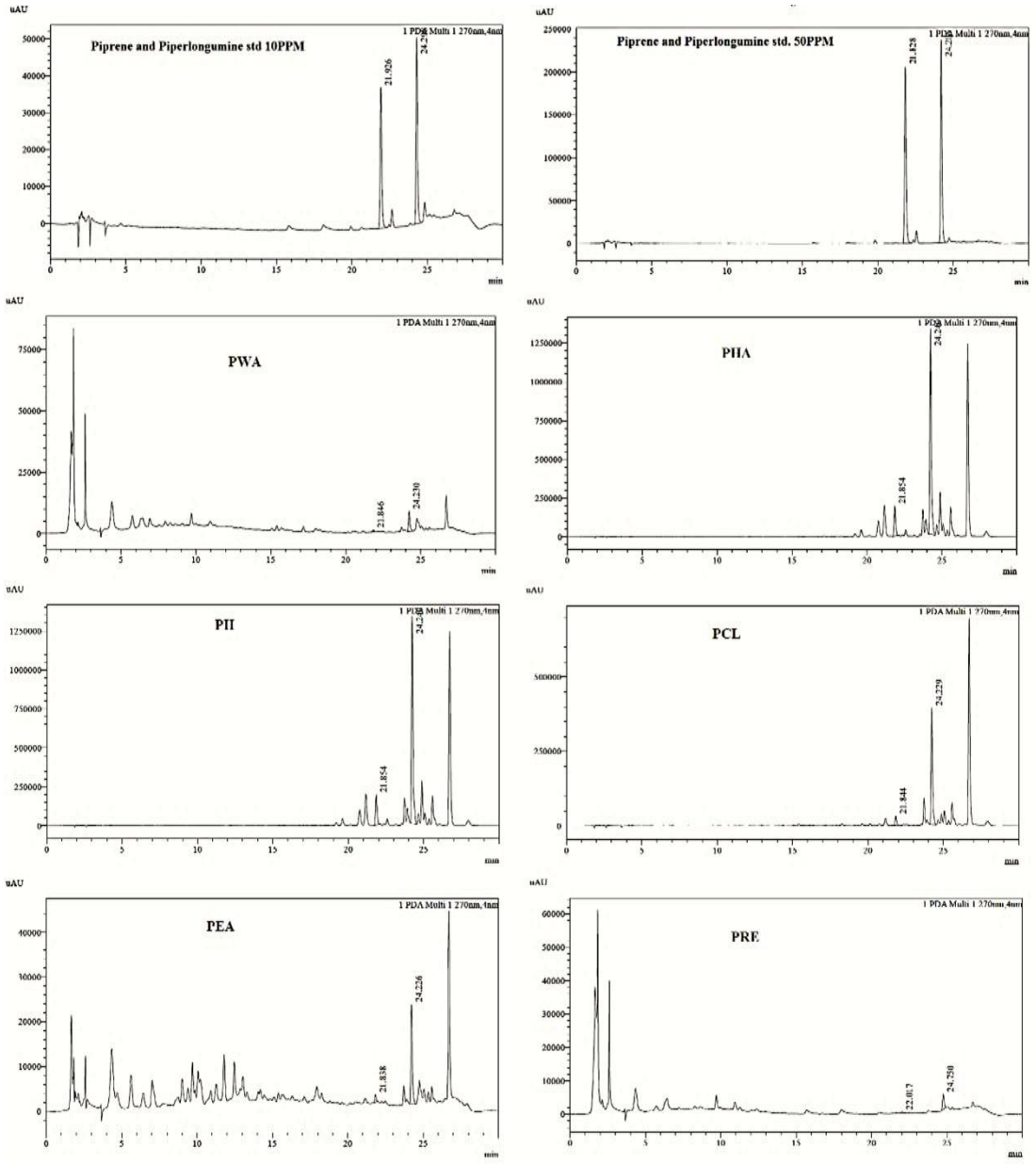

Figure 2: HPLC Chromatogram of extracts and fractions with Analytical markers compounds.

PHA- Hydro-alcoholics, PW- Water, PH- Hexane, PCL- Chloroform, PEA- Ethyl acetate, PRE- Remaining extract

Table 1: HPLC quantification of Piperine and Piperlongumine in *Piper longum* fruits, extracts and PHA fractions

Extracts	**Piperine %**	**Piperlongumine %**
PWA	0.051 ± 0.009	0.008 ± 0.001

PHA	0.565 ± 0.005	0.061 ± 0.001
Fractions Obtained from Hydro-alcoholic (PHA) extract		
PH	11.882 ± 0.091	2.883 ± 0.028
PCL	14.569 ± 0.113	1.768 ± 0.021
PEA	3.805 ± 0.029	0.463 ± 0.021
PRE	0.004 ± 0.0001	0.001 ± 0.0001

2.3. **Comparative Antioxidant Study of Extracts and Fractions with Standard Ascorbic acid.**

Antioxidant activity was evaluated using different models. The models were selected for a range of screening. Following are the experimental models.

2.3.1. DPPH assay.

Antioxidant evaluation by DPPH assay of extracts and fractions with standard ascorbic acid was done to determine IC_{50} values (Inhibition control 50%) and to determine concentration of sample at which 50% inhibition of free radical was obtained. Ascorbic acid showed IC_{50} value at 4.164 µg/ml. Among extracts PWA IC_{50} was found at 0.207±0.011 mg/ml, which was more potent than PHA IC_{50} value 0.614±0.009 mg/ml. Among the fractions of PHA, ethyl acetate fraction PEA showed higher IC_{50} at 0.058±0.001 mg/ml, followed by Chloroform extract PCL 0.063±0.001 mg/ml. The hexane fraction PH showed value at 0.259±0.013 mg/ml, whereas lowest reducing activity was found in extract remaining after fractionation PRE at 0.371±0.021 mg/ml. Graphical response of extracts, fractions and standard ascorbic acid for DPPH assay is shown in Fig. 3.

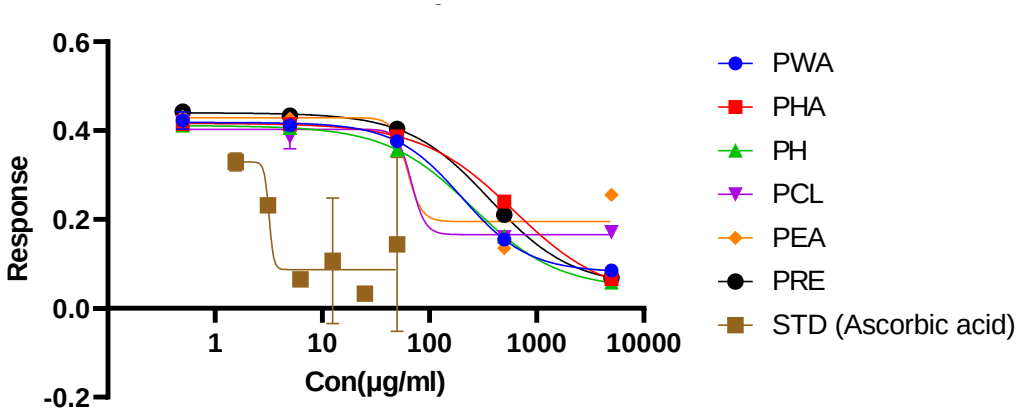

Figure 3: DPPH assay of *Piper longum* fruit extract and fractions

2.3.2. ABTS assay.

Antioxidant evaluation by ABTS assay of extracts and fractions with standard ascorbic acid was done to determine IC_{50}. Ascorbic acid showed IC_{50} value at 5.597 µg/ml. Water extracts PWA IC_{50} was found at 0.636±0.083 mg/ml, which was more potent than hydroalcoholic extract PHA with IC_{50} value at 3.317±0.079 mg/ml. Among the PHA fractions PEA showed highest ABTS reducing activity with IC_{50} value at 0.413±0.117 mg/ml, followed by PCL 0.503±0.015 mg/ml and PH 1.218 ± 0.318 mg/ml, whereas lowest reducing activity was found in PRE 3.6003±0.334 mg/ml respectively. Graphical response of extracts, fractions, and standard ascorbic acid for ABTS assay is shown in Fig. 4.

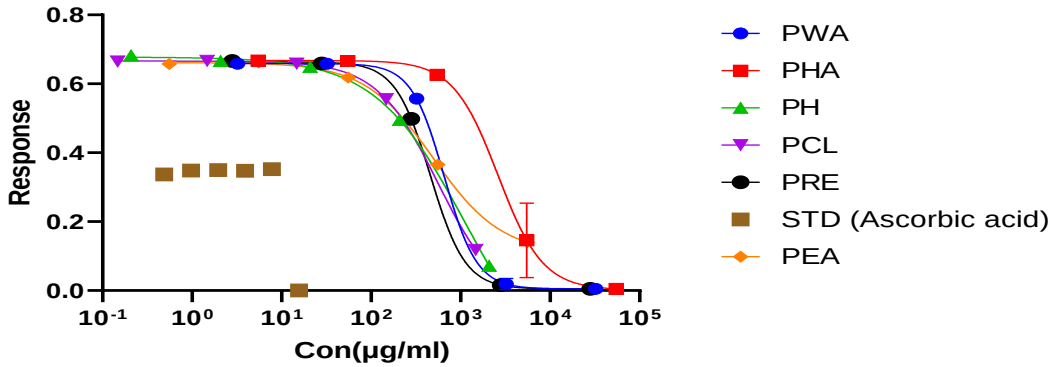

Figure 4: ABTS assay *of Piper longum* fruit extract and fractions

2.3.3. Total Reducing assay.

Antioxidant activity by TRA was done similar to DPPH and ABTS. The intensity of blue color formation was measured to signify IC_{50} value. Standard ascorbic acid showed IC_{50} value at 156.68 µg/ml. whereas in extracts PHA was more active and showed IC_{50} value at 1.793±0.057 mg/ml, whereas for PWA was 4.272±0.651 mg/ml. Among the PHA fractions PCL showed more potent IC_{50} value

at 0.375±0.037 mg/ml followed with PEA 0.418±0.005, PRE 3.93±0.328 mg/ml and PH IC_{50} at 4.086±0.651 mg/ml showed lowest reducing activity respectively. Comparative graphical representation of PWA, PHA, PH, PCL, PEA and PRE is represented in Fig. 5.

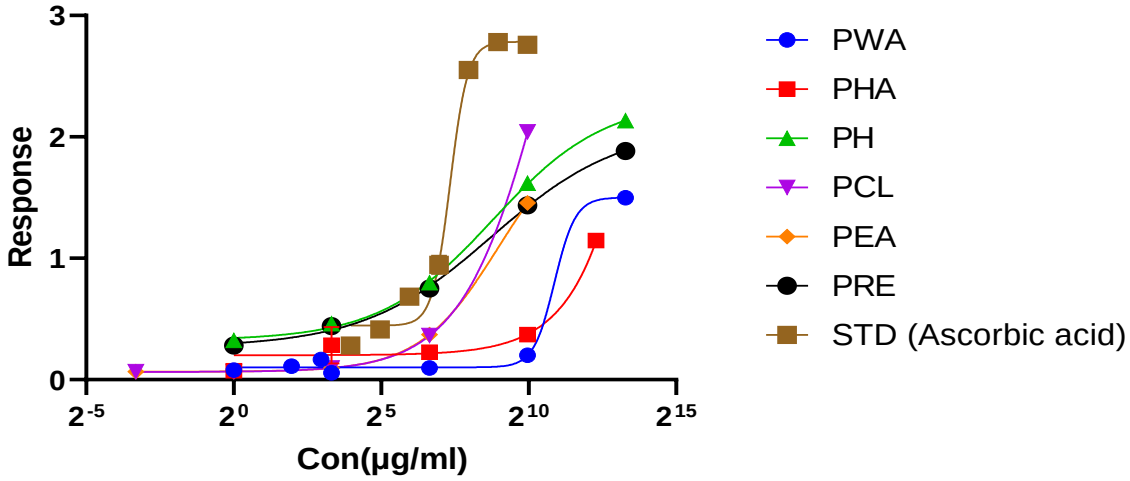

Figure 5: TRA assay of *Piper longum* fruit extract and fractions

2.3.4. FRAP assay.

FRAP value of all extracts and fractions were determine by line equation obtained from standard curve of ascorbic acid against FRAP reagent. FRAP value of PWA, PHA, PH, PCL, PEA and PRE were obtained in terms of µmol/10mg AAE. PWA showed higher FRAP value of 20.806±0.658 µmol/10mg AAE than PHA 20.362±0.205 µmol/10mg AAE. Similarly, among the fractions PEA showed highest FRAP value 24.23±4.052 µmol/10mg AAE followed with PH 16.091±1.168 µmol/10mg AAE, PCL 14.229±1.819 µmol/10mg AAE and PRE showed lowest FRAP value at 1.370±0.016 µmol/10mg AAE as compared with others fractions. Comparative hologram representation of activity in FRAP assay shown in Fig 6.

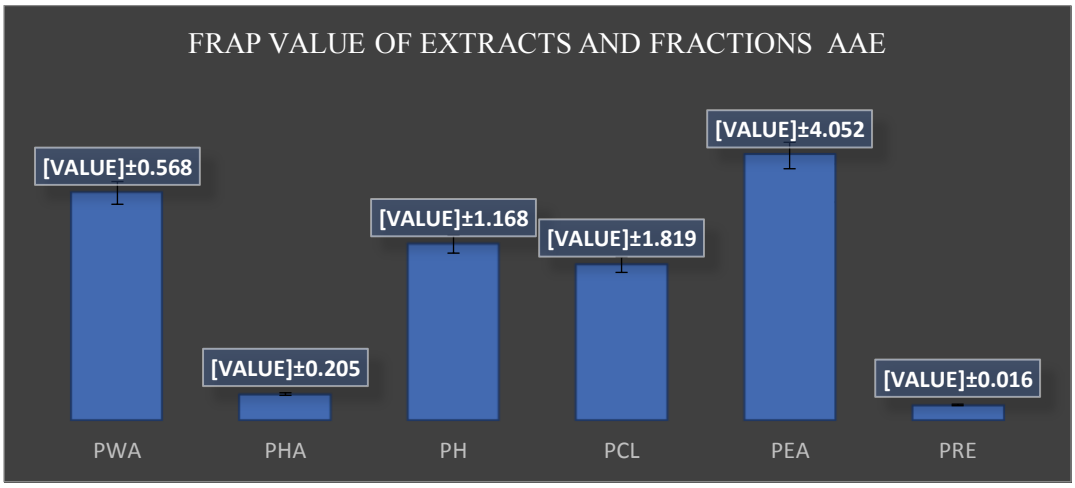

Figure 6: Comparative representation of FRAP value of extracts and fractions

2.3.5. Total antioxidant capacity (Phosphomolybdate assay).

TAC value of all extracts and fractions were determined by using line equation obtained from standard graph of ascorbic acid against phosphomolybdate reagent. TAC value of PWA, PHA, PH, PCL, PEA and PRE were determined in terms of µmol/10mg AAE. In extracts PHA showed higher TAC value 1.279±0.244 µmol/10mg AAE than PWA 0.630±0.143 µmol/10mg AAE. Similarly in fractions, PCL showed highest TAC value 2.946±0.338 µmol/10mg AAE followed with PH 2.539±0.349 µmol/10mg AAE, with PEA 2.249±0.229 µmol/10mg AAE and PRE showed very low TAC value at 0.895±0.107 µmol/10mg AAE as compared with others fractions respectively. Comparative hologram representation of extracts and fractions in TAC assay is shown in Fig 7.

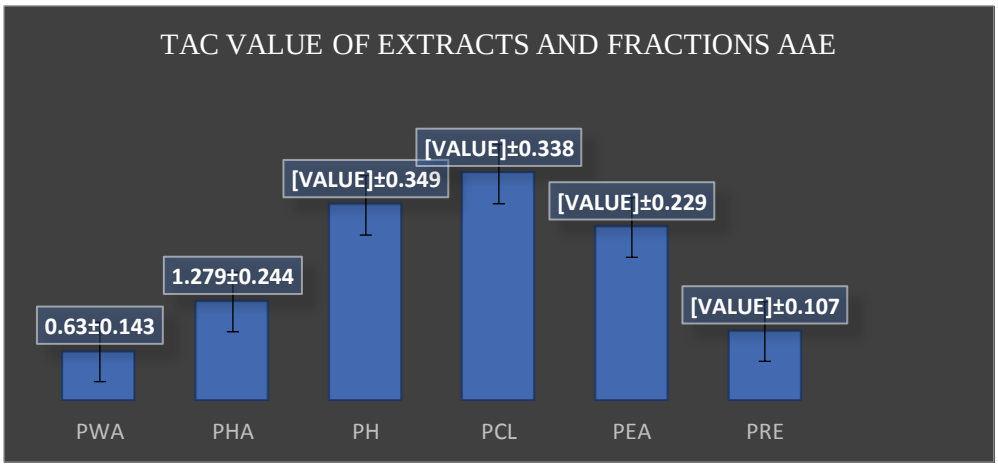

Figure 7: Comparative representation of FRAP value of extracts and fractions.

3. Experimental.

3.1.Collection, Identification and Processing of Plant Material.

Piper longum fruits were collected locally from Bageshwar district of Uttarakhand India, Herbariums of specimen was prepared and identified at Botanical Survey of India, Dehradun identified with accession no 173. Shade dried fruit materials was converted in course powder for extraction by using a milling machine and passed the powder through copper sieve. Extraction of dried course powdered was done with distilled water (PWA) and hydroalcoholic solvent (50:50) (PHA) by using the reflux condensation method. Both extracts were dried on rotatory vacuum evaporator and finally on water bath and kept in desiccators for further study. Fractionation of PHA with Hexane (PH), Chloroform (PCL), Ethyl acetate (PEA), was done in the manner of higher nonpolar to mild polar respectively and extract remaining after fractionation was termed (PRE). Finally, the yield of both extracts (PWA, PHA) and fractions (PH, PCL, PEA PRE) was calculated and recorded.

3.2.HPLC screening and quantification analytical markers compounds.

HPLC Screening of *Piper longum* for determining piperine and piperlongumine content in fruits extracts and fractions was done by using Shimadzu Prominance I instrument. Determination of piperine and piperlongumine by HPLC method was performed as per the reference method for piperine determination by HPLC method[5]. Chromatographic system for method is presented in Table 2 and Gradient programming of flow rate of mobile phase A and Mobile phase B is shown in Table 3.

Table 2: HPLC Chromatographic system for determination of Piperine and Piperlongumine

Experimental configuration of method in HPLC instrument.	
Column type and size	C_{18} Octa decile silane 25cm x 4.5mm, 5um internal pore size
Flow rate	1.5ml/min.
Absorbance (ʎ max)	270nm.
Injection volume	20µl.
Mobile Phase A	2.5pH potassium dihydrogen phosphate buffer
Mobile Phase B	Acetonitrile

Table 3: Gradient elution programming of mobile phase in HPLC programming for Screening of *Piper longum* fruits extracts and fractions.

Time	Mobile phase A 2.5 pH potassium dihydrogen phosphate	Mobile phase B Acetonitrile
0	95%	5%
18	55%	45%
25	20%	80%
30	95%	5%

3.3.In-vitro antioxidant assay of extracts and fractions of *Piper longum* fruits.

3.3.1. DPPH assay

2,2 Diphenyl-1-picryl hydrazyl (0.2 mM) solution in Methanol was taken as a stock solution. Stock solution of both extracts (10 mg/ml), all fractions (10 mg/ml) and Ascorbic acid (1 mg/ml) were prepared and serially diluted in methanol to set a concentration to test IC_{50} (inhibition control 50%) value against 0.1 mM DPPH at 517 nm[6, 7].

3.3.2. ABTS assay:

ABTS reagent solution was prepared using 14 mM of ABTS (2,2′-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) and 4.8 mM potassium persulphate. The solution was kept as stock solutions and allowed to stand for 16 hours in dark place at room temperature. The stock of ABTS reagent solution was diluted with methanol to obtained a control absorbance under 0.7 at 734 nm. All extracts and fractions of *Piper longum* fruits were dissolved in methanol and filtered. Each extract and fraction of stock solution were serially diluted in ratio of 1:10 with methanol. 1ml each of serially diluted extract and fractions were mixed with 1ml ABTS reagent and absorbance of each was recorded at 734 nm. IC_{50} of all extracts and fractions were determined. Similarly ascorbic acid was taken as standard and IC_{50} of standard were determined [6, 7, 8,9].

3.3.3. Total reducing assay

All the extracts, fractions and Ascorbic acid Standard were dissolved in methanol to prepare 10 mg/ml as a stock solution, serially diluted all extracts and fractions such to record IC_{50} value at 700 nm. 2.5 ml of each dilution of extracts and fractions in glass test tube and 2.5 ml of sodium phosphate buffer (0.2 mM pH 6 .6)

and 2.5 ml of potassium ferricyanide (1%) were added into the test tube and each test tube was incubated at 50ºC for 20 min. After incubation 2.5 ml 10% trichloroacetic acid was added into each test tube. Each test sample was centrifuged at 3000 rpm for 10 min, and each test tube supernatant was half-fold diluted by distilled water, 1 ml of 1% $FeCl_3$ was added in each dilution. Formation of blue colour was measured at 700 nm [6, 7].

3.3.4. Ferric reducing antioxidant potential (FRAP) assay

FRAP reagent solution were prepared by adding25 ml of acetate buffer (30 mM, pH 3.6) to 2.5 ml of TPTZ [2,4,6-Tirs-(2- pyridyl)-s-triazine] solution (10 mM in 40 mM hydrochloric acid) and finally added 2.5 ml freshly prepared ferric chloride solution (20 mM). The solution was allowed to incubate at 37 ºC for 15 min before use. Ascorbic acid was serially diluted to obtain a uniform graph for FRAP reagent at 593 nm. All the extracts and fractions were dissolved in methanol to prepare the stock solutions of 10 mg/ ml. The concentration of extracts and fractions were diluted to obtain a significant absorbance for the determination of FRAP value in Ascorbic acid equivalent (AAE) from standard graphs[6, 7].

3.3.5. Total antioxidant capacity assay (Phsophomolybdate assay)

For total antioxidant capacity assay Phosphomolybdate reagent (solution of 4 mM ammonium molybdate, 28 mM sodium phosphate and 0.6 M sulphuric acid) was prepared. All extracts and fractions were dissolved in methanol to prepare a stock solution of 10 mg/ml. Ascorbic acid was used as standard and was dissolved in methanol to prepare 10 mg/ml stock solution. Finally, 1 mg/ml was used as working solution serially diluted to prepare a graph against phosphomolybdate reagent at 695 nm. All extracts and fractions were dissolved in methanol to prepare a stock solution of 10 mg/ml. The concentration of extracts and fractions were diluted to obtain a significant absorbance for the determination of total antioxidant capacity (TAC) value AAE from standard graph[6, 7].

4. Conclusion.

Antioxidant potential of *Piper longum* and its constituents is well established[10]. Leaf and fruit extracts of *Piper nigrum* and *Piper longum* possess very good antioxidant properties. Various studies have suggested that the presence of phytochemical like flavonoids and phenols may be responsible for free radical

scavenging activity of the plants suggesting their use as a potential natural antioxidant[11]. Talking about extraction, in the present study hydroalcohlic extract of *Piper longum* gave higher yield than water extract with higher content of piperine and piperlongumine. Regarding antioxidant assay, PWA showed higher activity for DPPH, ABTS and FRAP assay than PHA. Comparing the results with previous analysis it was seen that some small chain of carboxylic acid were present in PWA *Viz*. malic acid, citric acid, quinic acid, gentisic acid, benzoylmailc acid. Some specific molecules are also reported in literature *Viz*. Piperyline; 4,5-dihydropiperlonguminine; (E,E)-piperlonguminine; piperanine; piperine; piperittine; dehydropipernonaline; pipernonaline; pipercide; piperolein B; piperundecalidine; guinesine. In PWA[12] apart from them presence of alkaloids piperine, piperlongumine and their derivatives shows that it contains a wide range of molecules responsible for higher therapeutic activity than PHA in DPPH, ABTS, FRAP assay[12]. Qualitative analysis also showed the presence of Proteins, sugars content higher than other non-polar solvents[13]. Among the fraction of PHA, PEA showed higher activity for DPPH, ABTS, FRAP assay as compared to other fractions. Some of previous studies have shown that the presence of higher content of polyphenols in ethyl acetate solvents of *Piper longum* fruits[14] are responsible. Thus, it can be said that higher content of piperine and piperlongumine alone is not responsible for higher antioxidant activity against both radical scavenging assay and ferric reducing antioxidant potential assay in *Piper longum* fruit extracts and fractions. However, higher content of piperine in extract and fraction showed higher activity against total reducing assay and total antioxidant capacity assay. Hence PHA is more effective for TRA assay and TAC assay with higher yield, whereas for DPPH assay, ABTS assay and FRAP assay PWA showed better result. Similarly, among fractions PCL was effective in TRA and TAC assay, whereas for DPPH assay ABTS assay and FRAP assay PEA showed better result.

Conflict of Interest:

None.

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Author's Contribution:

KU and DSJ had the Idea and DSJ designed and conducted the experiments. DSJ and KU organized the data and DSJ wrote the manuscript TK did review of manuscript. KU did final revisions. All the authors read and approved the final manuscript.

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Data Availability: All relevant data are included in the article.

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