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Original Article

ANTIOXIDANT AND ANTI CLASTOGENIC POTENTIAL OF *PIPER LONGUM* L.

ARCHANA D., DIXITHA M., SANTHY K. S.*

*Department of Zoology, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu, India 641043
Email: santhyanandan@gmail.com

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ABSTRACT

Objective: Present study was aimed to evaluate the antioxidant and anti clastogenic potential of methanol extract of *Piper longum* L (MEPL).

Methods: Chromatographic analysis was carried out using thermo GC-Trace Ultra Ver: 5.0 GC-MS. Antioxidant activities were assessed by DPPH free radical scavenging assay and reducing power assay. Based on the antioxidant activity, micronuclei formation in peripheral blood lymphocytes was analyzed. The protection afforded by *Piper longum* L. against the cytotoxicity of peripheral blood lymphocytes were confirmed by the micronucleus (MN) assay.

Results: The GC-MS analysis provides different peaks determining the presence of eighteen phytochemical compounds with different therapeutic activities. The methanol extract at a concentration of 40 µg/ml showed the highest antioxidant activity by DPPH assay (68.42%) comparable to standard, ascorbic acid (73.68%). The reducing power observed was in the order of 40 µg/ml>20 µg/ml>10 µg/ml. MEPL treatment decreased the frequency of MN in a concentration dependent manner.

Conclusion: A substantial amount of bioactive components are present in *Piper longum* L. A good correlation of the antioxidant capacity of the plant was established by different assay methodologies. MN test confirmed the anti clastogenic potential in peripheral blood lymphocytes.

Keywords: *Piper longum* L., MEPL, GC-MS, DPPH, reducing power, Micronuclei.

INTRODUCTION

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years. This resulted in an intense global search for plant extracts and their constituents for health care. *Piper longum* L is one of the medicinally important species of the family Piperaceae, commonly known as long pepper [1]. Indian long pepper is mostly derived from the wild plants but also occurs in hotter parts of India, from central Himalayas to Assam, Khasi and Mikir hills, lower hills of West Bengal and evergreen forests of Western Ghats from Konkan to Kerala and Nicobar Islands [2].

The fruiting spikes of the species are used in many medicaments of Indian traditional systems of medicine and in tribal and folk medicines [3]. It was reported as a good remedy for treating gonorrhoea, menstrual pain, and tuberculosis, sleeping problems, respiratory tract infections, chronic gut–related pain and arthritic conditions [4].

In recent years, there is an increasing trend of screening medicinal plants for bioactive compounds as a basis for further pharmacological studies. Several studies have shown that plant derived antioxidants scavenge free radicals and modulate oxidative stress. The chemistry of free radical is complicated and it caused a major limitation in the identification of free radical scavenging activity. To withstand this problem the potential antioxidant substance is tested in *in vitro* model and such approaches expand the scope of antioxidant activity [5]. Therefore, we have examined fruit extracts of *Piper longum* L. for antioxidant activity by DPPH free radical scavenging assay and reducing power assay.

The protocols and guidance documents are provided by international agencies discussed on the assessment of the safety of herbs for use in both foods and medicines. The types of testing described in these guidance documents represent the ideal type of information that could be obtained in order to adequately characterize the toxicity of a specific herb or a finished herbal product ready for the market place [6].

The major challenge in geno toxicity testing resides in developing methods that can reliably and sensibly detect the vast array of damages or a general cellular response to geno toxic insult. It is recognized that no single test can detect every genotoxin, therefore the concept of tests battery has been implemented and in the last decades, numerous damage signalization and repair mechanisms, complex and extremely efficient, have been unraveled [7].

The term clasto genicity is used for agents giving rise to structural chromosome aberrations. A clastogen can cause breaks in chromosomes that result in the loss or rearrangements of chromosome segments. The mammalian cell micronucleus test is the only *in vitro* test that can efficiently detect both clastogens and aneugens. The detection of micronuclei, manual or automated [8] provides a readily measurable index of chromosome breakage and loss.

An *in vitro* lymphocyte assay to investigate micronucleus formation has been widely recommended for studies of the cytotoxicity and geno toxicity of drugs [9] Therefore, in the present study, an analysis of micronuclei formation, proliferative status of viable cells was conducted in peripheral blood lymphocytes.

MATERIALS AND METHODS

Collection and preparation of sample

Piper longum L. fruits were collected in November and December 2014 from the local areas of Coimbatore. The fruits were cleaned thoroughly and dried at room temperature for 5-7 d in the shade. The dried samples were powdered using an electrical grinder. The powdered samples were stored in screw cap bottles until further analysis.

Preparation of extract

One hundred grams of powders from the whole dried fruits of *Piper longum* L. was taken, to which 500 ml of methanol was added, mixed, and kept for four days. The contents were periodically shaken using an electric shaker. After four days, the contents were filtered through a Buchner funnel in a conical flask and it was further concentrated by evaporation by keeping the filtrate in a round-bottomed flask, till the solvent completely evaporated and the extract settled down to the bottom.

Chemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH) was procured from Sigma, USA. Trichloroacetic acid (TCA) and ferric chloride were obtained from Hi media and Merck. Ascorbic acid was obtained from SD Fine Chem. Ltd., Biosar, India. All other reagents and solvents were of analytical reagent grade.

Analysis of bioactive compounds

Chromatographic analysis was carried out using thermo GC-Trace Ultra Ver: 5.0 GC-MS (Model Thermo MS DSQ II gas chromatograph). A fused-DB35-MS Capillary standard Non-polar Column Dimension (30 min, ID: 0.25 mm, FILM: 0.25µm) was used. The GC temperature program was as follows: initial temperature was 75 °C, held for 2 min,

increased to 150 °C at a rate of 2 °C/min, then to 220 °C at a rate of 3 °C/min, and finally to 260 °C at a rate of 6 °C/min and held for 10 min. The split ratio was 1:12, injection temperature was 250 °C, transfer line temperature was 270 °C, and the mass spectrometer was operated at 70 eV in run time 29 min.

Antioxidant activity

DPPH free radical scavenging activity [10]

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. The diluted working solutions of the test extracts were prepared in methanol. About 3 ml of graded concentration (10-40 µg/ml) of extracts and standard were taken in different test tubes, then 1 ml of 0.1 mM DPPH methanol solution was added to these test tubes and shaken vigorously. After 30 min incubation of samples at 25°C in the dark, the absorption was measured at 517 nm. Ascorbic acid was used as reference and prepared using methanol solvent instead of extract. The change in absorbance of each sample with standard was determined and the scavenging activity was calculated by the following equation.

Radical scavenging activity (%) = Abs (control)–Abs (sample)/Abs (control) x 100.

Where, Abs (control) is the absorbance of DPPH radical with methanol and

Abs (sample) is the absorbance of DPPH radical with a sample extract or standard.

Reducing power assay [11]

Reaction mixtures were prepared by adding 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%) and varying concentrations of extracts (10-40 µg/ml). After, the reaction mixtures were incubated at 50 °C in water bath for 30 min, allowed to cool at room temperature (28 °C), and 2.5 ml of 10% TCA (Tri chloro acetic acid) were added to each reaction mixture, and then centrifuged at 2000 rpm for 10 min. The supernatant (2.5 ml) was separated in the test tube and added with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1.0%), and allowed to react for 10 min at room temperature and the absorbance was measured at 700 nm. Ascorbic acid solution was used as standard.

Anticlastogenic activity

Cytotoxicity study of MEPL in cultured peripheral blood lymphocytes

In order to determine the concentration of MEPL which would allow the evaluation of the DNA damage without affecting the cell cycle or inducing cell death, cellular viability tests were performed using a concentration response curve before carrying out the micronucleus assay. The cytotoxicity of MEPL on lymphocytes was assayed using the Trypan blue dye exclusion method after incubation of cells with samples of *Piper longum* L. at concentrations of 10, 20, 40, 60, 80 and 100 µg/ml for 24 h. Viable cells were determined based on the ability of cells to exclude the dye.

Determination of MN formation in breast cancer lymphocytes

Culture the lymphocytes in sterile bottles using RPMI 1640 medium containing 15 % foetal calf serum. Lymphocytes stimulate to divide with PHA and incubated for 72 h at 37 °C; 44 hr after PHA stimulation, added cytochalasin B to the cultures to give a final concentration of 4.5 µg/ml. Twenty eight hours after addition of cytochalasin–B, transferred the whole content in to a sterile centrifuge tube and centrifuged for 10 min.; removed the supernatant and shaken the pellet in a cyclomixer. Added 10 ml of 0.075 M KCl solution to the cell button and kept at 37 °C for 10 min. After this, added 2 drops of freshly prepared fixative (Methanol: acetic acid) in the ratio 3:1. Again centrifuged for 10 min at 1000 rpm. The cell suspension (7-8 drops) was dropped on pre cleaned, labelled and chilled slides from a particular height. Stained the slides with 10-20%. Giemsa stain solution and allowed to remain for 10 min. The number of MN in number less than 1000 binucleated cells was scored and the distribution of MN among binucleated cells was recorded.

Statistical analysis

Experimental results were given as mean±SD. Significance level in different groups was analyzed by applying ANOVA and P values<0.01 were regarded as significant.

RESULTS AND DISCUSSION

Analysis of bioactive components

The GC-MS analysis leads to the identification of 18 pharmacologically important compounds from the fractions of the methanolic extract of *Piper longum* L. The relative concentrations of the volatile components identified are presented in table 1. The major constituents were (6R*,7R*)-6-Eyhenyl-2,6-dimethyltridec-11-en-7-ol (64.91%), 2-Chloro-1,3-(dipyrrolidino)-1-butene (14.16%), Methyl 4/5-oxoaraquidate (10.19%), 5A,14B-Pregnane-3,11-dione (3.30%), Hexadecanoic acid (2.12%), Methyl stearate (1.23 %) and Corydine (0.86%).

From the gas chromatogram, a total of 8 peaks were identified. Some peaks were broad or mixture and some were very small which were not considered in an analysis. Among the 8 peaks, the highest peak at retention time 26.01 min. possesses a basic signal at 95 in mass spectrum (fig. 1).

Analysis of the extract of *Piper longum* showed qualitative as well as quantitative differences. From table 1 it is evident that there is significant variation in the composition of volatile oil in fruit with respect to major constituents. On comparing our results with those earlier reported for the oils of Bangladesh origins were quite different. They contained eugenol (33.11%), caryophyllene (9.29%), cinnamyl acetate (5.91%) and β-pinene (4.74%) as major constituents [12].

Table 1: Major bioactive compounds present in methanol extract of *Piper longum*. L

S. No.	Compound name	Molecular formula	Molecular weight	Area %
1	Rhodoviolascin	C42H60O2	596	0.05
2	Hexadecanoic acid	C17H34O2	270	2.12
3	Octadecanoic acid	C18H36O2	284	0.21
4	Heptadecanoic acid	C17H34O2	270	0.18
5	Hexadecanoic acid,	C19H40O2S	328	0.12
6	Methyl 4/5-oxoaraquidate	C21H40O3	340	10.19
7	Methyl stearate	C19H38O2	298	1.23
8	(6R*,7R*)-6-Eyhenyl-2,6-dimethyltridec-11-en-7-ol	C17H32O	252	64.91
9	2-Chloro-1,3-(dipyrrolidino)-1-butene	C12H21ClN2	228	14.16
10	Eicosanoic acid	C21H42O2	326	0.44
11	9-Octadecenamide	C18H35NO	281	0.21
12	Crinamine, 1,2-dihydro-6-hydroxy-11-oxo-	C17H19NO5	317	0.15
13	(-)-Corlumine	C21H21NO6	383	0.15
14	4,9,13,17-Tetramethyl-4,8,12,16-octadecatetraenal	C22H36O	316	0.21
15	Corydine	C20H23NO4	341	0.89
16	Tridec-4-en-2-yna	C13H20O	192	0.22
17	5A,14B-Pregnane-3,11-dione	C21H32O2	316	3.30
18	d-Glycero-d-galacto-heptose	C15H20O10	360	0.10

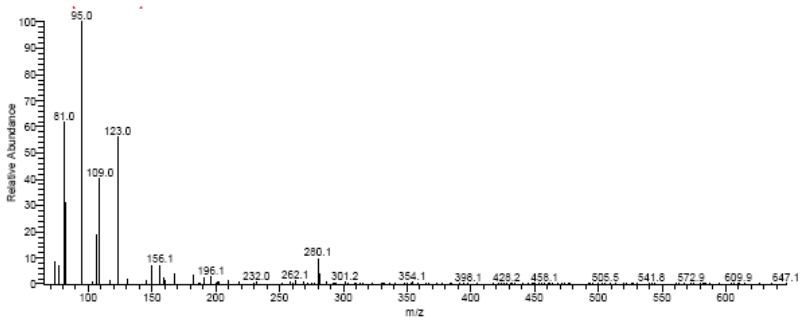

Fig. 1: Mass spectra of (6R*, 7R*)-6-Eyhenyl-2, 6-dimethyltridec-11-en-7-ol

On the other hand, oils obtained from plants grown in Indian origin contained aliphatic compounds predominated with n-pentadecane (15.8%) as the major constituent [3]. It was noticed that the chemical composition of volatile oil reported previously from different locations showed variation in the components which may be attributed to differences in the response to geographic, climatic or edaphic patterns and variation in the maturity of raw material.

Antioxidant activity

DPPH assay is a valid accurate, easy and economic method to measure the ability of compounds to act as free radical scavengers or hydrogen donors. The assay is based on the measurement of the scavenging capacity of antioxidants towards a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH). The method is unique in carrying out the reaction of the sample with DPPH in methanol/water, which facilitates the extraction of antioxidant compounds from the sample [13].

The results of the assay are expressed in scavenging ability of DPPH free radical expressed in percentage. The ability of radical scavenging activity of the extract at different concentrations increases with increasing concentrations. Out of the three different concentrations, the scavenging activity observed was in the order of 40 µg/ml>20 µg/ml>10 µg/ml with their percentage inhibition ranging from 5.26 to 68.42% (table 2). From the present results, it may be postulated that the methanol extracts of *Piper longum* reduces DPPH radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principles.

Table 2: DPPH radical scavenging activity

Drug	Concentration (µg/ml)	Absorbance (nm)	Scavenging ability (%)
MEPL	10	0.18±0.01	5.26
	20	0.16±0.02	15.78
	40	0.09±0.03	68.42
ABA	10	0.15±0.00	21.05
	20	0.14±0.00	26.31
	40	0.05±0.02	73.68

MEPL: Methanol Extract of *Piper longum*, ABA: Ascorbic acid

These results are in accordance with phyto chemical and antioxidant activity of *Nelumbo nucifera, Acorus calamus,* and *Piper longum* L. by solvent extraction method [14]. Results showed that the hexane fraction of *N. nucifera* exhibited the highest radical scavenging activity followed by methanolic fraction of *Piper longum* L. and *A. calamus*. The methanolic fraction of *Piper longum* L. showed a dose dependent increase in radical scavenging activity.

Also in another study *Piper longum* L. and *Piper chaba* Hunter was evaluated using DPPH free radical scavenging assay. The results showed that the antioxidant activity was higher for *Piper longum* L. fruits as compared to *P. chaba* fruits. The more decrease in absorbance in *Piper longum* L. reveals that it is a potent antioxidant [3].

The reducing ability is generally associated with the presence of reductones which breaks the free radical chain by donating a hydrogen atom [15]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. The reducing power of different concentrations of *Piper longum* L. was found to be remarkable and the absorbance of each concentration was found to rise as the concentration gradually increases. The reducing power observed was in the order of 40 µg/ml>20 µg/ml>10 µg/ml as shown in the table 3.

Table 3: Reducing power assay of *Piper longum* L.

S. No.	Conc. of MEPL (µg/ml)	Absorbance	
		Ascorbic acid	Methanol extract
1	10	0.41±0.01	0.44±0.01
2	20	0.43±0.04	0.48±0.02
3	40	0.50±0.01	0.51±0.01

Anti clastogenic activity

The protective effect against the DNA damage may be due to one or more of the following: antioxidant action, trapping of free radicals, formation of complex with mutagen, modulation of mutagen metabolism or by adsorbing the xenobiotics. This is feasible because many naturally occurring compounds are known to exhibit discrete mechanisms of protection [16].

Among the various techniques used to detect genetic and geno protective effects, the MN test is simple, cheap, and less cumbersome and allows convenient and easy application. Many studies are available in the literature on the application of Micronucleus test, evaluated as baseline level [17] and after *in vitro* challenge, involving more than 1000 cases of breast cancer patients and subjects with known or putative cancer predisposition.

Table 4: Viability of lymphocytes after treatment with MEPL

S. No.	Conc. of MEPL (µg/ml)	Viable cells (%)
1	10	71
2	20	72
3	40	75
4	60	45
5	80	42
6	100	41

Table 5: Frequency of MN in peripheral blood lymphocyte cultures exposed to MEPL

S. No.	Conc. of MEPL (µg/ml)	Freq. of MN (MN±SEM)
1	Control	12.8±0.03
2	10.00	12.12±0.005*
3	20.00	11.57±0.12*
4	40.00	11.06±0.18*

*significantly different from control ($p<0.01$): ANOVA, MN: Micronuclei; SEM: Standard Error Mean

In the present study, a concentration dependent decline was seen in the survival of peripheral blood lymphocytes exposed to MEPL for 24 h. The concentration up to 40 µg/ml did not affect the viability of lymphocyte cells during the 24 h exposure, but at the concentration of 60 µg/ml, the cell viability decreased below 50 percentages (table 4).

The protection afforded by *Piper longum* L. against the cytotoxicity of peripheral blood lymphocytes were confirmed by MN assay. When MN formation was analyzed after treatment with different concentration of methanol extracts of *Piper longum* L., significant changes in the frequency of MN were detected for 10, 20 and 40 µg/ml ($p<0.01$) when compared to control samples (table 5). MEPL treatment decreased the frequency of MN in a concentration dependent manner.

CONCLUSION

The results revealed the presence of substantial amount of bioactive constituents in *Piper longum*. The free radical scavenging activity has shown that the efficiency of plant species differ depending on the particular assay methodology, reflecting the complexity of the mechanisms involved in total antioxidant capacity. The protection afforded by *Piper longum* L. against the cytotoxicity of peripheral blood lymphocytes was confirmed by MN assay and it was observed that different concentrations of MEPL showed significant ($p<0.01$) changes in the frequency of micronuclei in a concentration dependent manner.

CONFLICT OF INTERESTS

Declared None

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