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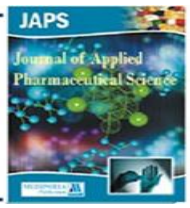

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Phytochemical Evaluation and Antioxidant activity of *Piper cubeba* and *Piper nigrum*

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ABSTRACT

Indian spices that provide flavor, color, and aroma to food also possess many therapeutic properties. Ancient Indian texts of Ayurveda, an Indian system of medicine, detailed the medicinal properties of these plants and their therapeutic usage. Recent scientific research has established the presence of many active compounds in these spices that are known to possess specific pharmacological properties. The therapeutic efficacy of these individual spices for specific pharmacological actions has also been established by experimental and clinical studies. The medicinal effects traditionally ascribed to Indian spices are validated by modern pharmacological and experimental techniques, thus providing a scientific rationale to their traditional therapeutic usage. Many plant-derived molecules have shown a promising effect in therapeutics. Among the plants investigated to date, one showing enormous potential is the Piperaceae. Piperine is an alkaloid found naturally in plants belonging to the pyridine group of Piperaceae family, such as *Piper nigrum* and *Piper cubeba*. It is widely used in various herbal cough syrups and it is also used in anti inflammatory, anti malarial, anti leukemia treatment. So the present study was aimed to extract the phytochemical compounds in different solvent system in *Piper nigrum* and *Piper cubeba*. In preliminary screening and confirmatory test it was identified as alkaloid. High antioxidant activity was found in *Piper cubeba* ethanol extract i.e. 77.61±0.02% in comparison to *Piper nigrum* extracts with 74.61±0.02% with IC_{50} values10.54±0.12µg/mg and 14.15±0.02 µg/mg respectively.

Keywords: *Piper nigrum, Piper cubeba,* Phytochemical, Antioxidant activity, DPPH.

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INTRODUCTION

Many plant derived molecule have shown a promising effect in therapeutics (Lokhande *et al*., 2007). Spices and herbs are recognized as sources of natural antioxidants and thus play an important role in the chemoprevention of diseases and aging. Among the plants investigated to date, one showing enormous potential is the pepper family otherwise known as Piperaceae (Dodson *et al*., 2000). *Piper nigrum* and *Piper cubeba* are the two flowering vine in the family Piperaceae. *Piper nigrum* (black pepper) it is a monocious or decorous climbing vine native to southern India and Srilanka and is extensively cultivated there and elsewhere in tropical regions. The shout climbing stem are very flexible with leathery blackish green leaves, they are widely cultivated in tropics. They have several uses such as they help in pain relief, rheumatism, chills, flu, colds, muscular aches and fever. Externally it is used for its rubefacient and as a local application for relaxed sore, throat and some skin disorder. It has antimicrobial (Dorman and Deans, 2000), antimutagenic (El-Hamss *et al*., 2003), antioxidant and radical scavenging property (Gulcin, 2005) and inhalation of black pepper oil increase the reflexive swallowing movement (Vijayakumar *et al*., 2004). Cubeb (*Piper cubeba*), or tailed pepper, is a plant in genus Piper,

cultivated for its fruit and essential oil. It is mostly grown in Java and Sumatra, hence sometimes called Java pepper. This is a perennial plant, with a climbing stem, round branches, about as thick as a goose-quill, ash-colored and rooting at the joints. The leaves are from four to six and a half inches long by one and a half to two inches broad, ovate-oblong, acuminate and very smooth. Flowers arranged in spikes at the end of the branches; fruit, a berry rather longer than that of black pepper. It is used to treat gonorrhea, dysentery, syphilis, abdominal pain and asthma (Eisai, 1995) and has also inhibitory effect on hepatitis C virus protease. Choi and Hwang (2003) demonstrated anti inflammatory and analgesic activity of methanol extract from the fruit of *Piper cubeba* it accumulates lignans and essential oil in a relatively high amount.

The alkaloids, of which some 5,500 are known, comprise the largest single class of secondary plant substance. Alkaloids are often toxic to man and many have dramatic physiological activities; hence their wide use in medicine. They are usually colorless, often optically active substances, most are crystalline but a few (e.g. nicotine) are liquids at room temperature. Piperine is an alkaloid found naturally in plants belonging to the pyridine group of Piperaceae family, such as *Piper nigrum* and *Piper longum*. Piperine is the Trans stereoisomer of 1-piperoylpiperidine. It is also known as (E, E)-1- piperoylpiperidine and (E, E)-1- [5-(1, 3-benzodioxol-5-yl)-1-oxo-2, 4-pentdienyl] piperidine. Piperine is the alkaloid responsible for the pungency of black pepper and long pepper, along with chavicine (an isomer of piperine). It has also been used in some forms of traditional medicine and as an insecticide. Majeed (1999) reported that piperine is widely used in various herbal cough syrups for its potent anti-tussive and bronchodilator properties. It is used in anti inflammatory, anti malarial, anti leukemia treatment. Recent medical studies have shown that it is helpful in increasing the absorption of certain vitamins, selenium, β-cartene, also increase the body's natural thermogenic activity.

The dried cubeb berries contain essential oil consisting monoterpenes (sabinene 50%, α-thujene, and carene) and sesquiterpenes (caryophyllene, copaene, α- and β-cubebene, δ-cadinene, germacrene), the oxides 1,4- and 1,8-cineole and the alcohol cubebol. About 15% of a volatile oil is obtained by distilling cubebs with water. Cubebene, the liquid portion, has the formula $C_{15}H_{24}$. It is a pale green or blue-yellow viscous liquid with a warm woody, slightly camphoraceous odor (Lawless and Julia, 1995). After rectification with water, or on keeping, this deposits rhombic crystals of camphor of cubebs. In India, Sanskrit texts included cubeb in various remedies. Charaka and Sushruta prescribed a cubeb paste as a mouthwash, and the use of dried cubebs internally for oral and dental diseases, loss of voice, halitosis, fevers, and cough. Unani physicians use a paste of the cubeb berries externally on male and female genitals to intensify sexual pleasure during coitus. Due to this attributed property, cubeb was called "Habb-ul-Uruus" (Khare, 2004). In traditional Chinese medicine cubeb is used for its alleged warming property. In Tibetan medicine, cubeb (ka ko la in Tibetan) is one of bzang po drug, six fine herbs beneficial to specific organs in the body, with cubeb assigned to the spleen (Stearns and Cyrus, 2000). Arab physicians of the Middle Ages were usually versed in alchemy, and cubeb was used, under the name Kababa, when preparing the water of al butm (Patai and Raphael, 1995).

In the present study an attempt was made to screen different multi solvent extracts prepared from dried fruits of *Piper nigrum* and *Piper cubeba* to study the antioxidant activity on basis of their phytochemical significance.

MATERIALS AND METHODS

Plant material

The dry fruits of *Piper nigrum* and *Piper cubeba* were collected from local tribal people of Koraput, Mayurbhanj District, Odisha. Then they were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were subsequently dried in a hot air oven at 40 ^{0}C for 48h, powdered and used for extraction.

Preparation of crude extract

50gms of dry powdered fruits of *Piper nigrum* and *Piper cubeba* were extracted successively with double distilled water, ethanol and methanol (each 400ml.) for 10-12 hrs. through Soxhlet apparatus method. Then collected solutions were filtered through Whatman No-1 filer paper. The extracts were evaporated to dryness under reduced pressure at 90^{0}C by Rotary vacuum evaporator to obtain the respective extracts and stored in a freeze condition at −18^{0}C until used for further analysis.

Qualitative screenings of phytochemicals

The qualitative screenings of powdered crude drugs for their active ingredients were carried out using the following standard procedures (Trease and Evans, 1983; Indian Pharmacopeias, 1996; Mukherjee, 2002; Horborne, 2005).

Phenolic estimation

The total phenol content of plant extracts were determined by using Folin-Ciocalteu Spectrophotometric method according to the method described (Kim *et al*., 2007). Reading samples on a UV-vis spectrophotometer at 650 nm. Results were expressed as Catechol equivalents (µg/mg).

Antioxidative activity

The evaluation of radical scavenging activity (antioxidant activity) was conducted by the method of (Blois, 1958) with modifications. The following concentrations of extracts were prepared 40µg/mL, 80µg/mL, 120µg/mL, 160µg/mL and 200µg/mL. A stock solution of the sample (100mg/ml) was diluted for 5 concentrations. Each concentration was tested in triplicate. The portion of sample solution (0.5ml) was mixed with 3.0ml of 0.1mM 1,1-Diphenyl-2-2picrylhydrazyl (DPPH, in 95% distilled ethanol) and allowed to stand at room temperature for 30 minute under light protection. The absorbance was measured at 517nm.The scavenging activity of the samples at corresponded intensity of quenching DPPH. Lower the absorbance of the

reaction mixture indicates higher free radical scavenging activity. The different in absorbance between the test and the control (DPPH in ethanol) was calculated and expressed as (%) scavenging of DPPH radical. The capability to scavenge the DPPH radical was calculated by using the following equation.

$$\text{Scavenging effect (\%)} = (1\text{-As/Ac}) \times 100$$

As is the absorbance of the sample at t =0 min.
Ac is the absorbance of the control at t=30 min.

In the DPPH test, antioxidants were typically characterized by their IC_{50} value (Inhibition Concentration of Sample required to scavenge 50% of DPPH radicals). The results were obtained by linear regression analysis of the dose response curve plotted using % inhibition and concentration.

RESULTS AND DISCUSSION

Phytochemical screening

In the present study, preliminary phytochemical testing shows (Table-1), the presence of high amount of glycosides, alkaloids, tannins, phenolics and other all the principal secondary metabolites were detected in ethanolic extract of *Piper nigrum* and *Piper cubeba.* The living system is protected from this by enzymes such as superoxide dismutase, glutathione peroxidase and catalase and certain endogenous antioxidant such as α–tocopherol, ascorbic acid, β–carotene and uric acid, since the endogenous antioxidants acting as intracellular defense systems protecting cells from free radicals damage and extensive lyses (Sies and Stahl., 1995; Pietta, 2000). Scavenging and diminishing the formation of oxygen-derived species are not 100% efficient, micro nutrients or antioxidants taken as supplements are particularly important in diminishing the cumulative oxidative damages (Vani *et al*., 1997). Atal *et al.*, (1985) showed that biochemical basis enhanced drug availability by piperine. The phytochemical screening and quantitative estimation of the percentage crude yields of chemical constituents of the plants studied showed that the leaves and stems were rich in alkaloids, flavonoids, tannins and saponins. They were known to show medicinal activity as well as exhibiting physiological activity (Sofowara, 1993). Steroids and phlobatannins were found to be present in all the plants. It has been found that some of these investigated plants contained steroidal compounds.

Table-1: Preliminary Phytochemical screening of *Piper nigrum* and *Piper cubeba.*

Plant's Name	Alkaloid	Glycosides	Terpenoid	Steroid	Flavonoid	Tannins	Reducing Sugar	Anthra -quinones
Piper nigrum	+	+	+	+	+	+	+	+
Piper cubeba	+	+	--	+	+	+	--	+

(+) Denotes average and (--) denotes absent

The effect of different solvents on the yields of crude extracts

The significant variation in the yields of *Piper nigrum* and *Piper cubeba* extracts were shown using various fraction solvents.

The yield of extracts using Water, Methanol and Ethanol in case of *Piper nigrum* were 2.80gm, 2.45gm and 3.35gm respectively. Likewise the *Piper cubeba* extract also followed the same order as the *Piper nigrum* extracts, and they were 1.51gm, 1.75gm and 2.56gm. The variation in yield may be due to the polarity of the solvents used in the extraction process (Table-3).

Total phenolic content

Medicinal plants are an important source of antioxidants (Rice-Evans, 2007). Natural anti-oxidants increase the anti-oxidant capacity of the plasma and reduce the risk of certain diseases (Prior and Cao, 2000). Polyphenols are the major plant compounds with anti-oxidant activity. Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids and flavonoids (Demiray *et al*., 2009). It is reported that the phenolics are responsible for the variation in the anti-oxidant activity of the plant (Luo *et al*., 2004). They exhibit anti-oxidant activity by inactivating lipid free radicals or preventing decomposition of hydro peroxides into free radicals (Pokorny 2001; Pitchaon *et al*., 2007). *Piper cubeba* showed the highest Phenolic content i.e. 123.1±0.05(µg/g) in comparison to *Piper nigrum* with 62.3±0.08(µg/g) (Table-2).

Table 2: Phenol content of *Piper cubeba* and *Piper nigrum* fruits in Ethanolic extracts.

Solvent	Total phenols (µg/g)	
	Piper cubeba	*Piper nigrum*
Ethanol	123.1±0.05	62.3±0.08

Table-3: Crude extracts and IC_{50} Values *Piper nigrum* and *Piper cubeba* in different solvent extracts.

	Piper cubeba		*Piper nigrum*	
Solvent used	**Crude Extracts (gm)**	**IC_{50} Value (µg/ml)**	**Crude Extracts (gm)**	**IC_{50} Value (µg/ml)**
Water	4.30	-------	3.51	-------
Methanol	4.15	-------	3.75	-------
Ethanol	4.35	10.54±0.12	3.56	14.15±0.02

DPPH free radical scavenging activity

The stable radical DPPH has been used widely for the determination of primary anti-oxidant activity (Brand-Williams *et al*., 1995; Katalinic *et al*., 2004). DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva *et al*., 2002). It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen donating ability (Chen *et al*., 1995). The collected fruit extracts exhibited remarkable DPPH free radicals scavenging ability at different concentrations. From these, the % inhibition concentrations and IC_{50}'s were calculated.

Table-4 shows the results of the free radical (DPPH) scavenging activity in % inhibition. The result revealed that the ethanol fraction of *Piper cubeba* exhibited the highest radical scavenging activity with 77.61±0.02 followed by its methanolic extract with 69.84±0.05and aqueous extract with 45.84±0.05. In

Table-4: DPPH scavenging activity *Piper cubeba* and *Piper nigrum* in different solvent extracts.

Conc. of extracts (µg/ml)	Antioxidant activity (%)					
	Water		Methanol		Ethanol	
	Piper cubeba	*Piper nigrum*	*Piper cubeba*	*Piper nigrum*	*Piper cubeba*	*Piper nigrum*
50	35.38±0.02	28.15±0.01	58.07±0.04	53.07±0.04	68.69±0.04	61.69±0.04
100	37.69±0.08	31.00±0.03	60.92±0.06	56.92±0.06	70.23±0.05	62.23±0.05
150	40.00±0.10	34.30±0.04	63.00±0.09	60.00±0.09	72.76±0.04	66.76±0.04
200	42.30±0.07	36.61±0.03	67.53±0.07	61.53±0.07	74.30±0.03	69.30±0.03
250	45.84±0.05	39.92±0.02	69.84±0.05	63.84±0.05	77.61±0.02	74.61±0.02

comparison to *Piper cubeba* and *Piper nigrum* extract shows less scavenging activity. The *Piper nigrum* extract of obtained from ethanol shows 74.61±0.02 i.e. highest scavenging activity followed by its methanolic extract with 63.84±0.05and aqueous extract with 39.92±0.02. In overall comparison the ethanolic extract of both *Piper cubeba* and *Piper nigrum* seeds show the highest scavenging activity followed by the aqueous and then methanol. Methanol and ethanol has been proven as effective solvent to extract phenolic compounds. Among solvents used in this study ethanol has showed the best effectiveness extracting phenolic components. Ethanol is preferred for the extraction of antioxidant compounds mainly because its lowers toxicity Karadeniz *et al.*, (2005). The antioxidant activities of the individual compounds may depend on structural factors, such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups and other structural features (Patt *et al.*, 1990). Piper species, commonly used in diet and traditional medicine, were assessed for their antioxidant potential. Catalase activity predominated in *Piper longum* Linn., followed by *Piper cubeba* Linn., green pepper, *Piper brachystachyum* Linn. and *Piper nigrum* Linn. Black pepper (*Piper nigrum* Linn.) was richest in glutathione peroxidase and glucose-6-phosphate dehydrogenase, green pepper was richest in peroxidase and vitamin C, while vitamin E was greater in *Piper longum* Linn. and *Piper nigrum* Linn. *Piper brachystachyum* Linn. and *Piper longum* Linn. were rich sources of vitamin A (Aqil *et al*., 2006). The antioxidant and radical scavenging activities of black pepper (*Piper nigrum* Linn.) seeds have been well reported (Gulcin, 2005). Both water extract and ethanol extract of black pepper exhibited strong antioxidant activity. Antioxidant, superoxide, dismutase, and catalase activities of *Piper cubeba* Linn have been reported (Aqil *et al.*, 2006; Karthikeyan and Rani, 2003; Choi and Hwang, 2005).

IC_{50} value

IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds. Results showed in Table-3 reports no IC_{50} value in water and methanol extraction of both the plants. Only ethanolic extract of the two plants showed an IC_{50} value of 27.34µg/mg in case of *Piper nigrum* and 14.51µg/mg in case of *Piper cubeba*. The ethanolic extract of *Piper nigrum* exhibited significant activity with low IC_{50} value in comparison to *Piper cubeba.* The antioxidant activity of *Piper nigrum* and *Piper cubeba* extracts rise with the rising of polyphenol content of the extract. Similar results were obtained by Nooman *et al*., 2008, in which the crude methanolic extracts of *Piper cubeba* Linn. and *Piper nigrum* Linn. showed antioxidant activity, with IC_{50} values 11.3 ± 0.3µg/ml and 144.1 ± 2.2µg/ml respectively. A linear relationship between the reciprocal of IC_{50} value and the total polyphenol content of *Piper nigrum* and *Piper cubeba* was observed in this study, indicating that increasing the polyphenol content strengths the antioxidant activity. This finding is similar to that reported by Katsube *et al*. (2004).

CONCLUSION

The phytochemical tests indicated the presence of alkaloids, glycosides, tannins, and flavonoids in the crude ethanolic extract. Several of such compounds are known to possess potent antioxidant activity. Some of these constituents have already been isolated from this plant. The results of antioxidant activity indicate higher free radical scavenging activity in ethanolic extracts of *Piper cubeba* in comparison to *Piper nigrum* due to presence of phytochemical constituents especially polyphenols. This experiment supports that these fruits can be used in pharmaceutical industries as natural antioxidants.

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