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VOLATILE CONSTITUENTS AND ANTIOXIDANT PROPERTY OF ESSENTIAL OIL FROM *PLECTRANTHUS AMBOINICUS (LOUR)*

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ABSTRACT

Aim: To investigate the *in vivo* and *in vitro* antioxidant activities of *P. amboinicus* essential oil having important volatile constituents such a Carvocrol, Thymol, Cis/Trans - Caryophyllene, and P-Cymene. Methods**:** For *in vitro* studies different concentration of essential oil ranging from 5 - 100µg/ml was used and the assay involves the DPPH free radical scavenging activity, Hydroxyl radical activity and reducing power activity. The *in vivo* study on antioxidant activity, was carried out in a lung cancer mice model induced by injecting 100µl of $B_{16}F_{-10}$ melanoma metastatic cell line suspension of about 1×10^6 cells/ml in PBS into the tail-vein of C57BL/6 mice. The essential oil was given in the dose of 50µg/ml/dose for 21 days to each animal. The *in vivo* study involves the assay of the level of Catalase, Superoxide Dismutase, Glutathione Peroxidase, Lipid peroxidation, Nitric oxide and reduced glutathione. Finally, Lung was dissected out for histopathology study to confirm the lung metastasis. Results: The essential oil exhibited significant inhibition in DPPH free radical and hydroxyl radical formation. Similarly, it has got a significant reducing power towards such reactive molecules. In animal studies, the level of antioxidant enzyme activity were found to be normalized in the essential oil treated group for Catalase, Superoxide dismutase, glutathione peroxidase and Lipid peroxidation. Whereas, the toxic molecule nitric oxide level has been reduced upon essential oil treatment. The non-enzymatic antioxidant reduced glutathione was found to be increased, in the essential oil of *P. amboinicus* treated mice. The volatile compounds identified in GC – MS analysis in the essential oil were Carvocrol, Thymol, Cis – Caryophyllene, Trans – Caryophyllene, p- cymene as the major compounds with 96% comparison with the wilily and NBS library. Conclusion: The result obtained for both the *in vitro* and *in vivo* study shows that the essential oil of *P. amboinicus* has a significant antioxidant activity due to the presence of important bioactive compounds and hence, it can be subjected for pharmaceutical drug formulations.

KEY WORDS: Antioxidants, Essential oil, Free radicals, *Plectranthus amboinicus (Lour)*, Volatile compounds.

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INTRODUCTION

World's major population depends on traditional medicine for primary healthcare. Medicinal plants have been used in an extensive manner as a rich source of medicinal value since they contain organic compounds with therapeutic value.[1] The properties of the medicinal plants have been known for centuries and most of the medicines used now are derived from the plant origin i.e., from traditional medicinal plants.[2] Report states that according to WHO 70 - 80% of the World's population and even developed countries are using some forms of alternative or complementary medicine which are traditionally plant products. Hence, the exploration of the traditionally used medicinal plants is necessary on the basis of bioactivity, pharmacological activities and their constituents responsible for bioactivities.[3] *P. amboinicus* belongs to the family Lamiaceae, widely cultivated in Africa and almost in all tropical countries. Medicinal plants have been used for centuries as remedies for human diseases because they contain chemical components of therapeutic value. In South India (Tamil Nadu) this herb is traditionally applied externally for burns and insect bites, while internally it is used as a carminative and to control asthma. *P. amboinicus* is a medicinal plant having diverse pharmacological properties such as anti – tumourogenic and anti-inflammatory.[4] In general majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals. The treatment for these diseases and disorders implicating antioxidant therapy is found to be a promising remedy. [5] In recent years, researches are focused towards finding the naturally occurring antioxidants of plant origin like flavonoids, isoflavones, flavones, anthocyanins, lignans, catechins and isocatechins. These antioxidants have been reported in many research data that they have the ability to prevent oxidative damage caused by free radicals and Reactive oxygen species and thereby control the occurrence of diseases like cancer, cardiovascular diseases and premature aging.[6] Many works has been carried out in the recent years using different solvent extract of *Plectranthus amboinicus (Lour)* for treatment against inflammation[7] bacterial and Fungal[8] infections. The plant (Leaves, Stem, Flowers etc..,) has also showed a very good antioxidant property which was assessed by different assays namely DPPH, reducing power and *in –vivo* antioxidants such as SOD, CAT etc.., The plant's essential oil is found to be the most usable and effective source of bioactive compounds than the extract.[9] The present study is therefore focused on the extraction of essential oil from *P. amboinicus* and evaluating the antioxidant activity of the essential oil components present in it by both *in – vitro* and *in – vivo* assays using experimental lung cancer bearing mice.

MATERIALS AND METHODS

Collection and Authentication of plants

Fresh leaves of the selected plant *P. amboinicus* having medicinal value were collected from Western Ghats of Siruvani hills of Coimbatore, India. The plant materials were taxonomically identified and authenticated by the Botanical Survey of India and the voucher specimen (No.BSI/SC/5/23/09-10/TECH.1449) was retained in our laboratory for future reference.

Extraction of essential oil from P. amboinicus

Extraction of essential oil from *P. amboinicus* is done by Hydro distillation method using Clevenger-type apparatus for 3 hours. Plant material (leaves) was immersed directly in a round bottom flask filled with water. This was then brought to boil. Vapours were condensed on a cold surface using condenser attached to it. Essential oil gets separated based on difference in density and immiscibility, is then collected and dried over anhydrous sodium sulphate and stored in vial at low temperature until analysis.[10]

Essential oil analysis for bioactive compounds

Essential oils were extracted from *P. amboinicus* based on hydro distillation method for GC-MS analysis. GC-MS analysis was performed using SHIMADZU GC – MS QP 2010 using CARBOWAX capillary column and Helium as carrier gas to quantify the major phytochemicals. 0.2µl of essential oil was injected in to the column at the flow rate of 1µl/minute. The injector was operated at 250ºC and the oven temperature was programmed as follows; 60ºC for 15minutes, then gradually increased to 280ºC at 3minutes. The identification of components were based on comparison of their mass spectra with those of WILEY and NBS libraries and those described by Adams as well as comparison of their retention indices. Eleven compounds were obtained and out of these compounds Carvocrol, Thymol, Cis – Caryophyllene, Trans – Caryophyllene, p- cymene were found to be the major compounds. The data was published earlier by our research group.[11]

IN VITRO ANTI - OXIDANT STUDY

DPPH free radical scavenging activity

The free-radical scavenging activity of the essential oil of *P. amboinicus* was measured as a decrease in the absorbance of methanol solution of DPPH. A stock solution of DPPH (33 mg in 1 L) was prepared in methanol, which gave initial absorbance of 0.49 and 5 ml of this stock solution was added to different concentrations of essential oil (5, 10, 25, 50, 100µg/ml). After 30 min, the pale pink colour developed was measured at 517 nm and compared with standards (5-100µg/ml ascorbic acid) **[12].** Scavenging activity was expressed as the percentage inhibition calculated using the following formula:

$$\% \text{ Anti-radical activity} = \frac{\text{Control Absorbance - Sample Absorbance}}{\text{Control Absorbance}} \times 100$$

Hydroxyl radical scavenging activity

The essential oil of *P. amboinicus* with different concentrations was placed in test tubes. To which, 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of 0.018% EDTA, 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) and 0.5 ml of 0.22% ascorbic acid were added to each tube. The tubes were capped tightly and heated in a water bath at 80–90°C for 15 min. The reaction was terminated by adding 1 ml of ice-cold TCA (17.5% w/v). 3 ml of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and distilled water was added to make up total volume of 1 L) was added to each tube, which were left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured at 412 nm against blank.[13] Percentage inhibition was determined by comparing the results of the test and standard compound (ascorbic acid) by using the formula:

$$\% \text{ inhibition activity} = \frac{\text{Control Absorbance - Sample Absorbance}}{\text{Control Absorbance}} \times 100$$

Reducing power assay

The reducing power of essential oil of *P. amboinicus* was determined by adding 1ml of different concentrations of essential oil (5, 10, 25, 50, 100µg/ml) with 2.5 ml of PBS and 2.5ml ferrous cyanide and the mixture was incubated at 50°C for 20 minutes. 2.5ml of TCA was added to the mixture which was centrifuged at 3000rpm for 10 minutes. Finally 2.5ml of supernatant solution was mixed with 2.5ml distilled water and 0.5ml ferric chloride. Absorbance was measured at 700nm in a visible spectrophotometer against blank and compared with standard (Gallic acid).[14]

Increased absorbance of the reaction mixture indicates stronger reducing power.

IN VIVO ANTI-OXIDANT STUDY

Experimental Animals

C57BL/6 (30-35g) of male sex mice (6animals/group/cage) were purchased from National Institute of Nutrition (Hyderabad, India). The animals were housed in ventilated plastic cages and maintained at 12hour Light/12Hour Dark Cycle with free access to food and water. All the experiments involving animals were performed according to the standard protocols from National Institute of Nutrition (NIN) guidelines, after getting proper approval.

Cell line

The B16F-10 melanoma cell line was purchased from National Centre for Cell Science (NCCS, Pune, India). The cells were maintained in RPMI 1640 medium buffered with 2g/L of HEPES and sodium bicarbonate, and supplemented with dextrose, penicillin, streptomycin and 10% of fetal bovine serum. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. When needed for experiments (or during routine passaging steps), the cells were harvested with trypsin: EDTA (0.05: 0.03 [W/V] solution, and then washed in phosphate buffered saline (PBS, pH 7.4). For the animal experiments, the recovered cells were adjusted to 1×10^6 cells/ml in PBS and then 100µl of the suspension was injected into the tail-vein of the mice develop with lung metastasis.[15]

In vivo acute drug toxicity study

Overnight-fasted C57BL/6 mice of male sex weighing 25-35g were used and the doses ranging from 10 - 100µg were used. Animals were divided into 5 groups of 3 animals each. Each group of animals was given different doses of drug ranging from 10, 25, 50, 75 and 100µg/kg via i.p. The toxicological effects were observed in terms of mortality and expressed as LD_{50}. The total period of observation was about for 72h.[16]

Experimental Design for in vivo antioxidant study

Group 1: Normal. (n = 6)

Group 2: Cancer alone [1 million cells (10^6 cells) of B16F-10 cell line injected by tail vein] (n = 6)

Group 3: Treated [Cancer + 50µg/dose i.p., Essential oil of *P. amboinicus* for 21 days] (n = 6)

Sample Collection

At the end of the experimental period (22nd day), the animals were sacrificed with excess of anesthetic agent chloroform. Lung and liver tissues were immediately excised out and placed in the ice cold saline, blotted dry and then weighed. The weighed lung and liver tissues were homogenized in 0.1 M Tris-HCL buffer and then centrifuged at 2500rpm for 10minutes and then the supernatant obtained was separated and used for the estimation of peroxidase, catalase, protein, glutathione, nitric oxide and superoxide dismutase. The blood was collected by cardiac puncture and then the serum was separated by centrifugation at 2000rpm for 10minutes and used for the estimation of above mentioned assays.

Assay of Reduced glutathione (GSH) level in Serum, Lung and Liver tissues

Estimation of reduced glutathione was carried out based on the method described by Hafeman et al [17]. Briefly, 0.5ml of tissue homogenate/serum was precipitated with 125µl of 25% TCA and cool in ice for 5 min. The mixture was diluted with 0.6ml of 5%TCA and centrifuged for 10min at 5000 rpm. 0.3ml of the supernatant was separated and added with 0.7ml of phosphate buffer and 2ml Ellman's reagent. The yellow colour developed was read in a colorimeter at 412nm. A series of standards (20 – 100 µg) were treated in a similar manner along with a blank containing 1ml buffer. The amount of GSH was expressed in µg/mg protein for tissue and µg/ml serum.

Nitric oxide (NO) radical scavenging activity in Serum, Lung and Liver tissues

Estimation of nitrite was carried out based on the method described by Stuehr et al [18]. Briefly, nitrite in the serum and Lung/Liver tissues was determined by incubating 100µl sample with equal amount of Griess reagent (one part of 0.1% N (1-naphthyl) – diamine dihydrochloride in distilled water and 1 part 1% sulphanilamide in 5% concentrated H_3PO_4) for 10 minutes at room temperature. Absorbance was measured at 540nm and the amount of nitrite was calculated from the $NaNO_2$ standard curve. The amount of nitric oxide was expressed in µl/ml for serum and µm/gm tissue.

Assay of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) in Serum, Lung and Liver tissues

Estimation of lipid peroxidation was carried out based on the method described by Ohkawa et al [19]. Briefly, to 0.1ml of tissue homogenate/serum, 0.2ml of SDS, 1.5ml of acetic acid solution and 1.5ml of aqueous solution of thiobarbituric acid (TBA) were added. The mixture was made up to 4.0ml with distilled water and heated in a water bath at 95°C for 60 minutes. After cooling with tap water, 1.0ml of distilled water and 5.0ml of a mixture of n-butanol and pyridine (15:1) were added and shaken vigorously. After centrifugation at 3000 rpm for 15minutes, the organic layer was removed and read its absorbance at 532nm. The values are expressed in nmoles/l for serum and nmoles/mg protein for tissue.

Assay of Superoxide Dismutase (SOD) in lungs and liver tissue

The assay of enzyme Superoxide Dismutase was carried out by McCord et al [20]. Briefly, 1ml of tissue homogenate, 0.25ml of chloroform and 0.5ml of ethanol were added and mixed vigorously with vortex mixer. This mixture was centrifuged at 1800rpm for 6mins. 100µl of supernatant was taken and transferred into a test tube. The volume was made up to 2.25ml with phosphate buffer (pH 7.8). Then 0.2ml of EDTA/ Na CN, 0.1ml NBT (nitro blue tetrazolium), and 0.5 ml riboflavin were added in the mixture and the reading were taken at 560nm. After taking the initial reading the tubes were kept for illumination inside the chamber for 15 min. After 15 min the tubes were taken out and again taken the OD value at 560nm. The difference between initial and final reading was recorded. The specific activity of the enzyme was expressed as Units/mg protein.

Assay of Catalase (CAT) in Serum, Lung and Liver tissues

Estimation of Catalase was carried out based on the method described by Sinha et al [21]. Briefly, to 0.9ml phosphate buffer, 0.1ml tissue homogenate/serum and 0.4ml H_2O_2 were added. The reaction was arrested after 60 sec by adding 2.0ml dichromate-acetic acid mixture (ratio 1:4). The tubes were kept in a boiling water bath for 10mins, cooled and the colour developed was read at 590nm. Standards in the concentration range of 20-100µM were used. The specific activity of the enzyme was expressed as Units/ml for serum and Units/mg protein for tissue.

Assay of Glutathione Peroxidase (GPx) in Serum, Lung and Liver tissues

Estimation of Glutathione Peroxidase was carried out based on the method described by Rotruck et al [22]. Briefly, to 0.2ml of Tris-HCL buffer, 0.2ml EDTA, 0.1ml sodium azide and 0.2ml tissue homogenate/serum were added and mixed well. To this 0.2ml reduced GSH followed by 0.1ml of H_2O_2 were added. The contents were mixed well and incubated at 37°C for 10mins along with a tube blank containing all reagents except the sample. After 10mins, reaction was arrested by the addition of 0.5ml of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH utilization by colorimetric reading at 340nm. The enzyme activity was expressed in Units/ml for serum and Units/mg protein for tissue.

Histopathological Analysis of Lung

Lung tissues (Tumor nodules) were fixed in 10% formaldehyde, dehydrated and embedded in paraffin wax. 4 µm sections were then stained with Hematoxylin and Eosin (H & E) and mounted in DPX and examined under a microscope for histopathological changes of lung cancer.[23]

Statistical Analysis

Data was statistically analyzed using one – way ANOVA as primary test followed by Dunnett's test using Graph pad InStat3.0 software. All the results were expressed as mean ± S.D for 6 animals in each group. Data are expressed as mean ±S.D and considered significant when P<0.05*, P<0.01** and P<0.001***.

RESULTS

The total compounds identified in GC – MS analysis were 11, out of these compounds Carvocrol, Thymol, Cis – Caryophyllene, Trans – Caryophyllene, p- cymene were found to be the major compounds with 96% comparison with the wilily and NBS libraries.

In Vitro Antioxidant study

The free radical scavenging activity of the essential oil was dose dependent and the result obtained was compared with standard antioxidant such as Ascorbic acid and Gallic acid with the different concentration such as 5, 10, 25, 50, 100µg/ml. Table 1, shows the DPPH free radical scavenging activity of *P. amboinicus* essential oil. The standard antioxidant used for comparison was Ascorbic acid. The percentage of inhibition of DPPH free radical was dose dependent and the values were observed as 4.58 (± 0.58), 13.61 (± 0.60), 28.87(± 0.60), 43.21 (± 0.47), 63.09 (± 0.47) respectively which were found to be less when compared with that of ascorbic acid 8.94 (± 0.23), 19.04 (± 0.06), 32.59 (± 0.33), 56.66 (± 0.03), 85.32 (± 0.48) respectively. The IC 50 value calculated for the essential oil was found to be 68µg/ml whereas for standard ascorbic acid it was found to be 48µg/ml.Table 2, shows the percentage inhibition of hydroxyl radical scavenging. Here, the activity is found to be more or less same with standard ascorbic acid. The IC 50 value calculated for the essential oil was found to be 65µg/ml whereas for standard ascorbic acid it was found to be 50µg/ml.Table 3, shows the Reducing power activity of the essential oil where, increased absorbance was observed with increase in the concentration of the essential oil and the results obtained were compared with the standard Gallic acid.

In Vivo antioxidant study

Various antioxidant systems were assayed in cancer induced C57BL/6 mice. From the results of acute drug toxicity study a dose of 50µg was used for *in – vivo* study which did not show any observable toxic effects in C57BL/6 mice. Table 4 shows the GSH & NO levels in the essential oil treated group. The increase in GSH level upon treatment was statistically significant when compared with cancer induced group and normal group. Whereas, the level of Nitric oxide was found to be decreased in the essential oil treated group as (27.18 ** ±0.211 - Lung tissue, 31.33**±0.449 Liver tissue, 26.40** ±0.398 Serum) and increased in the cancer induced group (35.58 ±0.012 - Lung tissue, 42.49±0.393 - Liver tissue, 37.32 ±0.342 - Serum) respectively on 22nd day of the experiment. Table 5 shows the increase in the SOD level (3.446**±0.128 - Lung tissue, 5.776**±0.173 - Liver tissue) in treated group whereas in the cancer induced group the level of SOD was found to be decreased and the results obtained for treatment group were statistically significant when compared with the cancer induced and normal group. It was also noted that the lipid peroxidation was found to be decreased in the cancer induced group (2.568 ±0.136 – Lung tissue) and decreased in the liver tissue (5.73** ± 0.083) respectively which was statistically significant when compared with the cancer induced and control group on 22nd day.

Table 6 shows that the essential oil of *P. amboinicus* increases the level of Catalase

in the lung (1.845** ±0.016), liver tissues (5.743** ±0.243) and in the serum (0.65** ±0.022) on the 22nd day. Similarly, the level of GPx in the serum (0.96** ±0.009) lung (2.33** ±0.040) and liver (6.14** ±0.023) were also found to be increased in the essential oil treated group when compared with the cancer induced group and control group and this was found to be statistically significant. The Hematoxylin and Eosin stained sections of lung tissues are shown in Figure 1. The lung from healthy normal animal shows normal architecture of lungs with bronchioles, alveoli and interstitium(Fig: A). The lungs of positive control (Cancer alone) animals showed massive tumor growth around the bronchioles and infiltration of metastatic colonies of melanoma in the interstitium of the lung. Increased fibrosis reduces alveolar space, which leads to reduction in vital capacity of the lung (Fig: B). Simultaneous administration of essential oil of *P. amboinicus* at 50µg/ml/animal showed significant reduction in tumor mass and regeneration of alveolar passage with ciliated columnar epithelial cells. Lungs of the essential oil treated animals were almost similar to the healthy normal lung (Fig: C).

Table 1
DPPH free radical scavenging activity of P. amboinicus essential oil

S. No	Substance	Concentration (µg/ml)	% Inhibition
1	Ascorbic acid (Standard)	5	8.94 (± 0.23)
		10	19.04 (± 0.06)
		25	32.59 (± 0.33)
		50	56.66 (± 0.03)
		100	85.32 (± 0.48)
IC_{50} Value of Ascorbic acid (standard) - 48 µg/ml			
2	*P. amboinicus*	5	4.58 (± 0.58)
		10	13.61 (± 0.60)
		25	28.87 (± 0.60)
		50	43.21 (± 0.47)
		100	63.09 (± 0.47)
IC_{50} Value of *P. amboinicus* - 68 µg/ml			

Table 2
Hydroxyl radical scavenging activity of essential oil of P. amboinicus

S. No	Substance	Concentration(µg/ml)	% Inhibition
1	Ascorbic acid (Standard)	5	20.25 (± 0.37)
		10	35.20 (± 1.17)
		25	49.66 (± 0.57)
		50	50.23 (± 1.01)
		100	82.99 (± 0.52)
IC_{50} Value of Ascorbic acid (standard) - 50 µg/ml			
2	*P. amboinicus*	5	12.47 (± 0.73)
		10	23.02 (± 0.04)
		25	32.00 (± 2.64)
		50	43.82 (± 0.65)
		100	72.56 (± 0.58)
IC_{50} Value of *P. amboinicus* - 65 µg/ml			

Table 3
Reducing power activity of *P. amboinicus* essential oil

S. No	Substance	Concentration(µg/ml)	Absorbance (700 nm)
1	Gallic acid (Standard)	5	0.34 (± 0.003)
		10	0.44 (± 0.001)
		25	0.58 (± 0.001)
		50	0.72 (± 0.002)
		100	0. 77 (± 0.001)
2	*P. amboinicus*	5	0.26 (± 0.005)
		10	0.31 (± 0.003)
		25	0.49 (± 0.003)
		50	0.52 (± 0.007)
		100	0.61 (± 0.002)

Table 4
Effect of essential oil of P. amboinicus on reduced glutathione and Nitric oxide in serum, lung and liver tissue

Treatment	Reduced Glutathione			Nitric Oxide		
	Lung Tissue	Liver Tissue	Serum	Lung Tissue (µg/gm tissue)	Liver Tissue (µg/gm tissue)	Serum (µl/ml)
Group:1	0.144 ±0.004	0.226 ±0.002	7.50 ±0.194	24.78 ±0.099	26.22 ±0.223	29.37 ±0.398
Group:2	0.116[a] ±0.001	0.164[a] ±0.004	1.86[a] ±0.011	35.58[a] ±0.012	42.49[a] ±0.393	37.32[a] ±0.342
Group:3	0.126[b] ±0.002	0.206[b] ±0.001	3.74[b] ±0.021	27.18[b] ±0.211	31.33[b] ±0.449	26.40[b] ±0.398

Note: Each value is expressed as mean ± S.D. for six mice in each group. GSH unit: µg/mg protein for tissues and µl/ml for serum and NO unit: µg/gm Tissue for tissue and µl/ml for serum. (a) - Statistically compared Treated with Cancer, P<0.01**, (b) - Statistically compared Treated with Normal, P<0.01**.

Table 5
Effect of essential oil of *P. amboinicus* on lipid peroxidation and Superoxide Dismutase

Treatment	Lipid peroxidation			Superoxide Dismutase	
	Lung Tissue	Liver Tissue	Serum	Lung Tissue	Liver Tissue
Group:1	0.995 ±0.002	1.036 ±0.001	0.026 ±0.002	4.665 ±0.216	6.408 ±0.124
Group:2	2.186 ±0.001[a]	1.956 ±0.001[a]	0.057 ±0.001[a]	2.568[a] ±0.1366	5.732[a] ±0.083
Group:3	1.545[b] ±0.002	1.439[b] ±0.001	0.037[b] ±0.001	3.446[b] ±0.128	5.776[b] ±0.173

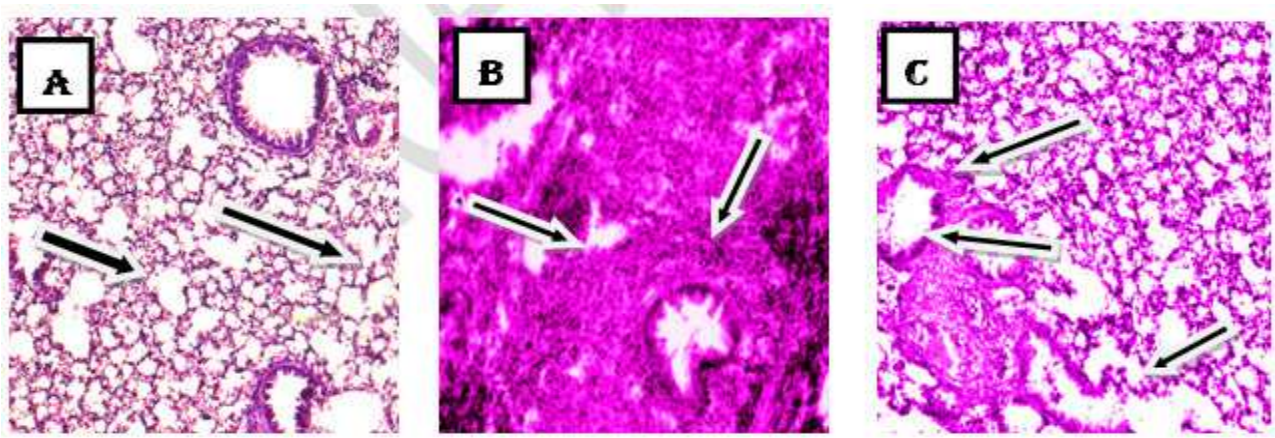
Note: Each value is expressed as mean ± S.D. for six mice in each group.LPO unit: n moles/mg protein for tissues and n moles/L for serum and SOD unit: Units/mg protein for tissue (a) - Statistically compared Treated with Cancer, P<0.01**, (b) - Statistically compared Treated with Normal, P<0.01**.

Table 6
Effect of essential oil of *P. amboinicus* on Catalase and Glutathione Peroxidase in serum, lung and liver tissues

Treatment	Catalase			Glutathione Peroxidase		
	Lung Tissue	Liver Tissue	Serum	Lung Tissue	Liver Tissue	Serum
Group:1	84.41 ±0.209	87.56 ±0.068	36.27 ±0.009	3.473 ±0.120	7.251 ±0.030	1.043 ±0.022
Group:2	62.85 [a] ±0.037	47.62 [a] ±0.062	25.62 [a] ±0.088	1.845 [a] ±0.016	5.743 [a] ±0.243	0.65 [a] ±0.022
Group:3	72.53 [b] ±0.118	66.48 [b] ±0.148	32.21 [b] ±0.125	2.33 [b] ±0.040	6.14 [b] ±0.023	0.96 [b] ±0.009

***Note:** Each value is expressed as mean ± S.D. for six mice in each group. Catalase unit: Units/mg protein for tissues and Units/ml for serum and Glutathione peroxide unit: Units/mg protein for tissue and Units/ml for serum. (a) - Statistically compared Treated with Cancer, P<0.01**, (b) - Statistically compared Treated with Normal, P<0.01**.*

FIGURE 1
HISTOPATHOLOGICAL ANALYSIS OF LUNG



- ***A- Normal [Shows normal architecture of lungs with bronchioles, alveoli and interstitium]***
- ***B- Cancer control [Shows massive growth around the bronchioles and infiltration of melanoma in the interstitium of the lung]***
- ***C- Essential oil Treated [shows significant reduction in tumor mass and regeneration of alveolar passage with ciliated columnar epithelial cells]***

DISCUSSION

Major tests used for antioxidant assays are based on the evaluation of lipid peroxidation or on the measurement of free radical scavenging capacity (hydrogen – donating ability). The radical scavengers donate hydrogen to free radicals, which leads to non toxic species and therefore inhibits the propagation phase of lipid oxidation.[24] DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers.[25] In our study the IC_{50} obtained for the essential oil of *P. amboinicus* was 68µg/ml which was compared with the standard ascorbic acid 48µg/ml. This result clearly shows that the free radical scavenging activity (DPPH) of the essential oil was dose dependent and when compared with the standard, a high concentration of essential oil is needed for scavenging the free radicals which indicates that the essential oil of *P. amboinicus* pocesses a moderate scavenging activity. Hydroxyl radicals are the major active oxygen

species causing lipid peroxidation and enormous damage to the biological system.[26] From, the present study the essential oil of *P. amboinicus* shows an IC_{50} value of 65µg/ml whereas for the standard ascorbic acid shows 50 µg/ml which clearly indicate that the essential oil has a better hydroxyl activity than the standard used.

Reducing power activity is performed for measuring the electron-donating capacity of an antioxidant[27]. The reducing power properties are generally associated with the presence of reductones, which shows antioxidant activity by breaking the chain reaction by donating a hydrogen atom[28]. These reductones have been already reported to react with certain precursors of peroxide, thus preventing peroxide formation. [29] In the present study, the reducing power activity of the essential oil was found to be increasing with the increase in the concentration of the essential oil and when compared with the standard Gallic acid the reducing power was found to be more or less same. The higher reducing power of essential oil may be attributed due to the presence appreciable amount of Carvocrol[30] and Thymol [31] which has been already reported for its antioxidant activity by many researchers.[32] The result may be due to the electron donating nature of the compounds as they are terpenoid derivatives. Generally, for all the vertebrates Liver plays the major site of all the antioxidant actions and is the principal organ which has the capacity of converting the drugs into readily eliminatable forms of drugs from the body.[33] Glutathione participates in the detoxification during different levels and scavenges free radicals. [34] Glutathione serves the cell with multiple defenses not only against ROS but also against their toxic products.[35] Alterations in antioxidant enzyme activities and tissue GSH concentration were reported in cancers. Decreased alterations in cancer may be caused by different pathways including, increased sorbitol synthesis leading to NADPH depletion and deficiency of this limits the reduction of GSSH to GSH catalyzed by glutathione reductase, decreased activity of HMP shunt enzyme and transport of GSSH through erythrocyte membranes due to oxidative stress induced membrane damage.[36] Hence, in the present study, increased GSH level in the tissue and serum in the treated group with essential oil of *P. amboinicus* indicates its control over oxidative stress caused in lung cancer development.

Nitric oxide (NO) is a free radical produced in all mammalian cells, which is involved in the regulation of various physiological processes and the excess production of NO is correlated with several diseases. NO is an unstable species under aerobic condition.[37] It reacts with oxygen to produce stable by product nitrate and nitrite. [38] In the present study, the level of NO was found to be high in cancer group and in the essential oil treated group it was found to be reduced in both the tissues and serum which indicates that the major compound Carvocrol present in the essential oil pocesses significant antioxidant activity which was proved already by different researchers.[39] The reaction between free radicals and membrane lipids arise the lipid peroxidation and this reaction is considered as an important feature of cell injury. Many studies have suggested that lipid oxidation products ingested with food or produced endogenously results in health risk. [40] It has been already reported that the measurement of malondialdehyde [MDA] has been used as an indicator of lipid peroxidation. This reactive species occurs naturally and is a bio-marker for oxidative stress. [41] Our study, reveals that the essential oil of *P. amboinicus* treated group could decrease the release of MDA, whereas in the cancer control group the level of MDA released was found to be high. Superoxide radicals are generally generated both by spontaneous and during enzyme – catalyzed oxidations. This radical is catalytically scavenged by Superoxide dismutase (SOD) which is an important defense mechanism during stress condition.[42] The results obtained in our study, shows that the essential oil of *P. amboinicus* has the capacity to release superoxide dismutase in to the system thereby it has an ability to scavenge the superoxide radical produced

during cancer development. Catalase is a common enzyme found in nearly all living organisms which is get exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen.[43] Moreover, one Catalase molecule can convert millions of molecules of H_2O_2 to H_2O and O_2 each second.[44]

H_2O_2 is a harmful by-product of many normal metabolic processes for the prevention of damage to cells and tissues; it must be quickly converted into other, less dangerous substances.[45] Our study report states that, in cancer induced group the level of catalase was found to be low whereas in the treated group with the essential oil of *P. amboinicus* the level of Catalase produced was found to be increased and hence the H_2O_2 produced may be scavenged and converted into water and oxygen. This activity of *P. amboinicus* may be due to the major compounds Carvocrol and Thymol present in the essential oil which has been already reported for its significant antioxidant activity. However, it was reported that during autoxidation of lipids at ambient temperature, thymol is a more effective and more active antioxidant than Carvocrol.[46] Carvocrol and Thymol as an antioxidant differ in the mechanism of their inhibiting action which depends on the character of the lipid medium.[47] Thymol is a monoterpene phenol derivative Cymene, isomeric with Carvocrol. Thymol has been reported for its microbial activity because of its phenolic structure.[48] Glutathione peroxidase (GPx) is an enzyme family with peroxidase activity with the help of GSH. The major biological role is to protect the organism from oxidative damage and reduce lipid hydro peroxides to their corresponding alcohols and to reduce hydrogen peroxide to water.[49]

Till now eight different isoforms of glutathione peroxidase have been identified in humans. [50] GPx 1 is found abundantly in cytoplasm of all mammalian tissues and its preferred substrate is hydrogen peroxide.[51] GPx 3 is found abundantly in plasma.[52] Report states that GPx 1 is not critically essential for survival but GPx 4 deficiency leads to the death of knock out mouse during early embryonic development. [53] The present study indicate that in the cancer induced group the level of GPx produced was low due to the development of stress and free radicals get released in the system. Whereas, in the essential oil treated group it was found that the drug used was able to increase Glutathione peroxidase level and thereby this may scavenge the free radicals and reduces the hydrogen peroxide into water.

CONCLUSION

The essential oil of the study plant *P. amboinicus* possess a significant antioxidant property against stress created in cell line induced lung cancer model due to the presence of the phytochemical compounds such as Carvocrol and Thymol. On the basis of our results, the essential oil may be used as an alternative medicine for stress related diseases. In addition to it can be used for the treatment of acne and cancer in combination therapy. Hence the use of essential oil of *P. amboinicus* will be a cheaper and natural drug formulation, to replace the commercially available chemical drug and also without any side effects.

Declaration of interest

The authors declare no conflicts of interest.

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