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Antioxidant activities, phenolics and piperine contents in four *Piper* species from India

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Abstract
The present investigation was carried to compare the antioxidant activities, phenolics and piperine contents in fruits of four *Piper* species namely *P. longum*, *P. mullesua*, *P. nigrum* and *P. peepuloides*. Hydroalcoholic extract of fruits of selected *Piper* species was prepared and total phenolics content in extract samples was determined by Folin-Ciocalteu method. Three assays namely DPPH free radical scavenging activity, ABTS free radical scavenging activity and reducing power were used to assess the antioxidant activities of extracts *in vitro*. Ascorbic acid was used as positive control for comparing the antioxidant activities of the extracts. Piperine content was estimated using a reversed phase high performance liquid chromatography-photo diode array detection (RP-HPLC-PDA) method. Extract yield (%) was maximum for *P. peepuloides* (26.56) and it was followed by *P. nigrum* (21.58), *P. mullesua* (15.86) and *P. longum* (13.34), respectively. Piperine could be quantified in *P. longum* (2.43±0.13%) and *P. nigrum* (0.73±0.04%) extracts only. Antioxidant activity was found to be concentration dependent in all three assays. Also, extracts of all four species exhibited weak antioxidant activity in all three assays.

Keywords: *Piper*, piperine, *P. longum, P. mullesua, P. nigrum, P. peepuloides*

1. Introduction
The genus *Piper* (Piperaceae) consisting of about 2000 species, is a medicinally important group of plants. *Piper* species have been used in folk medicine since ancient time. The pepper plant has formed the basis of a large number of traditional formulations of Indian System of Medicine since thousands of years. Also, pepper and its constituents find vital importance in health care of traditional systems of medicine of many other countries [1]. *Piper* species mostly grows in wild in tropic and subtropical climates in north eastern region and southern parts of India [2]. More than ninety *Piper* species are reported in tropical evergreen forests and semi deciduous forests of North Eastern region of India. *P. nigrum* is the most widely recognised species of this genus followed by *P. longum* [3]. *P. longum* is commonly known as Long *Piper* or Indian long *Piper*. *P. nigrum* is known as king of spices and "Black gold" is one of the important foreign exchange earners for India [4]. *P. mullesua* (Hill *Piper*) is used for removing blood stasis. *P. peepuloides* (Wild pepper) is used in a variety of *Ayurvedic* preparations and local people of Meghalaya use its dried seed powder with honey and egg yolk for the treatment of severe cough [5]. Piperine (1-piperoylpiperidine, Figure 1), an alkaloid, commonly occurs in fruits of *Piper* species. Piperine and its derivatives are effective anticonvulsant.

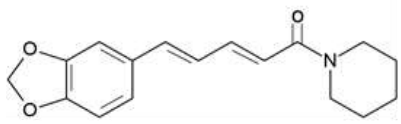


Fig 1: Chemical structure of piperine

Antiepilepsirine, one of the derivatives of piperine, is used as an antiepileptic drug in treating different types of epilepsy [1]. Piperine is one of the first purified natural molecules with bio-enhancer properties [6]. It increases the bioavailability of drugs by inhibiting the biotransformation processes occurring in liver and intestine, due to its ability to inhibit the activity of various metabolizing enzymes [6, 7, 8].

2. Materials and Methods

2.1 Plant materials

Dried fruit spikes of *P. nigrum and P. longum* were collected from Thrissur, Kerala in April 2017. *P. mullesua* was collected from Bamunkhal village, Manash National Park, Baksa district, Assam in September 2018. *P. peepuloides* was collected from Herbal Garden, ICAR-DMAPR, Boriavi, Anand in March 2019. Taxonomical identification of collected fruits was confirmed with the help of a taxonomist. The fruits were sorted an infected and mechanically damaged fruits were discarded (Figure 2). The dried fruit rind pieces were then pulverised to a coarse powder and stored in airtight container free from moisture.

Fig 2: Fruits of *Piper* species: (A) *P. longum*, (B) *P. mullesua*, (C) *P. nigrum* and (D) *P. peepuloides*

2.2 Solvent and standard

HPLC grade solvents methanol was purchased from Merck, Mumbai, India. Deionized water used throughout the experiment was obtained using a Millipore water purification system (Millipore, gradient- 0.22 µm). Reagents such as Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical, 2, 2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ascorbic acid were purchased from Sigma-Aldrich, Mumbai, India. Analytical grade potassium ferricyanides, trichloroacetic acid and ferric chloride from SRL, Mumbai were used. Piperine was purchased from Sigma-Aldrich, Bangalore, India.

2.3 Extract preparation

Dried samples (10 g) were finely powdered. Extract of the samples were prepared by refluxing the powdered samples with ethanol (96%, v/v) on a water bath [9]. Sample and solvent ratio was 1:20 and extraction time was 8 h. The extracts were filtered and concentrated under reduced pressure using a rotary evaporator (Heizbad Hei-VAP, Heidolph, Schwabach, Germany, temperature = 40±5 °C). All extracts were stored in glass vials and away from direct sunlight.

2.4 Determination of total phenolic content

The dried extracts were dissolved in distilled water (1 mg/mL) and were sonicated before use. TPC in extracts was determined by a colorimetric method using Folin-Ciocalteu [10]. Briefly, extract solution (0.5 mL), Folin-Ciocalteu reagent (0.5 mL) and distilled water (7.5 mL) were mixed in a test tube and further mixed vigorously by using a Vortex mixer. Test tubes were kept at room temperature for 10 min and thereafter sodium carbonate (20%, 1.5 mL) was added to test tube mixture. The resultant mixture was allowed to incubate in a water bath at 40 ºC for 20 min. The intensity of the blue colour developed was measured by recording the absorbance at 755 nm using a UV–visible spectrophotometer (UV-5704SS, Electronic Corporation of India). The reagent blank was also prepared using distilled water. For quantification of TPC in the extracts, a standard calibration curve was prepared using gallic acid. TPC of the extract samples was expressed as gallic acid equivalent (GAE).

2.5 Determination of DPPH free radical scavenging activity

Free radical scavenging activity of extracts was evaluated using DPPH free radical scavenging assay. Different concentrations of the extracts were taken in test tubes. The total volume was adjusted to 8.5 mL by the addition of methanol. 5.0 mL of methanolic solution of DPPH (0.1 mM) was added to these tubes and mixed thoroughly using a Vortex mixer. Thereafter, tubes were kept at room temperature for 20 min. The blank was prepared in the same as described above but without the extract and methanol was used for the baseline correction. Changes in the absorbance of the extract samples were measured at 517 nm using UV–visible spectrophotometer.

Radical scavenging activity (RSA) was expressed as the inhibition percentage and was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = \frac{(\text{absorbance of blank} - \text{absorbance of sample})}{\text{absorbance of blank}} \times 100$$

2.6 Determination of ABTS free radical scavenging activity

Free radical scavenging activity was determined by ABTS radical cation decolorization assay [11]. ABTS was dissolved in water to get a 7 µM concentration and radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS solution with 2.45 µM potassium persulphate at room temperature in dark (12–16 h) before use. For assay, $ABTS^{+}$ solution was diluted with water to an absorbance value of 0.700±0.02 at 734 nm. After

addition of 3.0 mL of diluted $ABTS^+$ solution to 100 µL of extract solutions, absorbance was recorded after 6 min.

$$\text{ABTS radical scavenging activity (\%)} = \frac{(\text{absorbance of blank} - \text{absorbance of sample})}{\text{Absorbance of blank}} \times 100$$

2.7 Determination of reducing power

The total reducing power of standard antioxidants and extracts were determined as described by Oyaizu [12]. Different concentrations of extracts were mixed with distilled water (2.5 mL), phosphate buffer (2.5 mL; 0.2 M; pH 6.6) and potassium ferricyanide (2.5 mL; 1%). The resulting mixture was incubated at 50 ºC for 20 min in a water bath. After cooling, trichloroacetic acid (2.5 mL; 10 %) was added to the mixture. The upper layer of solution (2.5 mL) was taken and mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL; 0.1%). The absorbance was recorded using a UV-visible spectrophotometer at 700 nm. The increasing absorbance value was interpreted as increased reducing activity [13].

2.8 Calculation of IC_{50} concentration

The extract concentration corresponding to fifty percent inhibition (IC_{50}) was calculated from the curve. Ascorbic acid was used as standards. Each sample was assayed in triplicate for each concentration.

2.9 HPLC-PDA system for quantification of piperine in extract samples

HPLC system for chromatographic analysis consisted of a separation module (Waters 600E) equipped with Empower software (Waters) and comprising of quaternary pump, an in-line vacuum degasser and a photodiode array detector (Waters 2996).

Dried extract was reconstituted in methanol for HPLC—PDA analysis. Standard stock solution of piperine (1000 µg/mL) was prepared in HPLC grade methanol. The chromatographic separation was carried out in an isocratic elution mode on RP-18 column (250 × 4 mm, 5µm Merck, India). The mobile phase was a mixture of solvents: methanol and water (80:20, v/v). The solvent flow rate was 1.4 mL/ min and the injection volume was 20 µL. The photo diode array detector wavelength was set at 342 nm.

2.10 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD) for three replications of each sample. Linear regression was carried out using Microsoft Excel 2010 to establish a correlation of antioxidant activities and extract concentration.

3. Results

3.1 Extract yield and TPC of *P. longum, P. mullesua, P. nigrum* and *P. peepuloides*

The extract yield (%) of *P. longum, P. mullesua, P. nigrum* and *P. peepuloides* was 13.34, 15.86, 21.58 and 26.56, respectively. TPC (%) varied in the following sequence: *P. peepuloides* (4.94±0.09) > *P. mullesua* (1.98±0.04) > *P. longum* (1.71±0.07) > *P. nigrum* (1.07±0.05).

3.2 Anti-oxidant properties of *P. longum, P. mullesua, P. nigrum* and *P. peepuloides* extracts

The free radical scavenging activity of *P. longum, P. mullesua, P. nigrum* and *P. peepuloides* extracts were evaluated by DPPH method. Ascorbic acid was used as control. The percentage of DPPH free radical scavenged was plotted against the concentration of extracts of *P. longum, P. mullesua, P. nigrum* and *P. peepuloides* (Figure 3).

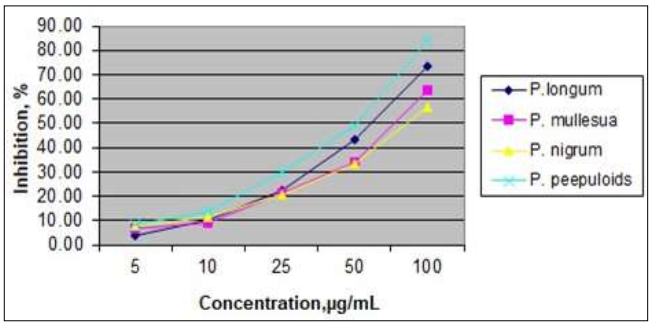


Fig 3: Graph of concentration and percentage inhibition for DPPH assay of *Piper* extracts

Concentration (µg/ mL) corresponding to 50 percent inhibition (IC_{50}) was calculated. For reference antioxidant ascorbic acid IC_{50} (µg/ mL) was 2.79. For extracts of *Piper* species IC_{50} (µg/mL) value varied in the following sequence: *P. nigrum* (85.35 ± 3.45) > *P. mullesua* (76.49 ± 3.09) > *P. longum* (64.70 ± 2.61) > *P. peepuloides* (54.52 ± 2.20). In ABTS assay of *P. longum, P. mullesua, P. nigrum* and *P. peepuloides* extracts, IC_{50} (µg/mL) value showed similar trend as it was in DPPH assay (Figure 4).

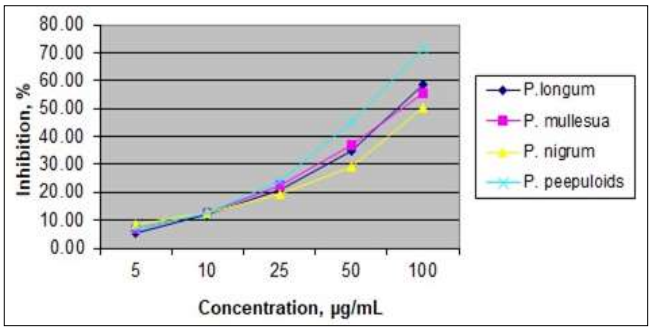


Fig 4: Graph of concentration and percentage inhibition for ABTS assay of *Piper* extracts

It varied in the following sequence: *P. nigrum* (98.84 ± 3.99) > *P. mullesua* (84.24 ± 3.40) > *P. longum* (81.79 ± 3.31) > *P. peepuloides* (64.72 ± 2.62). Ascorbic acid had IC_{50} (µg/mL) was 5.81. For measuring the reducing power of extract, the capability of the extract to convert ferric ion (Fe^{+3}) into ferrous ion (Fe^{+2}) was measured by using reducing power assay. Here, IC_{50} (µg/mL) value of extract of *P. longum* (67.35 ± 2.72), *P. mullesua* (19.09 ± 0.77), *P. nigrum* (43.53 ± 1.76) and *P. peepuloides* (25.12 ± 1.02) was higher than IC_{50} (µg/mL) of reference antioxidant ascorbic acid (Figure 5**)**.

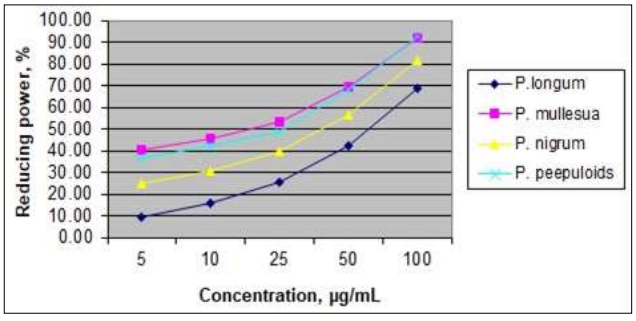
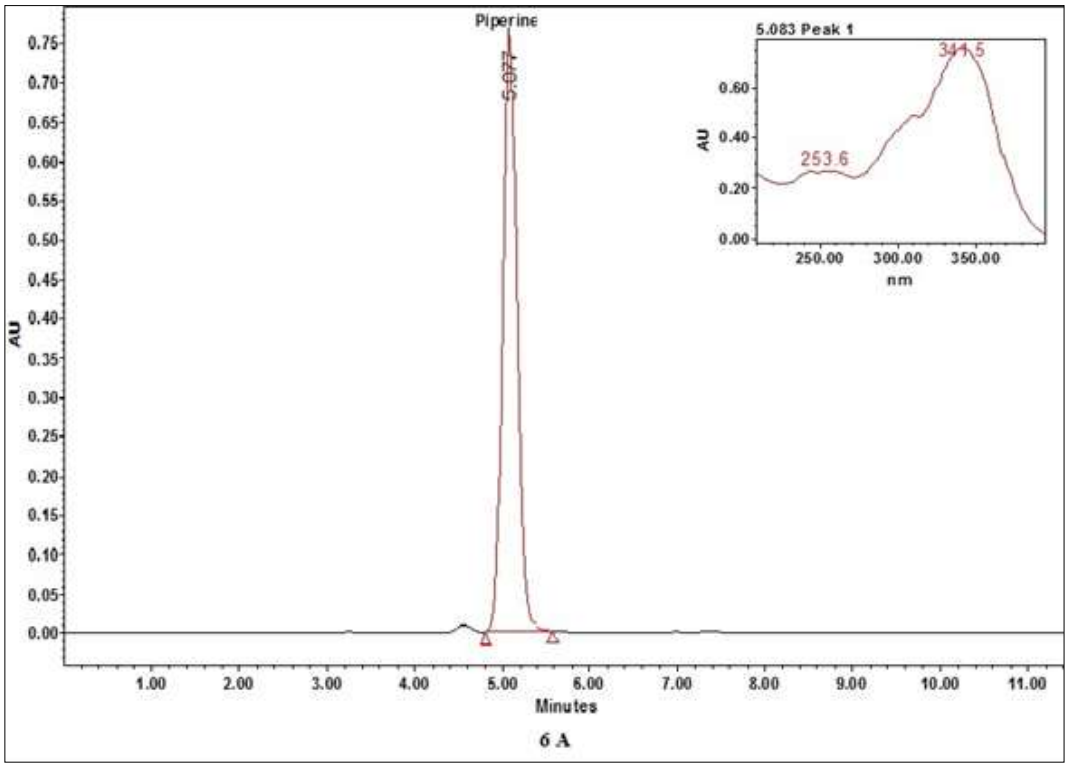


Fig 5: Graph of concentration and percentage inhibition for reducing power assay of *Piper* extracts

3.3 Estimation of piperine content in extracts of *P. longum, P. mullesua, P. nigrum* and *P. peepuloides* using HPLC-PDA method

Peak of piperine in extracts of samples were identified on the basis of retention time of peak and matching its PDA spectrum at 342 nm. Concentration of piperine in extract samples was calculated by comparing the integrated peak area of the individual samples with that of a standard curve prepared for standard piperine [14]. Peak of piperine was eluted at 5.2 min was detected in *P. longum* and *P. nigrum* only (Figure 6).



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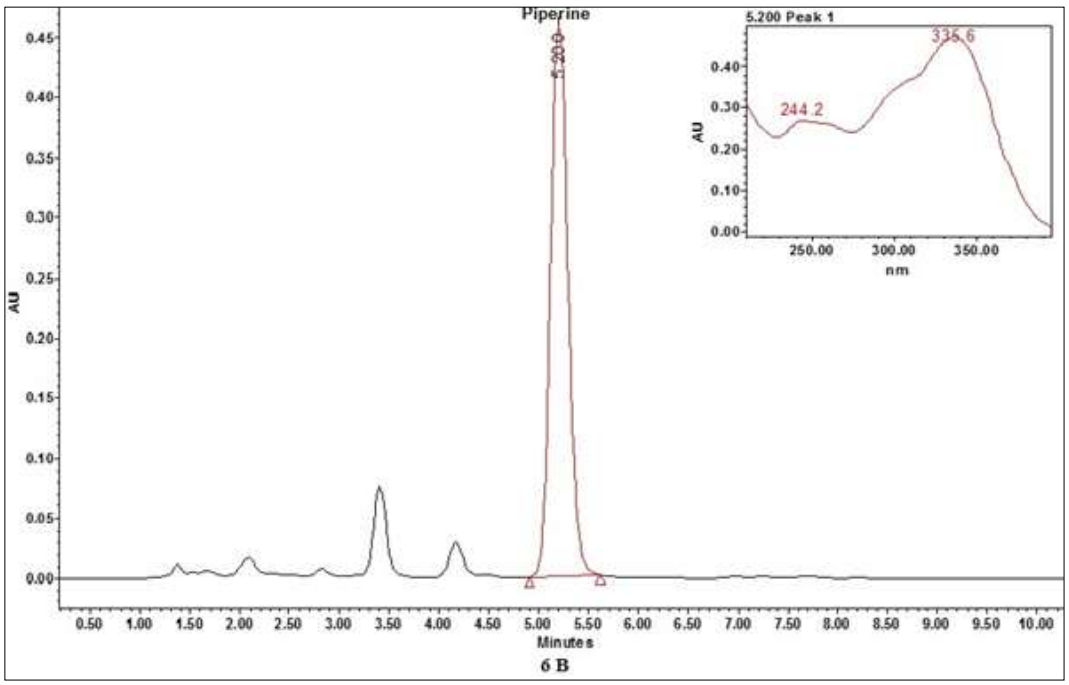

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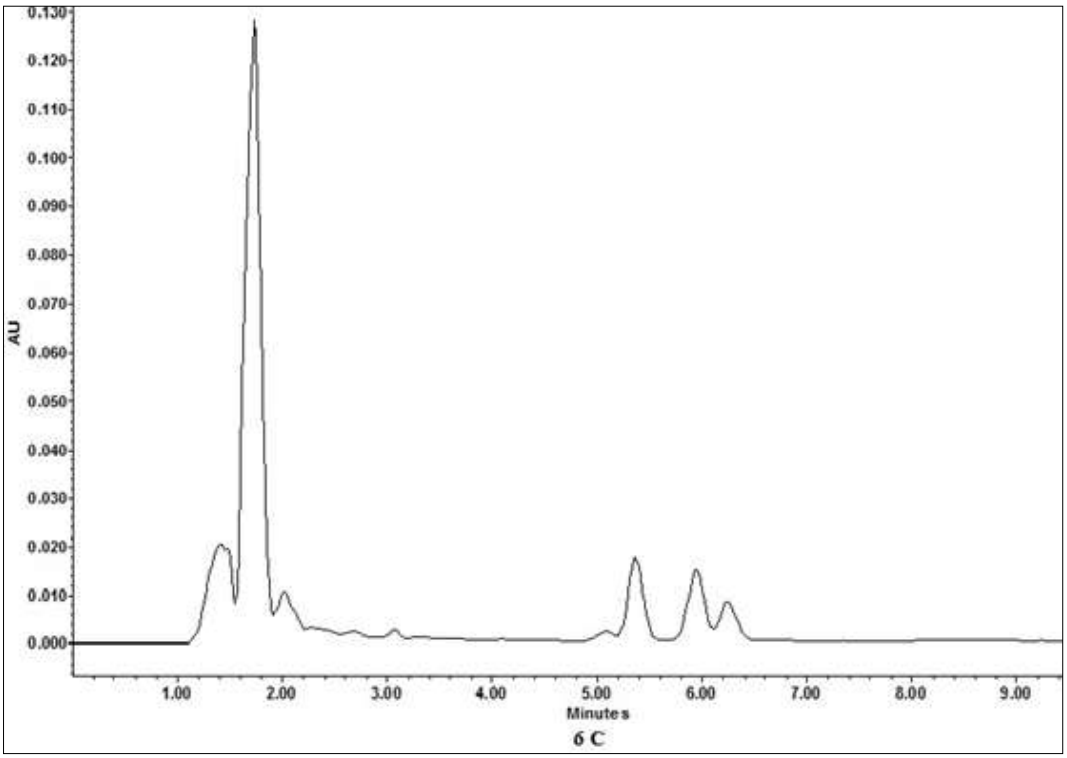
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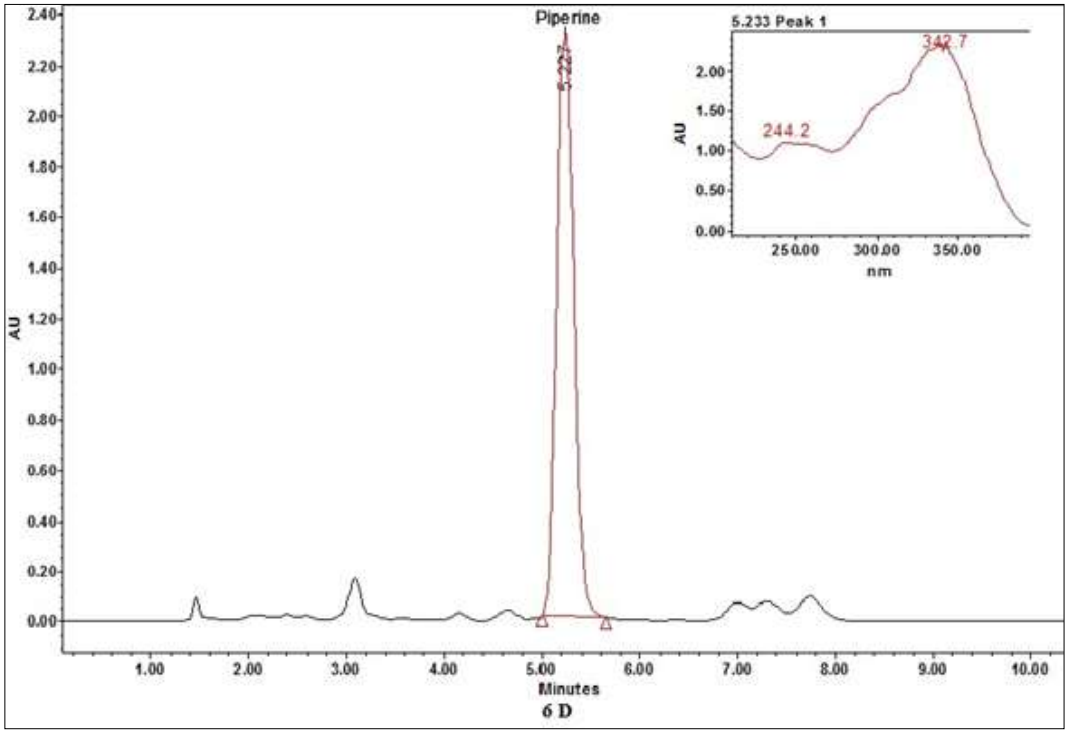
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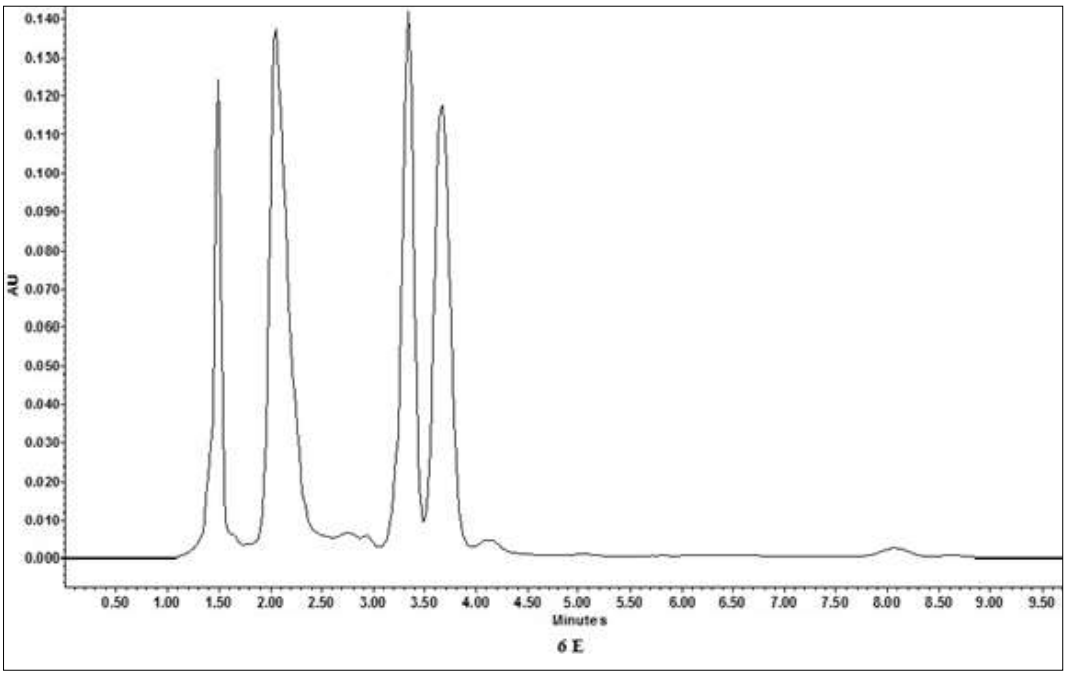

Fig 6: HPLC-PDA chromatogram of (A) Piperine standard and extract of (B) *P. longum*, (C) *P. mullesua*, (D) *P. nigrum* and (E) *P. peepuloides*

Absence of peak of piperine in extracts of *P. mullesua* and *P. peepuloides* was further confirmed by the spiking of standard solution of piperine in these extracts. Piperine content (%) in dried samples of *P. longum* and *P. nigrum* was 2.43 ± 0.13 and 0.73 ± 0.04. HPLC-PDA chromatogram of *P. longum* showed that several others peaks were eluted before peak of piperine. Although, peak of piperine was not detected in *P. mullesua* extracts, however, several peaks could be observed before and after the retention time, i.e. 5.2 min, of piperine. Similarly, in chromatogram of *P. nigrum* also, many peaks were observed before and after the retention time of piperine. In case of *P. peepuloides*, four major peaks could be observed before the retention time of piperine.

4. Discussion

Very scanty research activities have been carried out for *P. peepuloides* in spite of high market demand. Little information about its habit, economic aspects, conservation, harvesting and processing is available in literature [15-16]. Whole plants of *P. mullesua* are used in Chinese Folk Medicine to treat bone fractures, injuries from fall, rheumatoid arthritis, rheumatic arthralgia, acroanesthesia, asthma, cold, stomach aches, abdominal pain, toothaches, swelling and pain of furuncles, dysmenorrhea, menoxenia, empyrosis, snake and insect bites [17, 18].

The dried fruiting spikes of *P. longum* appear very similar to that of *P. sylvaticum* and *P. peepuloides*, which are therefore often incorrectly identified as the former [2]. Ahmad *et al*. [19] reported the extractive values of *P. nigrum* fruits. The extractive values (%) for solvents of varying polarity were as follows: petroleum ether (10.51 ± 0.62), chloroform (8.25 ± 0.56), methanol (12.56 ± 1.32) and n-butanol (5.34 ± 0.87). Ramesh *et al* [20] reported that methanolic extract yields (%) of *P. nigrum* (fruit), *P. chaba* (root) and *P. longum* were 14.12 ± 1.89, 11.47 *P.* ± 1.18 and 1.47± 1.32, respectively. Akbar *et al*. [21] reported extract yield of *P. longum* and *P. nigrum* collected from two different locations of Bangladesh. Extract was prepared with ethanol and water. The extract yield (%) of *P. longum* for ethanol was 9.68 and 10.32. Similarly, extract yield (%) of *P. longum* for water was 7.76 and 8.04. In case of *P. nigrum*, for ethanol extract yield was 11.80 and 10.92. For water, extract yield was 8.6 and 8.32.

The nature and distribution of phenolic compounds in *Piper* species are important. In addition to their contribution to astringent taste and thus overall flavour of foods, phenolics are known to contribute to browning/blackening [22, 23]. Variyar *et al*. [24] classified phenolic constituent of *P. nigrum* into two groups: enzyme inactive and enzyme active based on their ability to act as substrates towards pepper polyphenol oxidase (PPO). Enzyme inactive group comprised of nine phenolic acids, namely, protocatechuic, gentisic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, ferulic, synapic and salicylic acids and enzyme active group consisted of two novel phenolic compounds namely, 3,4-dihydroxyphenylethanol glucoside and 3,4-dihydroxy-6-(*N*-ethylamino) benzamide. Phenolics from enzyme active acted as highly efficient substrates for pepper PPO [24]. These enzyme-active phenolics were found to be highly unstable and completely converted to brown or black oxidized products during drying processes. It is likely that the proportional distribution of these two novel phenolics in different varieties of green pepper plays a significant role in the degree of brownness or blackness of commercial, finished black pepper with respect to which their qualities are graded [25].

In the present study TPC (%) values for *P. longum, P. mullesua, P. nigrum* and *P. peepuloides* was in the range of 1.07 ± 0.05- 4.94 ± 0.09. Ahmad *et al*. [19] reported that TPC of methanolic extract of *P. nigrum* was 1.728 ± 0.0490 mg/g gallic acid equivalent. Akbar *et al*.[22] also reported TPC (mg/g GAE) of *P. longum* and *P. nigrum* fruits grown in Bangladesh. TPC for ethanol extracts of *P. longum* and *P. nigrum* varied from 81.24 ± 0.32 – 121.47 ± 0.57 and 140.50 ± 0.38 – 174.92 ± 0.33. TPC for water extracts of *P. longum* and *P. nigrum* varied from 34.64 ± 0.22 – 105.51 ± 0.82 and 32.83 ± 0.15 – 34.64 ± 0.22, respectively.

Piperine is endowed with numerous pharmacological activities but the most important being the enhancement of bioavailability inhibition of cytochrome P450 as well as mammalian p-glycoprotein. Its potential immune-modulatory activity has also been reported [6, 7, 26, 27]. Piperine content in fruits of five *Piper* species namely, *P. nigrum*, *P. longum*, *P. retrofractum* Vahl, *P. cubea* Hunter, *P. betle* and also in

processed *P. nigrum* (white pepper) was estimated by high performance thin layer chromatography method by Rajopadhye *et al*. [28]. Fruits of *P. nigrum* had maximum concentration of piperine and it was minimum in *P. betle*. The following trend was reported for piperine content: *P. nigrum* > *P. longum* > processed *P. nigrum* > *P. cubea* Hunter > *P. betle*. Further investigation is needed for identification and quantification of the bioactive compounds in *P. mullesua*, *P. peepuloides*. Khound *et al*. [14] reported piperine content in ten accessions including check variety of *P. longum* collected from different states of North Eastern region of India. Piperine content in fruits of *P. longum* varied from 1.33- 7.85 %.

Plants are high in numerous antioxidant compounds as well as enzymes with antioxidant activity. Animal cells have a much more limited de-novo antioxidant production. Oxidative damage accumulates in animal cells when the critical balance between generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and antioxidant defence is unfavourable [29,30,31]. Oxidative stress reduction by dietary antioxidants has been regarded as the most likely candidate [31]. In the present study antioxidant properties of hydro-alcoholic extract of *P. longum*, *P. mullesua*, *P. nigrum* and *P. peepuloides* were evaluated by measuring DPPH free radical scavenging activity, ABTS free radical scavenging activity and reducing power. For DPPH free radical scavenging activity, IC_{50} (µg/mL) values varied from 54.52±2.20 - 85.35 ± 3.45. In case of ABTS free radical scavenging activity free radical scavenging activity IC_{50} (µg/mL) values varied from 64.72 ± 2.62 – 98.84 ± 3.99. Reducing power assay had high IC_{50} (µg/mL) values varying between 19.09 ± 0.77- 67.35 ± 2.72. High value of IC_{50} (µg/mL) for *P. longum*, *P. mullesua*, *P. nigrum* and *P. peepuloides* hydro-alcoholic extract showed their weak anti-oxidant properties. Similar results were reported by Akbar *et al*. [21] for *P. longum* and *P. nigrum* fruit extracts. IC_{50} (µg/mL) for ethanol extracts varied from 89.18 ± 0.24 – 448.20 ± 3.9. For water extracts, IC_{50} (µg/mL) varied from 118.29 ± 2.5 - 424.26 ± 3.5. In case of ABTS assay, IC_{50} (µg/mL) for ethanol extracts varied from 184.2 ± 1.92 – 224.3 ± 1.61. For water extracts, IC_{50} (µg/mL) varied from 154.02 ± 1.59 - 364.2 ± 2.66.

5. Conclusion

The morphological similarity between the species makes botanical authentication of plants of the *Piper* species difficult. For quality control purposes, discrimination of different species is important. Further research works need to be carried out for identification of constituents of *P. mullesua* and *P. peepuloides* to make better use of these two medicinal plants of folk medicine in order to serve for human health. Advance hyphenated technique such as liquid chromatography-mass spectrometry (LC-MS/MS) could serve as a better analytical method for characterization of identified as well as unidentified peaks in extract chromatograms of four *Piper* species selected for the present study.

6. Acknowledgements

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