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PHARMACOGNOSTIC EVALUATION AND FREE RADICAL SCAVENGING ACTIVITY OF ETHANOLIC EXTRACT OF *PIPER CUBEBA* FRUITS

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ABSTRACT

Ancient Indian system of medicine detailed the medicinal properties of several plants and their therapeutic usage. Recent scientific research has established to study pharmacognostic and preclinical parameters of herbal medicine rationale to specific pharmacological properties. A folk medicinal plant *Piper cubeba* L., Family-Piperaceae, commonly known as 'pepper' have been widely used to treat gonorrhea, dysentery, syphilis, abdominal pain, asthma. Thus, the present study was undertaken to evaluate *Piper cubeb* fruit for its pharmacognostic parameters and in-vitro free radical scavenging activity. The high nutritional value 353.95 Cal per100 g of powder was obtained. The phytochemical investigation revealed the presence of alkaloids, glycosides, tannins, diterpenes, phenols and flavonoids etc., in ethanolic extract of powdered fruit. Similarly, the massive extent of Vit. C (443.57 mg/gm) and Vit. E (321.14 mg/gm) of powdered fruit indicates its potent free radical scavenging activity. The IC50 values for DPPH, nitric oxide and hydroxyl radical scavenging was found to be 8.6, 11.9 and 14 µg/ml respectively.

KEYWORDS

Antioxidant, Microscopy, *Piper cubeba* and Phytochemical.

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INTRODUCTION

Traditional remedial applications are well recognized in modernistic medication system due to presences of the active phytochemicals in plants. The World Health Organization (WHO) estimated that, worlds 80% population exercise herbal medicines for elementary health care[1]. In the modern medicine approach, an herbal drug plays a great role to substitute the synthetic drugs with uncomplicated accessibility. This resulted in an intense global search for plant extracts and their

bioactive constituents as a basis for further pharmacological studies of health care[2]. Several studies shown that plant derived antioxidants have widely used to scavenge free radicals and modulate oxidative stress[3].

Piper cubeba L., is a flowering vine commonly known as tailed or java pepper belongs to family Piperaceae, genus Piper which is a folkloric plant and has been cultivated in many countries including India for its fruit and essential oil[4]. In India, Charaka and Sushrutatexts included cubeba in various remedies and in traditional Chinese medicine it is used for its alleged warming property. Arab physicians of the Middle Ages, the cubeba was used under the name of Kababa, when preparing the water of al butm[5]. The fruits are berry and are used to treat gonorrhea, dysentery, syphilis, abdominal pain, asthma[6]. The extract of *P. cubeba* has various pharmacological activities like anti-inflammatory and analgesic[7], anti-leishmanial[8], anti-proliferative[9] and anti-hepatitis C virus[10]. Thirteen different lignans including furanofuranlignans such as cubeb in, hinokinin, yatein and isoyatein were appeared in the dried fruit of *P. cubeba*[11]. About 15% of a volatile oil is obtained by distilling cubebs with water[12].

International agencies provided protocols and guidance documents on the assessment of the safety use of herbs. Therefore, in order to adequately characterize the toxicity of a specific herb, complete pharmacognostic evaluation, isolation and pharmacological screening techniques have emphasized the way to discover new drugs. In the present study an attempt was made to evaluate ethanolic extract of *Piper cubeba* for its pharmacognostic and free radical scavenging activity.

MATERIAL AND METHODS

Chemicals and Reagents

DPPH, Griess Reagent (Sigma, USA), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), EDTA, Silica gel (Merck Kiesegel 60),Sodium nitroprusside (Loba chemicals, India). All the reagents and solvents were of analytical grade and are prepared freshly before the experimentation.

Plant Material and Authentication

The dry fruits of *Piper cubeba* were acquired from local market (Dhorle and Sons Ayurveda) of Kolhapur District, Maharashtra, India. The herbarium plant was identified and authenticated by Dr. (Mrs.) Anuradha Upadhye, Scientist, Agharkar Research Institute Pune, India and the specimen (Auth.15-118) was deposited in Department of Biodiversity and Palaeobiology. The shade dried fruits were grounded to fine powder (sieve no 40) and stored in an airtight container for further use.

Macroscopic and Microscopic Analysis

A detailed macroscopic and microscopical study of the fruit was carried out by using dissecting microscope (Labomed, India).The sections were of the thickness of 20 ± 2 µ of fruit was taken to histochemical study by using reagents and stains like iodine, concentrated Sulphuric and hydrochloric acid, ferric chloride, Sudan III, ruthenium red and phloroglucinol with concentrated Hydrochloric acid. The organoleptic characters such as color, odor, size, shape, texture and taste were evaluated[13].

Proximate Analysis

The physicochemical parameters of the powdered drug such as total, water soluble, acid insoluble and sulphatedash values were determined for powder. The moisture content was determined by Loss on drying method. Similarly, extractive and soluble extractive values were determined[14].

The nutritive contents mainly ash, moisture, fat, fiber, protein, carbohydrate, Vit. C and Vit. E, of powdered fruit was determined[15].Percentage of carbohydrate was calculated by the following formula:

% carbohydrates = 100 - (% of ash + % moisture + % fat + % protein)

The nutritive value was calculated as per the formula used by Nile and Khobragade[16].

Nutritive value = (4 x percentage of protein) + (9 x percentage of fat) + (4 x percentage of carbohydrate)

The presence of nutritionally important minerals such as Na, K, Ca, Fe, Mg, and Zn along with toxic heavy metals like Hg, Pb, As and Se were done by using Inductively coupled plasma-Atomic Emission Spectroscopy (ICP-AES).The phosphorus content was estimated with biochemical method by using spectrophotometer at 410 nm and the amount of phosphorus was calculated using a standard curve of phosphorus[17].

Fluorescence analysis

The powdered drug sample was analyzed under visible light and UV light (254nm) after treating with various organic, inorganic solvents and reagents to study the fluorescence character[18].

Ethanol Extraction and Fractionation

500 gms of dry powdered fruits of *Piper cubeba* was extracted with ethanol (each 400ml.) for 10-12 hrs by using Soxhlet apparatus method. The extract was then filtered through Whatman (No-1) filter paper, dried on a rotary evaporator at 45°C under reduced pressure. The extract was preserved in airtight containers until further use.

Fractionation of ethanol extract was carried out according to procedure[19]. To obtain the fractions, the ethanol extract was dissolved in methanol: distilled water (1:1),the resulting solution was separated by column chromatography (1.5 x 30cm) on silica gel and eluted with increasing proportions with hexane, chloroform and ethyl acetate. The fractions obtained were concentrated under pressure using rotary evaporator.

Thin layer chromatography

Thin layer chromatography for extract was carried out with aluminium plate (5 × 9cm) pre-coated with silica gel. The plates were developed using ethanolic extract and R_f was calculated. Different combinations of solvent system were tried for better separation of constituents[20].

QUALITATIVE PHYTOCHEMICAL INVESTIGATION

The ethanol extract obtained was subjected to qualitative tests for their identification of various plant constituents like carbohydrates, amino acids, proteins, alkaloids, glycosides, steroids, sterols, saponins, tannins, diterpenes, phenols and flavonoids*etc*[21-23].

Test for carbohydrates: Molisch's test

2 ml of the extract and few ml of Molisch's reagent added along the sides of test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Test for amino acids: Ninhydrin test

2 ml of the extract was boiled with 2 drops of 5% Ninhydrin solution and observed to formation of blue or violet colour for the presence of amino acids.

Test for Proteins: Biuret test

The extract was treated with 1 ml of 10% NaOH and heated. Formation of purplish violet colour after adding drop of 0.7 % copper sulphate solution indicates the presence of proteins.

Test for alkaloids: Dragendorff's Test

2 ml of the extract was treated with a few drops of Dragendorff's reagent. Formation of orange brown precipitate indicates the presence of alkaloids

Test for Glycosides: Keller-Kiliani Test

The extract was mixed with 2 ml of glacial acetic acid containing 1 or 2 drops of 5% ferric chloride solution and was carefully poured into a test tube containing concentrated sulphuric acid along the sides. Formation of a brown ring at the junction indicates the presence of cardiac glycosides.

Test for steroids: Salkowski Test

The extract was added to 2 ml of chloroform solution. A reddish brown color formation after addition of sulphuric acid indicates the presence of steroids.

Test for sterols: Liebermann-Burchard Test

2 ml of the extract was mixed with a few drops of acetic anhydride. The solution then boiled and cooled. The concentrated sulphuric acid was added along the sides of the test tube. A brown ring at the junction of two layers and the upper layer turning green indicates the presence of sterols.

Test for Saponins: Foam Test

2 ml of the extract was mixed with 20 ml of distilled water in a graduated glass cylinder and shaken for10 minutes. Formation of 1 cm thick froth indicates presence of saponins in sample.

Test for Tannins: Ferric chloride Test

2 ml of freshly prepared 1% ferric chloride solution was added to 2 ml of the extract. Formation of dark blue or green or black colour indicates the presence of tannins.

Test for diterpenes: Copper acetate Test

2 ml of the extract was mixed with 2-3 drops of copper acetate solution. Shake vigorously. The formation of green colour indicates the presence of diterpenes.

Test for Phenols: Ferric chloride Test

2 ml of freshly prepared ferric chloride solution was added to 2 ml of the extract. The development of blue-green or black colour indicates the presence of phenols.

Test for Flavonoids: Shinoda Test

Small quantity of extract was dissolved in 5 ml of 95% ethanol and treated with few drops of conc. hydrochloric acid and 0.5 gm of magnesium turnings. Development of magenta colour is an indication of presence of flavonoids.

ANTIOXIDANT ACTIVITY OF *PIPER CUBEB*

DPPH Free radical scavenging activity

The free radical scavenging activity of ethanolic fraction of *Piper cubeb* was measured by using DPPH according to method of Blois (1958) by modification[24]. Briefly, 0.2 mM solution of DPPH in methanol was prepared and 100µl of this solution was added to test tubes containing various concentrations (6.25, 12.5, 25, 50,100 and 200 µg/ml) of plant extract. After 30 minutes incubation in a dark, absorbance was measured at 517nm by using UV Visible Spectrophotometer (Shimadzu 1800). The different in absorbance between the test and the control (DPPH in methanol) was calculated and the percentage inhibition was calculated by using the following equation.

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

As-is the absorbance of the test sample at t=0 min.

Ac-is the absorbance of the control at t=30 min.

The IC_{50} value was obtained by linear regression analysis of the dose response curve plotted using % inhibition and concentration.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of ethanolic extract of *Piper cubeb* was determined by using Deoxyribose assay[25]. The reaction mixture containing FeCl3 (100 µM), EDTA (104 µM), H_2O_2 (1 mM) and 2-deoxy- D-ribose (2.8 mM) were mixed with various concentrations (6.25-200 µg/ml) of plant extract in 1 ml final reaction volume made with potassium phosphate buffer (20 mM, pH 7.4) and incubated for 1 hr at 37 ^{o}C. The mixture was heated at 95 0C in water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5%). The reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm. The % hydroxyl radical scavenging activity of test sample was obtained and IC50 value was calculated.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was estimated according to method Garrat, 1964 by the using Griess reagent[26]. In the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different (6.25-200 µg/ml) concentrations of ethanol extract of plant was dissolved in methanol and incubated at 30^{0}C for 2 hours. After the incubation period, 0.5 ml of Griess reagent was added and the absorbance was immediately read at 550nm. The percentage inhibition was linearized and then IC50 value was calculated.

RESULTS AND DISCUSSION

Macroscopic and Microscopic Analysis

These macroscopic and microscopic parameters are considered quit inexpensive for the purpose of quality control, assessment of purity and identification of crude drug samples[27]. The dried *Piper cubeb* fruits are globular in shape, 4-6 cm in diameter. The berries appear black or grayish brown with stalks attached (Figure No.1). The dried pericarp is wrinkled and seed is hard, white and oily.

The T.S of fruit showed a well identified thick pericarp, testa and inner mass of perisperm and

enclosing a small embryo. The parenchymatous mesocarp and endocarp containing few stone cells were observed in pericarp. Elongated parenchymatous cells and oil cells seen in mesocarp. Perisperm contains few oil globules and starch grains (Figure No.2).

The organoleptic evaluation of the fruit and fruit powder showed that both were grayish black or dark brown in colour, with agreeable and aromatic odour and pungent taste.

Physicochemical Analysis

The results of physicochemical characters such as ash value, moisture content, extractive values, nutritive content and nutritive value were summarized in Table No.1. The total ash, water soluble ash, acid insoluble ash and sulphated ash value was obtained as 8.32, 2.10, 0.42 and 3.75 %w/w respectively. The high ash value indicates the amount of inorganics, minerals and silica components in the powdered sample. The moisture content was found about 4.53 %w/w. Extractives values; by using different organic solvents such as petroleum ether, chloroform, ethyl acetate, ethanol and water were obtained. The extractive value was found more in ethanol (18.71 %w/w) and is less in petroleum ether (3.86 %w/w). The higher amount of extractive value in ethanol for a powdered fruit is an indication of concentration of secondary metabolite present in it[28]. The solubility value was higher in ethanol (20.68 %w/w) than water (17.19 %w/w).

The nutritive contents reported as fat 1.07%, fiber 2.51% and protein 6.49% whereas carbohydrate content was estimated 79.59 %. The high nutritive value; 353.95 Cal per 100 g of powder was obtained. It is noted that, the value obtained was with the dried fruit powder, and may not necessarily mimic that of fresh fruit. The vitamin C and E was found to be 443.57 and 321.14 mg/gm of powder respectively which indicates the fruit has high degree of antioxidant activity.

Qualitative or quantitative determination of mineral contents present in plants is important as their presence may often be postulated on the label of a food. The minute quantities of minerals are needed for the proper functioning, growth and development of human body[29]. The results of nutritionally important macro minerals such as sodium, potassium, calcium, magnesium, phosphorous and micro minerals like iron and zinc was showed in Figure No.3. Sodium and potassium are major electrolytes of human body fluid. They also helps in the release of several chemicals which acts as nerve impulses, regulate contractility of tissue[30]. In fruit sodium 21.31ppm and potassium 511.23 ppm was reported. Calcium and magnesium plays important role in formation and function of bones, muscles and prevents disorders. They regulate many vital cellular activities. Calcium is one of the mineral assumed to be an important for regulating fruit storage quality[31]. The estimated calcium and magnesium content for fruit is 123.19ppm and 0.96 ppm respectively. Similarly, phosphorous, iron and zinc are also important for normal cell growth and repair. Iron performs a wide range of biological functions including synthesis of hemoglobin. Its deficiency may prevalent nutritional deficiency in humans. Zn helps to construct and maintain normal genetic material[32]. The results showed as phosphorous 19.52, iron 5.73 and zinc 0.27 ppm found in fruit.

The conclusion for of heavy metals estimation was reported in Table No.2. Mercury, lead, arsenic and selenium could not be detected with ICP-AES suggesting that the concentration of these heavy metals was beyond the lowest limit of detection (0.01 ppm) and hence are reported as absent.

Fluorescence analysis

Fluorescence is an essential parameter for first line standardization of crude drug. The ultraviolet light produces fluorescence in many natural products, which do not visibly fluoresce in day light. Analysis of fruit powder of *Piper cubeb* was carried out after treating with several reagents. Fluorescence was observed at 254 nm; comparing its change of colour in visible light. The results of fluorescence analysis were shown in Table No.3.

Extraction, fractionation and TLC

The ethonolic extract of fruit powder was carried out by continues Soxhelt apparatus. The percentage

yield was obtained about 9.17 %w/w. The extract showed sticky nature and clour varied from dark brown to black. The fraction yield of extract with hexane, chloroform, ethyl acetate and ethanol was found to be 0.32, 0.41, 0.75 and 0.91g.w/w respectively.

Similarly, now a days TLC has become widely adopted technique for separation of plant constituents and standardization of the herbal products. It is reliable monitoring of the identity and purity of drugs and also used for the detection of adulteration and substitution[33]. In the present study, TLC showed number of banding pattern suggesting presence of various chemical components. The solvent system hexane: ethanol (3:1) proved best for the separation of phytochemicals. Retention factor (Rf) was found to be 0.83.

Preliminary phytochemical investigation

The pharmacological action of the crude drug mainly depends on the metabolites present in it. Such a phytochemical analysis helpful in knowing the chemical nature of the drug[27,33]. In the present investigation, the qualitative screening of extract revealed the presence of a wide range of phytoconstituents (Table No.4) like carbohydrates, proteins, glycosides, saponins, diterpenes, flavonoids etc., and all other secondary metabolites. The reported phytoconstituents can be ascertained to various biological activities of *Piper cubeb* fruit and in turn its usage in traditional medicine.

Antioxidant Activity

Reactive oxygen species (ROS) such as superoxide anions, hydroxyl radical, hydrogen peroxide, nitric oxide etc., are representing an essential part of aerobic life and cellular metabolism. Increased generation of free radicals leads to oxidative stress; have been implicated in cause of ailments like cancer, inflammation, diabetes, liver cirrhosis, cardio vascular disease, Alzheimer's, Aging and acquired immunodeficiency syndrome[34]. Biological systems have developed a complex antioxidant network such as Catalase, superoxide dismutase, GSH, ascorbic acid, α-tocopherol, flavonoids constitute to counteract reactive species. Recently use of plant metabolites gain attention as potential antioxidants[35]. Phenolic compounds and flavonoids have been stated to be associated with antioxidative action in living systems, acting as scavengers of singlet oxygen and free radicals[36].

In the present study, the ethanolic fraction of *Piper cubeba* fruit powder showed potent in-vitro antioxidant activity (Table No.5 and Figure No.4) may be due to vast content of its phenols and flavonoids. The highest DPPH scavenging (93.88%), nitric oxide (89.06%) and hydroxyl radical (87.69%) scavenging activity was showed by plant extract. The IC50 values for DPPH, nitric oxide and hydroxyl radical scavenging was determined as 8.6, 11.9 and 14 µg/ml respectively. It was observed that, plant extract have more potent DPPH scavenging activity than hydroxyl radical scavenging.

Table No.1: Physicochemical evaluation of *Piper cubeb*

S.No	Parameters	Observation
Ash Values (% w/w)		
1	Total ash	8.32%w/w
2	Water soluble ash	2.10 %w/w
3	Acid insoluble ash	0.42 %w/w
4	Sulphated ash	3.75 %w/w
Moisture content (%w/w)		
1	Moisture content	4.53 %w/w
Extractive value (% w/w)		
1	Petroleum ether	3.86 %w/w
2	chloroform	4.12 %w/w
3	Ethyl acetate	9.39 %w/w
4	Ethanol	18.71 %w/w
5	Water	16.45 %w/w
Solubility Value(% w/w)		
1	Ethanol	20.68 %w/w
2	Water	17.19 %w/w
Nutritive content		
1	Fat	1.07 %
2	Fiber	2.51 %
3	Protein	6.49 %
4	carbohydrate	79.59 %
5	Nutritive value: Cal per 100 g of powder	353.95
6	Vit. C	443.57 mg/gm
7	Vit.E	321.14 mg/gm

Table No.2: Estimation heavy metals.

S.No	Heavy metals	Inference
1	Mercury (Hg)	-
2	Lead (Pb)	-
3	Arsenic (As)	-
4	Selenium (Se)	-

- indicates absent

Table No.3: Fluorescence analysis of *Piper cubeba* fruit powder

S.No	Reagents	Visible light	UV light (254nm)
1	Distilled water	Omaha orange	Light green
2	Petroleum ether	Light brown	Greenish brown
3	Chloroform	Light brown	Green
4	Methanol	Yellowish brown	Green
5	Acetic acid	Greenish brown	Greenish black
6	Conc. H_2SO_4	Yellowish brown	Brown
7	1N NaOH	Redish brown	Dark green
8	Picric acid	Yellowish brown	Fluoroscent green

Table No.4: Phytochemical investigation of *Piper cubeb* fruit extract

S.No	Phytoconstituents and Test	Observation
1	Carbohydrates: Molisch's test	++
2	Amino acids: Ninhydrin test	+
3	Proteins: Biuret test	+
4	Alkaloids: Dragendorff's Test	+
5	Glycosides: Keller-Kiliani Test	+
6	Steroids: Salkowski Test	+
7	Sterols: Liebermann-Burchard Test	+
8	Saponins: Foam Test	++
9	Tannins: Ferric chloride Test	++
10	Diterpenes: Copper acetate Test	+
11	Phenols: Ferric chloride Test	++
12	Flavonoids: Shinoda Test	+++

+ indicates present

Table No.5: Antioxidant activity of ethanolic fraction of *Piper cubeb*

S.No	Conc. of ethanolic fraction (µg/ml)	% Scavenging Activity		
		DPPH	Nitric oxide	Hydroxyl radical
1	6.25	37.32	24.76	28.86
2	12.5	59.15	52.24	49.22
3	25	77.47	69.43	61.08
4	50	82.56	76.99	72.51
5	100	91.04	84.86	82.03
6	200	93.88	89.06	87.69

Figure No.1: Macroscopic Characters of fruit

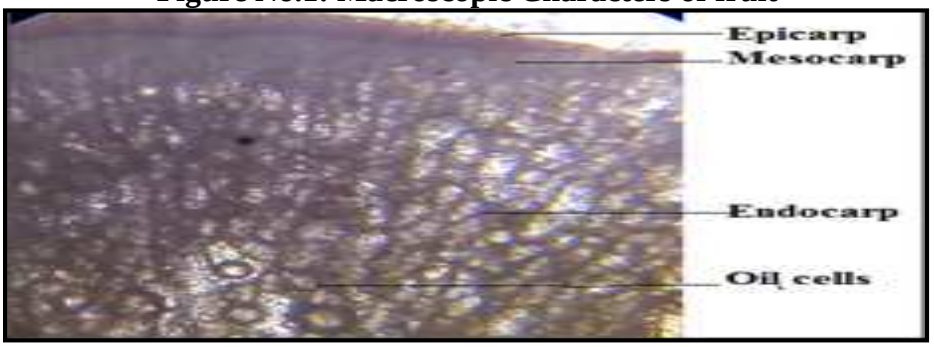

Figure No.2: Transverse Section of fruit

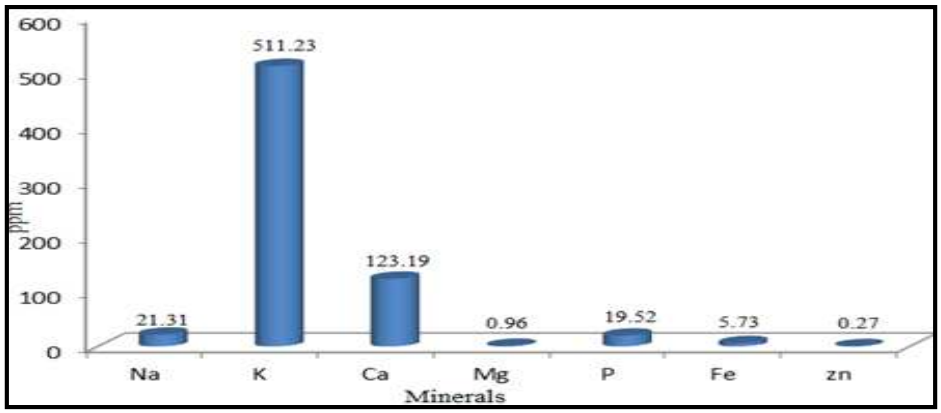

Figure No.3: Estimation of Mineral contents

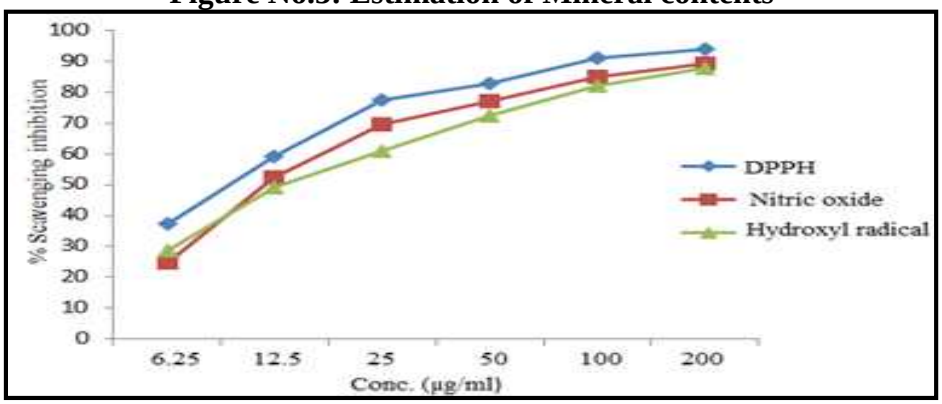

Figure No.4: Free radical scavenging activity of ethanolic fraction of *Piper cubeb*

CONCLUSION

Phytochemical and free radical scavenging study have been received increasing attention because of interesting new discoveries considering their biological activities. The present study confirms that the plant contains many bioactive compounds such as alkaloids, glycosides, tannins, and flavonoids in the crude ethanolic extract along with its high free radical scavenging activity. This experiment supports scientific authentication of traditional plant *Piper cubeb* fruits and it can be used in pharmaceutical industries.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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