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In vitro antioxidant activity and cytotoxicity of sequential extracts from selected black pepper (*Piper nigrum* L.) varieties and *Piper* species

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Abstract

Present study evaluated *in vitro* antioxidant activity and cytotoxicity of four important *Piper* species (*P. nigrum* L., *P. chaba* Hunter, *P. longum* L. and *P. colubrinum* Link.) and six black pepper varieties (Sreekara, Subhakara, IISR Malabar Excel, Panniyur-1, Panchami and IISR Thevam). It was performed with sequential extracts of the dried berries/fruits using n-hexane, chloroform, methanol and water in the order of increasing polarity. Concentrated extracts were tested for total phenolic content, *in vitro* antioxidant activity and cytotoxicity. Methanol and chloroform extracts showed high antioxidant activity than hexane and water extracts. Among black pepper varieties, methanol extract of IISR Malabar Excel followed by that of Panchami and among *Piper* species chloroform extract of *P. colubrinum* expressed highest antioxidant activity. Significant positive correlation between total phenol and antioxidant activity was noted for methanol and chloroform extracts. *In vitro* cytotoxicity of the extracts was tested on cervical cancer cell line CaSki by MTT assay and compared with that of synthetic anticancer drug Doxorubicin. Results showed more cytotoxicity with more extract and increased time of exposure with CaSki. Chloroform extract of *P. longum* and *P. colubrinum* were found to be highly toxic to CaSki than other extracts. By considering three time intervals, chloroform extract of IISR Malabar Excel was more toxic to CaSki than other black pepper varieties. To the best of our knowledge, this is the first report regarding *in vitro* antioxidant activity and cytotoxicity on CaSki for sequential extracts of *P.colubrinum* fruits cultivated in India. This is also the first report on variability in antioxidant activity and cytotoxicity (on CaSki) of sequential extracts from black pepper varieties selected for the study.

Introduction

Human body produces free radicals mainly Reactive Oxygen Species (ROS) as a part of normal metabolic processes. Mitochondria, peroxisomes and immune cells like leukocytes and macrophages are the main endogenous sources for free radical production in cells. Many acute and chronic diseases like cancer, diabetes, inflammation, arthritis, aging, atherosclerosis and various neurodegenerative disorders mainly arises from oxidative stress initiated by highly reactive and unstable free radicals. So, the oxidation and antioxidation balance should be maintained for a healthy biological system. This can be achieved by exploring compounds with antioxidant activity (Agbor *et al*., 2006; Krishnaswami *et al*., 2013). Plants are tremendous source for such antioxidants. Several studies revealed that various phytochemicals especially phenolic compounds possess remarkable antioxidant activity (Van Acker *et al*., 1996; Pietta, 2000; Williams *et al*., 2004; Chatterjee *et al.*, 2007). Thus, in recent years, researchers mainly focused on isolation and identification of such antioxidants from various plant species and looking for new leads to develop better drugs from these phytochemicals for various diseases by oxidative stress. It may help to reduce the risk of using synthetic antioxidant and anticancer drugs. *Piper* is one of such plant genera with diverse medicinal properties. *P. nigrum, P. longum, P. chaba* and *P. colubrinum* are the *Piper* species selected for the study.

P. nigrum (Black pepper) is an important spice valued for its aroma and pungency. Aroma is contributed by essential oil constituents and pungency by the alkaloid piperine. Black pepper and its alkaloid piperine is known for its therapeutic properties like antimicrobial, analgesic, antipyretic, antioxidant, anticancer and also for enhancement of bioavailability of drugs (Lee *et al*., 1984; Bano *et al*., 1991; Vijayakumar *et al.*, 2004; Karsha and Lakshmi, 2010; Pingili *et al*., 2012). *P. longum* (Long pepper) and *P. chaba* has been used in traditional medicine and are the major ingredients in many Ayurvedic

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systems. Their antimicrobial activity, antioxidant activity, antitumor effect and efficacy against respiratory tract disorders, rheumatic pains and diarrhoea were studied (Anshuman *et al.*, 1984; Yusuf *et al*., 1994; Srinivasa *et al.,* 2001; Sunila and Kuttan, 2004; Taufiq-Ur-Rahman *et al.*, 2005; Samudram *et al*., 2009; Rahman *et al*., 2011). *P. colubrinum* is an exotic *Piper* species native to Northern part of South America. It has great importance because of its resistance to *Phytophthora capsici* and *Radopholus similis* (Ravindran and Remashree, 1998). But it is to be noted that medicinal values of *P. colubrinum* Link. is under explored.

Present study is aimed to find *in vitro* antioxidant activity and cytotoxicity of sequential extracts from four *Piper* species *viz. P. nigrum, P. longum, P. chaba* and *P. colubrinum* and to find out correlation between total phenolics of different extracts of this species with different antioxidant assays. *In vitro* cytotoxicity of sequential extracts was checked on cervical cancer cell line CaSki since it is one of the least studied cancer cell line among these *Piper* species.

Materials and Methods

Collection of samples

Matured and dried fruits/berries of *P. nigrum* (wild), *P. longum, P. chaba, P. colubrinum* and six high yielding black pepper varieties (Sreekara, Subhakara, IISR Malabar Excel, IISR Thevam, Panchami and Panniyur-1) were collected from ICAR-Indian Institute of Spices Research (IISR) Experimental Farm, Peruvannamuzhi, Kerala, India and used for the study.

Chemicals

Folin-Ciocalteau reagent, ammonium molybdate, ascorbic acid, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, ferrozine, Ethylene diamine tetraaceticacid (EDTA) and dimethylsulphoxide (DMSO) were purchased from Sisco Research Laboratories (Mumbai, India). Gallic acid, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), butylated hydroxyanisole (BHA) and 3-(4,5--dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and antibiotic antimycotic mixture were purchased from Life Technologies (Carlsbad, CA, USA). All other reagents and chemicals used were of analytical grade and of highest purity.

Preparation of extracts

All the samples were powdered and extracted sequentially with n-hexane, chloroform, methanol and water in the increasing order of polarity using Soxhlet apparatus. After the completion of extraction with one solvent, the sample left was dried at room temperature and extracted with next solvent. Each extract was filtered and evaporated to dryness using a BUCHI rotary evaporator equipped with BUCHI rotavapor R-205 and BUCHI heating bath B-490. The concentrated extracts were stored at 4°C until analysis. Sequential extraction helps for effective and complete extraction of compounds with different polarity (Policegoudra *et al*., 2011).

Determination of total phenol content

Total phenolic content of all extracts was estimated by Folin-Ciocalteau method (Malick and Singh, 1980). In this method, phenolics reacts with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured molybdenum blue which is measured at 650 nm and the results were expressed as milligram gallic acid equivalents/g of extract (mg GAE/g of extract).

In vitro *antioxidant activity*

Antioxidant activity was tested using 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity, Phosphomolybdenum method, Ferric reducing power method and Ferrous chelating activity. Stock solution of all the extracts and synthetic antioxidant Butylated Hydroxyanisole (BHA) was prepared in methanol with a concentration of 10 mg/mL.

Free radical scavenging activity on 1,1-diphenyl-2-picryl hydrazyl (DPPH)

The antioxidant activity of the extracts was determined in terms of their hydrogen donating or radical scavenging ability and thus to scavenge DPPH free radical (Braca *et al.*, 2001). A working solution with a concentration range of 10 - 500 µg/mL for methanol and chloroform extracts and 100 - 2000 µg/mL for hexane and water extracts were prepared from the respective stock solutions. One mL of each aliquot was added into test tubes and final volume was made up to 4 mL with methanol. One mL of 0.004% DPPH was added to the samples. After proper mixing, samples were incubated at dark for 30 minutes and absorbance was taken at 517 nm with UV-VIS spectrophotometer (UV-1800, Shimadzu Corp., Japan). The synthetic antioxidant BHA was taken as positive control. Radical scavenging ability of each extract was expressed as IC_{50} value, which represents the amount of extract required to scavenge

50% DPPH free radicals. IC_{50} value was calculated using statistical software SAS 9.3 and expressed as µg/mL.

Total antioxidant activity by Phosphomolybdenum method

An aliquot of 50 µL for methanol and chloroform extracts and 100 µL for hexane and water extracts were taken from the corresponding stock solutions and the final volume was made up to 3 mL with methanol. One mL of phosphomolybdenum reagent (0.6 M sulphuric acid, 28 mM disodium hydrogen phosphate, 4 mM ammonium molybdate) was added into the tubes and incubated at 95°C for 90 minutes. After incubation, the samples were read at 695 nm using UV-VIS spectrophotometer (UV-1800, Shimadzu Corp., Japan). Ascorbic acid (0.2-1.0 mM) was used as standard for the preparation of calibration curve. The results were expressed as molar ascorbic acid equivalents/g of extract (M AAE/ g of extract). Synthetic antioxidant BHA was used for comparison (Prieto *et al*., 1999).

Ferric reducing power (FRP) method

The reductive capacity of extracts was estimated by the method described by Oyaizu (1986). 50 µL of methanol, chloroform and hexane extracts and 100 µL of water extract were taken from the corresponding stock solutions and the final volume was made up to 1 mL with distilled water. The samples were then mixed with phosphate buffer (0.2 M, pH 6.6). Potassium ferricyanide (2.5 mL, 1% W/V) was added to the mixture and incubated at 50°C for 30 min. The reaction was terminated by adding trichloroacetic acid (10% W/V) and the mixture was centrifuged at 3000 rpm for 20 minutes. The supernatant was mixed with distilled water and $FeCl_3$ (0.1% W/V) solution and the absorbance was measured at 700 nm using UV-VIS spectrophotometer (UV-1800, Shimadzu Corp., Japan). Ascorbic acid (0.25-1.0 mM) was used as standard for the preparation of calibration curve. Increased absorbance of the reaction mixture indicated greater reducing power and it was expressed in molar ascorbic acid equivalents/g of extract (M AAE/ g of extract). Synthetic antioxidant BHA was kept as positive control.

Determination of ferrous chelating ability

The ability of extracts to chelate ferrous ion was estimated by measuring the intensity of ferrous-ferrozine complex (Carter, 1971). An aliquot of 25 µL for methanol and chloroform extracts and 50 µL for hexane and water extracts from the stock solutions were taken into test tubes and made up the volume to 3 mL with methanol. All the test solutions were then treated with $FeCl_2$ (0.1 mL; 2 mM). After incubation for 5 minutes, 0.4 mL of 5 mM ferrozine was added to the above mixture. After incubation for 10 minutes at room temperature, the absorbance was recorded at 562 nm with UV-VIS spectrophotometer (UV-1800, Shimadzu Corp., Japan). EDTA (10-50 µg) was used as standard for the preparation of calibration curve. Ferrous chelating ability was expressed as milligram EDTA/g of extract (mg EDTA/g of extract).

In vitro *cytotoxicity analysis*

In vitro cytotoxicity study of sequential extracts was tested on cervical cancer cell line CaSki by MTT assay. Cervical cancer cell line CaSki was cultured as adherent monolayer as per earlier method (Freshney, 2007) and maintained in 90% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 5% CO_2. Stock solution (250 mg/mL) of each extract was prepared in DMSO and stored at -20°C until use.

MTT Assay

MTT (3-(4,5 Dimethylthiazol-2yl)-2,5-Diphenyl Tetrazolium Bromide) assay (Mosmann, 1983) is based on the ability of a mitochondrial dehydrogenase enzyme of viable cells to cleave the tetrazolium rings of the pale yellow tetrazole MTT to a dark blue formazan crystals. Formazan crystals are impermeable to cell membranes which results in its accumulation within healthy cells. The crystals can be solubilised by adding lysis buffer and its colour can be measured spectrophotometrically. The level of the coloured formazan product is directly proportional to the number of surviving cells. The stock solutions of extracts were diluted in 10% DMEM to get lower concentration of extracts (0.5 mg/mL). Cells harvested in the log phase of growth were counted and seeded (5×10^3 cells/ well) in 96 well micro titer plates and incubated over night at 37°C in a humidified 5% CO_2 incubator (NuAire, USA). The cells were then allowed to react with different amount of extracts (25, 50 and 100 µg) for 24, 48 and 72 hrs in a humidified 5% CO_2 incubator (NuAire, USA) at 37°C. Synthetic anticancer drug Doxorubicin served as positive control whereas 10% DMEM was taken as negative control. After incubation, the medium was discarded and wells were washed with PBS. 100 µL of the MTT (5% in 10% DMEM media) was added and incubated for 2 hours. MTT lysis buffer (100 µL) was added to solubilise the colored formazan crystals formed by the reduction of MTT. After incubated for 4 hrs, the absorbance was measured at 570 nm using a micro plate spectrophotometer (Bio-Tek, USA).

The percentage cytotoxicity was determined and IC_{50} (amount of extracts required for 50% cytotoxicity) values were calculated from the dose response curve for CaSki. Potential extracts were again put for MTT assay and their IC_{50} was calculated by using Microsoft Excel 2007 and compared with synthetic anticancer drug Doxorubicin.

Preliminary phytochemical analysis of screened extracts

The extracts screened for high antioxidant activity and *in vitro* cytotoxicity were subjected to preliminary phytochemical screening. The extracts were tested for phenolics, alkaloids, flavones, flavonols, steroids/triterpenes, saponins, fixed oils and fats, carbohydrates and protein by adopting standard protocols (Trease and Evans, 2002; Khandelwal, 2008; Kokate *et al*., 2008).

Statistical analysis

Data were combined and analyzed by analysis of variance (ANOVA). The ANOVA was performed with the MSTATC software (version 1.41). The Significant differences ($p<0.05$) was estimated by Duncan's multiple range test (DMRT) using 'RANGE' procedure and the correlation coefficient (r) was determined using the 'CORR' procedure of MSTATC. Values were expressed as Mean ± SD of three replicates and superscripts represent significant difference as per DMRT ($p < 0.05$).

Results and Discussion

Total phenolic content of extracts

It is reported that phenolics especially phenolic acids and flavonoids shows antioxidant activity and they are responsible for variation in antioxidant activity of plants (Luo *et al*., 2004; Demiray *et al*., 2009). Phenolics exhibit antioxidant activity by various mechanisms like radical scavenging activity, transition metal chelating activity and lipid peroxidation inhibition. Due to this multiple mechanism of antioxidant activity, phenolics became an interesting class of compounds for researchers to find natural health beneficial phytochemicals (Rice-Evans *et al*., 1997; Chen and Ahn, 1998; Yanishlieva and Marinova, 2001). Thus, estimation of total phenol from the selected extracts is crucial. Table 1 showed variability in total phenol content among four extracts from different black pepper varieties and *Piper* species. Chloroform and methanol extracts showed maximum phenol content than hexane and water extracts. Hexane extract of *P. colubrinum* also showed high phenolic content. Total phenol content was in the range of 3.14 ± 0.13 to 30.7±1.23, 14.02±0.56 to 100.6±4.02, 14.86±0.59 to 50.85±2.03 and 1.05 ±0.04 to 5.12±0.20 mg GAE/g of extract in hexane, chloroform, methanol and water extracts respectively. Chloroform extract of *P. colubrinum* followed by methanol extract of IISR Malabar Excel showed highest total phenol among all the extracts. Among black pepper varieties, methanol extract of IISR Malabar Excel showed highest total phenol content followed by methanol extract of Panchami. It was clear from the table 1 that the extracts showed significant variability in their total phenol content among black pepper varieties and also among *Piper* species.

DPPH free radical scavenging activity

Table 2 showed the inhibitory concentration of each extract required to scavenge 50% DPPH free radicals (IC_{50} in µg/mL). Though all the extracts exhibited free radical scavenging ability, chloroform and methanol extracts predominated. Hexane extract of *P. colubrinum* also showed high DPPH radical scavenging ability. IC_{50} value for chloroform and methanol extracts was found to be very low (27.4 ±1.1 to 228.9±9.2 µg/mL for chloroform extract and 29.42±1.2 to 153.9±6.2 µg/mL for methanol extract) and this indicated their high ability to scavenge DPPH free radicals. Among methanol extracts, IISR Malabar Excel and among chloroform extracts, *P. colubrinum* showed highest radical scavenging activity. Methanol extract of Panchami and *P. colubrinum* also showed high ability to scavenge DPPH. Hexane extracts (except *P. colubrinum*) and water extracts showed comparatively low DPPH radical scavenging ability with high IC_{50} values. Significant variation was observed among varieties and also among *Piper* species in DPPH radical scavenging ability. DPPH radical scavenging activity of BHA was also checked and compared with that of extracts. It was found that BHA was superior to extracts for scavenging DPPH free radicals (IC_{50} - 5.2 µg/mL).

DPPH free radical scavenging ability of samples is due to their hydrogen donating ability. DPPH inhibitory capacity of water and methanol extracts of black pepper was reported as 35.2 and 45.66% (Gupta *et al*., 2014). Ethyl acetate and water extract of black pepper showed a concentration dependent increase in their DPPH radical scavenging ability (Asimi *et al*., 2013). Methanolic extract of black pepper showed good DPPH scavenging ability with IC_{50} value of 144.1±2.2 µg/mL (Nooman *et al*., 2008). DPPH scavenging ability of ethanol extract of black pepper from Brazil was reported with EC_{50}

Table 1. Total Phenolic content from different extracts (mg GAE/g of extract)

Sample	Hexane	Chloroform	Methanol	Water
Sreekara	5.98±0.24^{c}	22.32±0.89^{c}	17.44±0.70^{de}	3.41±0.14^{c}
Subhakara	4.90±0.20^{de}	14.02±0.56^{e}	21.28±0.85^{c}	1.73±0.07^{e}
IISR Malabar Excel	7.74±0.31^{b}	35.26±1.41^{b}	50.85±2.03^{a}	1.12±0.04^{f}
IISR Thevam	4.26±0.17^{e}	21.14±0.85^{c}	20.72±0.83^{c}	5.12±0.20^{a}
Panniyur-1	6.98±0.28^{b}	17.26±0.69^{d}	19.33±0.77^{cd}	1.62±0.06^{e}
Panchami	7.43±0.30^{b}	23.64±0.95^{c}	38.74±1.55^{b}	1.12±0.04^{f}
P. nigrum (wild)	3.14±0.13^{f}	15.06±0.60^{de}	14.86±0.59^{f}	3.84±0.15^{b}
P. longum	5.41±0.22^{cd}	15.66±0.63^{de}	17.38±0.70^{de}	2.16±0.09^{d}
P. chaba	5.37±0.21^{cd}	16.50±0.66^{de}	16.53±0.66^{ef}	2.22±0.09^{d}
P. colubrinum	30.7±1.23^{a}	100.6±4.02^{a}	37.26±1.49^{b}	1.05±0.04^{f}

value 110±2 g spice/kg DPPH (Mariutti *et al*., 2008). Petroleum ether extract of black pepper was also subjected to DPPH scavenging activity and found that there is a concentration dependent increase in their scavenging activity and hence black pepper could be considered as a potent source of natural antioxidant (Singh *et al*., 2008). IC_{50} for DPPH radical scavenging activity of chloroform, ethyl acetate, hexane, ethanol, hydroethanol and aqueous extracts of *P. longum* were reported as 6,54, 70, 50, 26, 19.5 µg/mL respectively (Barua *et al*., 2014). A strong bioactive alkaloid Chabbarin was isolated from acetone extract of *P. chaba* and its strong ability to scavenge DPPH was reported with a very less IC_{50} value of 3 µg/mL (Biswas *et al*., 2012). Antioxidant activity of methanolic extract of *P. longum* and *P. chaba* was estimated by various assays including DPPH radical scavenging activity and showed that *P. longum* is superior to *P. chaba* (Ramesh kumar *et al*., 2011).

Total antioxidant activity by Phosphomolybdenum method

Table 2 showed total antioxidant capacity of each extract by Phosphomolybdenum method. The assay is based on the ability of sample to reduce Mo (VI) to Mo (V) and the resultant green colour was measured. Hexane extract was in the range of 0.41±0.08 to 0.75±0.09 M AAE/g of extract whereas chloroform, methanol and water extracts were in the range of 0.81±0.26 to 1.85±0.01, 0.65±0.13 to 1.87±0.01 and 0.09±0.01 to 0.49±0.13 M AAE/ g of extract. All methanol and chloroform extracts and also the hexane extract of *P. colubrinum* showed high total antioxidant activity whereas hexane extract of other samples and all water extracts expressed comparatively low activity. Methanol extract of IISR Malabar Excel and chloroform extract of *P. colubrinum* showed highest antioxidant activity. The result of extracts was compared with that of synthetic standard BHA and found that BHA showed high ability to reduce Molybdenum (4.6 Molar AAE/ g of extract). The ability of cold methanolic extract from *P. nigrum* and *P. longum* to reduce molybdenum was reported earlier (Prasad and Sushant, 2014).

Ferric reducing power (FRP) method

Antioxidant activity was also checked on the basis of the ability of antioxidants to reduce ferric (III) ion to ferrous (II) ion. Table 3 showed ferric reducing capacity of each extract. Among hexane extracts, *P. colubrinum* showed highest activity followed by IISR Malabar Excel and Panchami. Chloroform and methanol extracts were in the range of 0.54±0.01 to 1.02±0.16 and 0.50±0.01 to 1.18±0.02 M AAE/ g of extract respectively. For chloroform extract, *P. colubrinum* followed by IISR Malabar Excel and for methanol extract, IISR Malabar Excel followed by Panchami showed highest ferric reducing power. Water extract showed comparatively very low activity. Among all extracts, chloroform extract of *P. colubrinum* and methanol extract of IISR Malabar Excel showed highest ferric reducing power ability. Ferric reducing ability of different extracts from above selected *Piper* species were also reported by researchers (Kapoor *et al*., 2009; Gopalakrishna *et al*., 2010).

Ferrous chelating activity

The ability to chelate ferrous ion was in the range of 72.17±2.9 to 137±5.5, 228.5±9.14 to 320.1±12.8, 245.5±9.82 to 349.8±13.9 and 124.5±4.9 to 170.6±6.8 mg EDTA/g of extract for hexane, chloroform, methanol and water respectively (Table 3). Hexane and water extracts showed comparatively low ferrous chelating ability whereas it was found to be high for methanol and chloroform extracts. Methanol extract of IISR Malabar Excel was found to be superior for their ability to chelate ferrous ions. Chloroform extract of *P. colubrinum*, methanol extract of Panchami and methanol extract of *P. colubrinum* were also showed high ferrous chelating ability.

Secondary metabolites like phenolics and flavonoids can chelate metal ions and often decrease the prooxidant activity of metal ions. Metal chelating potency of phenolic compounds is dependent upon their unique phenolic structure and the number and location of the hydroxyl groups (Van Acker *et al*., 1998; Santoso *et al*., 2004). Metal chelating ability

Table 2. DPPH radical scavenging activity and Total antioxidant capacity of sequential extracts

DPPH scavenging activity of sequential extracts in terms of IC_{50} (µg/mL)				
Sample	**Hexane**	**Chloroform**	**Methanol**	**Water**
Sreekara	1068.0±42.7^{d}	148.2±5.9^{e}	62.45±2.5^{c}	935.47±37.4^{e}
Subhakara	1240.0±49.6^{c}	228.9±9.2^{a}	54.09±2.2^{d}	800.00±32.0^{f}
IISR Malabar Excel	776.54±31.1^{f}	99.61±3.9^{f}	29.42±1.2^{f}	1942.0±77.7^{a}
IISR Thevam	1430.0±57.2^{b}	154.2±6.2^{de}	54.04±2.2^{d}	765.20±30.6^{f}
Panniyur-1	1019.0±40.8^{de}	170.1±6.8^{bc}	54.58±2.2^{d}	1784.0±71.4^{b}
Panchami	970.00±38.8^{e}	104.6±4.2^{f}	40.14±1.6^{e}	1620.0±64.8^{c}
P. nigrum (wild)	1830.0±73.2^{a}	164.9±6.6^{bcd}	153.9±6.2^{a}	1025.0±41.0^{e}
P. longum	1400.0±56.0^{b}	174.1±6.9^{b}	130.4±5.2^{b}	1660.0±66.4^{c}
P. chaba	1800.0±72.0^{a}	159.9±6.4^{cd}	152.1±6.1^{a}	1900.0±76.0^{a}
P. colubrinum	122.70±4.91^{g}	27.40±1.1^{g}	42.28±1.7^{e}	1207.0±48.3^{d}
BHA	5.200±0.21			
Total antioxidant capacity by Phosphomolybdenum method (M AAE/ g of extract)				
Sample	**Hexane**	**Chloroform**	**Methanol**	**Water**
Sreekara	0.59±0.01^{bc}	1.19±0.19^{bc}	0.83±0.23^{d}	0.32±0.01^{bc}
Subhakara	0.53±0.06^{bcd}	1.08±0.04^{bc}	0.85±0.03^{d}	0.24±0.07^{cd}
IISR Malabar Excel	0.62±0.09^{b}	1.06±0.07^{bc}	1.87±0.01^{a}	0.09±0.01^{e}
IISR Thevam	0.51±0.04^{bcd}	0.81±0.26^{c}	1.13±0.04^{c}	0.49±0.13^{a}
Panniyur-1	0.55±0.01^{bcd}	0.83±0.02^{bc}	0.66±0.10^{d}	0.24±0.01^{cd}
Panchami	0.64±0.01^{ab}	1.24±0.12^{b}	1.52±0.21^{b}	0.15±0.01^{de}
P. nigrum (wild)	0.41±0.08^{d}	1.01±0.25^{bc}	0.73±0.08^{d}	0.45±0.06^{ab}
P. longum	0.43±0.06^{d}	1.16±0.34^{bc}	0.65±0.13^{d}	0.19±0.03^{de}
P. chaba	0.47±0.01^{cd}	0.85±0.06^{bc}	0.79±0.08^{d}	0.21±0.01^{cde}
P. colubrinum	0.75±0.09^{a}	1.85±0.01^{a}	1.15±0.03^{c}	0.33±0.01^{bc}
BHA	4.6±0.18			

of water and ethanolic extract of black pepper was reported as 84±2.20% and 83±4.36% respectively (Gulcin, 2005).

Correlation between total phenolic content and antioxidant activity

Several studies reported that phenolic constituents in spices and other plants have significant antioxidant properties (Shan *et al.*, 2005; Wu *et al*., 2006; Maizura *et al*., 2011). In the present study, chloroform and methanol extracts were screened for high antioxidant activity by different assays. So correlation of total phenolic content of these two extracts from all the samples with their antioxidant activity by each assay was performed. Result showed significant ($p<0.05$) negative correlation between total phenolic content of each extract and their IC_{50} value to scavenge DPPH free radicals. Correlation coefficient (r) for total phenolic content of chloroform extract and their IC_{50} for DPPH scavenging activity was -0.86 and that for total phenolic content of methanol extract and their IC_{50} for DPPH scavenging activity was -0.72. This indicated that, extract with high total phenol shows less IC_{50} and thus more ability to scavenge DPPH free radicals. Correlation coefficient (r) for total phenolic content of chloroform extract with their antioxidant activity by phosphomolybdenum method, FRP and ferrous chelating activity was +0.86, +0.81 and +0.95 respectively ($p<0.05$). Total phenolic content of methanol extracts of samples also showed significant ($p<0.05$) positive correlation with their antioxidant activity by phosphomolybdenum method (r=+0.94), FRP (r=+0.92) and ferrous chelating activity (r=+0.79). High ability of *P. colubrinum* hexane extract to scavenge DPPH may also be an indication of radical scavenging ability of phenolics since that extract contains more total phenol than other hexane extracts. Such linearity between total phenol of black pepper extracts and their antioxidant activity was reported by researchers (Gulcin, 2005; Nahak and Sahu, 2011).

In vitro *cytotoxicity of sequential extracts from* Piper *species and black pepper varieties.*

To screen the extracts with high cytotoxicity to CaSki, MTT assay was performed using all extracts with a mass range of 25-100 µg for three time intervals viz. 24, 48 and 72 hrs. All the extracts showed cytotoxicity (%) in a dose dependent and time dependent manner. Among all extracts tested, chloroform extracts of all samples and hexane extract of *P. colubrinum* expressed more cytotoxicity. IC_{50} value was calculated for chloroform extracts of black pepper and wild *P. nigrum* and they have categorised by Duncan's multiple range test (DMRT) to screen black pepper chloroform extract with

Table 3. Ferric reducing power (FRP) and ferrous chelating activity of sequential extracts

FRP Method (M AAE/ g of extract)				
Sample	**Hexane**	**Chloroform**	**Methanol**	**Water**
Sreekara	0.54±0.04^{cde}	0.64±0.04^{cde}	0.61±0.01^{f}	0.27±0.02^{ab}
Subhakara	0.57±0.04^{bcd}	0.54±0.01^{e}	0.75±0.01^{de}	0.23±0.01^{cd}
IISR Malabar Excel	0.63±0.05^{ab}	0.91±0.07^{ab}	1.18±0.02^{a}	0.22±0.01^{de}
IISR Thevam	0.59±0.01^{abc}	0.78±0.04^{bc}	0.81±0.05^{cd}	0.29±0.01^{a}
Panniyur-1	0.59±0.04^{abc}	0.73±0.07^{cd}	0.71±0.04^{e}	0.25±0.01^{bc}
Panchami	0.63±0.05^{ab}	0.76±0.01^{bc}	0.93±0.06^{b}	0.21±0.01^{e}
P. nigrum (wild)	0.46±0.01^{e}	0.55±0.12^{e}	0.52±0.02^{g}	0.26±0.01^{b}
P. longum	0.49±0.03^{e}	0.59±0.04^{de}	0.50±0.01^{g}	0.22±0.01^{de}
P. chaba	0.52±0.04^{de}	0.57±0.03^{de}	0.56±0.01^{fg}	0.22±0.01^{de}
P. colubrinum	0.65±0.04^{a}	1.02±0.16^{a}	0.84±0.05^{c}	0.21±0.01^{e}
BHA	3.46±0.14			
Ferrous chelating activity (mg EDTA/g of extracts)				
Sample	**Hexane**	**Chloroform**	**Methanol**	**Water**
Sreekara	130.5±5.22^{b}	232.2±9.29^{cd}	294.5±11.8^{cde}	167.7±6.7^{ab}
Subhakara	108.6±4.3^{d}	228.5±9.14^{d}	314.7±12.6^{bc}	157.8±6.3^{bc}
IISR Malabar Excel	131.7±5.3^{ab}	251.6±10.1^{b}	349.6±13.9^{a}	126.6±5.1^{e}
IISR Thevam	131.1±5.2^{b}	234.3±9.37^{bcd}	297.2±11.9^{cd}	170.6±6.8^{a}
Panniyur-1	133.6±5.3^{ab}	230.3±9.21^{cd}	316.5±12.7^{bc}	153.4±6.1^{cd}
Panchami	132.8±5.3^{ab}	249.3±9.97^{bc}	320.8±12.8^{b}	143.4±5.7^{d}
P. nigrum (wild)	72.19±2.9^{e}	241.6±9.66^{bcd}	245.5±9.82^{f}	166.2±6.7^{ab}
P. longum	111.4±4.5^{d}	249.1±9.96^{bc}	273.4±10.9^{e}	147.9±5.9^{cd}
P. chaba	123.5±4.9^{c}	243.3±9.73^{bcd}	290.8±11.6^{de}	153.2±6.1^{cd}
P. colubrinum	137.0±5.5^{a}	320.1±12.8^{a}	319.8±12.8^{b}	124.5±4.9^{e}
BHA	--			

highest cytotoxicity. Result showed that chloroform extract of IISR Malabar Excel showed lowest IC_{50} value and thus highest cytotoxicity to CaSki for all the three time intervals (Table 4 A). Thus chloroform extract of IISR Malabar Excel, *P. longum, P. chaba, P. colubrinum* and hexane extract of *P. colubrinum* were screened as potent cytotoxic extracts. All the potential extracts, along with synthetic anticancer drug Doxorubicin were again put for MTT assay with a mass range of 5- 100 µg for three time intervals (24, 48 and 72 hrs) and their IC_{50} was calculated and compared (Table 4 B). Results showed more cytotoxicity with more extract and increased time of exposure with CaSki. Chloroform extract of *P. longum* and *P. colubrinum* were found to be highly toxic to CaSki for all the three time intervals. Chloroform extract of IISR Malabar Excel was also toxic to CaSki for 24, 48 and 72 hrs. Hexane extract of *P. colubrinum* had almost similar toxicity to CaSki as that of chloroform extract of IISR Malabar Excel. *P. chaba* was less toxic in 24 and 48 hrs but highly toxic in 72 hrs. IC_{50} for Doxorubicin was <5µg for all the three time intervals. The IC_{50} value for certain extracts and Doxorubicin were beyond the adopted mass range. So, further experiment has to be performed to find out their exact IC_{50} values.

In vitro cytotoxicity was studied in black pepper extracts for esophageal squamous cell line TE-13 (Dwivedi *et al*., 2011), breast cancer cell lines like MCF-7, MDA-MB-231 and MDA-MB-468 (Sriwiriyajan *et al.*, 2014), in *P. longum* extracts for lung epithelial adenocarcinoma cell line HCC-827 (Sawhney *et al.*, 2011) and leukaemic cell line K562 (Joy *et al*., 2010) and in *P. chaba* extracts for large lung carcinoma cell line COR-L23, cervical cancer cell line HeLa and liver cancer cell line HepG2 (Ruangnoo *et al*., 2012). However, cervical cancer cell line CaSki was least studied in these *Piper* species.

Preliminary phytochemical analysis of screened extracts

In the case of *in vitro* antioxidant activity, methanol extract of IISR Malabar Excel and chloroform extract of *P. colubrinum* showed highest activity. The methanol extract of Panchami also showed high antioxidant activity. In the case of *in vitro* cytotoxicity, chloroform extract of *P. colubrinum*, IISR Malabar Excel, *P. longum, P. chaba* and hexane extract of *P. colubrinum* showed high activities. These screened extracts were tested preliminary for the presence of constituents which may contribute to their high activities. These extracts were tested for alkaloids, flavones, flavonols, phenolics, steroids/triterpenes, saponins, fixed oils and fats, carbohydrates and protein and results are shown in table 5. Alkaloids, phenolics and steroids/triterpenes were present in all the extracts whereas flavonols were present in all the methanol extracts and chloroform extract of *P. colubrinum*. Flavones were present in all samples except hexane extract of *P. colubrinum* whereas saponins were present only in methanolic extracts.

Table 4. A: IC_{50} value for chloroform extract of black pepper varieties and wild *P. nigrum*; B: IC_{50} value for potential extracts and Doxorubicin

A: IC_{50} (µg) for chloroform extract of black pepper varieties and wild *P. nigrum*

Sample	24 hrs	48 hrs	72 hrs
Sreekara	64 ± 2.56^{c}	58 ± 2.32^{a}	28 ± 1.12^{c}
Subhakara	64 ± 2.56^{c}	60 ± 2.40^{a}	28 ± 1.12^{c}
IISR Malabar Excel	60 ± 2.40^{c}	30 ± 1.20^{c}	25 ± 1.00^{d}
Panchami	72 ± 2.88^{b}	32 ± 1.28^{c}	26 ± 1.04^{cd}
Panniyur-1	84 ± 3.36^{a}	52 ± 2.08^{b}	32 ± 1.28^{b}
IISR Thevam	60 ± 2.40^{c}	32 ± 1.28^{c}	26 ± 1.04^{cd}
P. nigrum (wild)	86 ± 3.44^{a}	58 ± 2.32^{a}	36 ± 1.44^{a}

B: IC_{50} (µg) for potential extracts and Doxorubicin

Sample	24 hrs	48 hrs	72 hrs
IISR Malabar Excel (Chloroform extract)	60.00±2.4	30.00±1.2	25.00±1.0
P. chaba (Chloroform extract)	>100	100.0 ±4.0	<5
P. longum (Chloroform extract)	12.00±0.5	5.2.00±0.2	<5
P. colubrinum (Chloroform extract)	12.00±0.5	<5	<5
P. colubrinum (Hexane extract)	56.00±2.2	32.00±1.3	25.00±1.0
Doxorubicin	<5	<5	<5

Table 5. Preliminary phytochemical analysis of screened extracts

Phytochemicals Tested	Chemical Test	Screened extracts						
		MM	MP	CC	CM	CL	CH	HC
Alkaloids	Meyer's Test	+	+	+	+	+	+	+
Flavones	Test with NaOH	+	+	+	+	+	+	-
Flavonols	Shinoda's Test	+	+	+	-	-	-	-
Phenolics	Ferric chloride Test	+	+	+	+	+	+	+
Steroids/triterpenes	Salkowski Test & Liebermann-Burchard Test	+	+	+	+	+	+	+
Saponins	Foam Test	+	+	-	-	-	-	-
Fixed oils and fats	Saponification Test	-	-	-	-	-	-	+
Carbohydrates	Molisch's Test	+	+	-	-	+	+	-
Protein	Biuret Test	-	-	-	-	-	-	-

*MM- Methanol extract of IISR Malabar Excel, MP-Methanol extract of Panchami, CC-Chloroform extract of *P. colubrinum*, CM-Chloroform extract of IISR Malabar Excel, CL- Chloroform extract of *P. longum*, CH-Chloroform extract of *P. chaba*, HC-Hexane extract of *P. colubrinum.*

** (+) for presence, (-) for absence.

Fixed oils and fats were present only in hexane extract of *P. colubrinum*. Carbohydrates were present in all methanol extracts and chloroform extract of *P. longum* and *P. chaba*. None of the sample has given positive result for protein. Based on these qualitative analysis it was found that potent extracts are good source for various phytochemicals.

Conclusion

In vitro antioxidant activity and cytotoxicity of sequential extracts of four important *Piper* species viz. *P. nigrum, P. chaba, P. longum* and *P. colubrinum* and six black pepper varieties *viz.* Sreekara, Subhakara, IISR Malabar Excel, Panniyur-1, Panchami and IISR Thevam were performed. It can be concluded that methanol and chloroform extracts predominated for antioxidant activity. Considering the four *in vitro* antioxidant systems tested, methanol extract of IISR Malabar Excel and chloroform extract of *P. colubrinum* were screened as extracts with highest antioxidant activity. Significant positive correlation was obtained for total phenolic content of methanol and chloroform extracts with their antioxidant activity by all the four assays. *In vitro* cytotoxicity of the extracts on cervical cancer cell line CaSki showed that chloroform extracts of all the samples and hexane extract of *P. colubrinum* showed more cytotoxicity. Among the potential extracts, chloroform extracts of *P. longum* and *P. colubrinum* were highly toxic to CaSki. By considering both *in vitro* antioxidant activity and cytotoxicity, chloroform extract of *P. colubrinum* was found to be more active than other extracts. Qualitative analysis showed that chloroform extracts of *P. colubrinum* is a good source of high value constituents. Hence further studies can be

performed for identification of active phytochemical constituents from this extract. This is the first report regarding *in vitro* antioxidant activity and cytotoxicity on CaSki for sequential extracts of *P. colubrinum* fruits cultivated in India. This is also the first report on variability in antioxidant activity and cytotoxicity (on CaSki) of sequential extracts from black pepper varieties selected for the study.

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