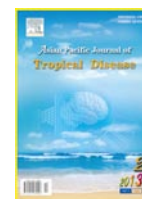




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Evaluation of antioxidant potential of leaves of *Leonotis nepetifolia* and its inhibitory effect on MCF7 and Hep2 cancer cell lines

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PEER REVIEW

ABSTRACT

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Comments

This is a good study and considered for publication in the journal. Because, it is a very basic study, it has very good scope to further continue this work with more advanced tools to prove their anticancer activity.

(Details on Page 109)

Objective: To investigate the antioxidant and antiproliferative potential of *Leonotis nepetifolia* (*L. nepetifolia*) leaves. **Methods:** The leaves of *L. nepetifolia* were subjected to extraction using three different solvents and the antioxidant potential of those extracts were tested by using various *in vitro* assays. Further, the best screened extract was analyzed for its phytochemical profile by both qualitative and quantitative assays. In order to determine its anti-proliferative activity, the best screened extract was treated with breast and laryngeal cancer cell lines such as MCF-7 cells and Hep2 cells, respectively. The cytotoxicity of the extract was also studied using MTT assay. The inhibitory effect of the extract of leaves of *L. nepetifolia* on the selected cell-line DNA was determined by DNA fragmentation assay. Also, the extract was subjected to TLC and bioautography analysis. **Results:** The DPPH assay showed methanol extract of *L. nepetifolia* leaves to be more significant in scavenging free radicals with inhibition percentage of 60.57%. From the data obtained, the methanol extract proved to be significant in all anti-oxidant assays and this effect was well comparable with the standard used in the study. The predominant phytochemicals such as phenols and flavonoids were further quantified as 0.107% and 0.089%. The cytotoxicity assay showed that the cell viability increased with increasing concentration of methanol extract. In addition, the extract caused dose dependent damage to the cancer cell lines MCF-7 and Hep2. **Conclusions:** Our study suggests that the leaves of *L. nepetifolia* were significant in scavenging free radicals and causing damage to proliferative cells. Further mechanistic studies would help in proving the efficiency of the selected plant under *in vivo* conditions.

KEYWORDS

Leonotis nepetifolia, antioxidant assays, MCF-7, Hep 2, Bioautography, DNA fragmentation

1. Introduction

Free radical-induced oxidative damage is involved in the pathogenesis of many chronic and degenerative diseases, such as cardiovascular disease, cancer, diabetes, neurodegenerative disease and ageing[1–3]. In recent years, much attention has been devoted to natural antioxidant and their association with health benefits[4]. Consumption of

antioxidant constituents reported to have protection against oxidative damage induced degenerative and pathological processes including ageing and cancer[5]. Harmful effects resulted from the disequilibrium in the antioxidant-prooxidant balance that can be largely prevented by the intake of antioxidant substances[6]. Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce

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the risk for chronic diseases including cancer and heart disease[7]. Oxidation processes are intrinsic in the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms[8]. Antioxidant properties have been studied in several plant species for the development of natural antioxidant formulations in the areas of food, medicine and cosmetics[9]. Health promoting effects of antioxidants in plants have been well documented. Antioxidants work by donating an electron to a molecule that has been compromised by oxidation, bringing it back into a state of proper function[10].

The present study has therefore been designed to determine the antioxidant and anticancer activities of *Leonotis nepetifolia* (*L. nepetifolia*) and to profile the active compounds present in the leaf extract. *L. nepetifolia*, also known as Klip Dagga or Lion's Ear, is a species of plant in the *Leonotis* genus and the Lamiaceae (mint) family. *L. nepetifolia* is known in Trinidad as shandilay and the leaves are brewed as a tea for fever, coughs, womb prolapse and malaria.

2. Materials and methods

2.1. Plant collection and extraction

Fresh plant *L. nepetifolia* was collected from the fields located in Melachery forest, Gingee, Tamil Nadu. The collected plant was taxonomically identified by Dr. N. Mathivanan, CAS in Botany, University of Madras, Tamil Nadu, Chennai. After identification, the leaves were separated, carefully washed with tap water, rinsed with distilled water left for drying under shade. Then they were ground into coarse powder and subjected to extraction following the method of Eloff[11], using solvents such as chloroform, ethyl acetate and methanol. The extract was further subjected to concentration by condensation in order to obtain a syrupy mass.

2.2. Evaluation of in vitro antioxidant potential

2.2.1. Radical scavenging assay (DPPH method)

The radical scavenging activity of the three solvent extracts of *L. nepetifolia* was studied using DPPH method as proposed by Harini *et al.* with minor modifications[8]. Varying concentrations of the leaf extracts (dissolved in DMSO) was added to 5 mL of methanolic solution of DPPH (0.1 mmol/L), shaken vigorously and allowed to stand for 20 min at 27 °C, and the absorbance was measured at 517 nm. Pure DPPH solution served as control. The radical scavenging activity (RSA) was expressed as the inhibition percentage of free radical by the sample which was calculated using the formula:

$$\%RSA = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100\%$$

Where, $\text{Abs}_{\text{control}}$ is absorbance of control at 517 nm, $\text{Abs}_{\text{sample}}$ is absorbance of sample at 517 nm.

2.2.2. Phosphomolybdenum assay

The phosphomolybdenum reducing potential (PRP) of methanol extract of *L. nepetifolia* leaves (MELL) was determined by using the assay method followed by Prieto *et al.*[12]. A total of 100 μL of MELL was combined with 1 mL of reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate, and 4 mmol/L ammonium molybdate) in a 4 mL vial, capped and incubated in a water bath at 95 °C for 90 min. After cooling the samples to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The result was expressed in terms of percentage inhibition:

$$\%PRP = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100\%$$

Where, $\text{Abs}_{\text{control}}$ is absorbance of control at 695 nm, $\text{Abs}_{\text{sample}}$ is absorbance of sample at 695 nm.

2.2.3 Super oxide anion radical scavenging activity

The superoxide anion radical scavenging efficiency of MELL was measured using the method of Nishikimi *et al.*[13]. The reaction mixture consisted of 1 mL of nitroblue tetrazolium (NBT) solution (156 $\mu\text{mol/L}$ NBT in 100 mmol/L phosphate buffer, pH 8.0), 1 mL of reduced form of nicotinamide-adenine dinucleotide (NADH) solution (468 $\mu\text{mol/L}$ in 100 mmol/L phosphate buffer, pH 8.0), 0.1 mL of MELL (10 mg/mL) and 100 μL of PMS (60 $\mu\text{mol/L}$ phenazine methosulphate in 10 mmol/L, phosphate buffer, pH 8.0). The reaction mixture was incubated 25 °C for 5 min and the absorbance was read at 560 nm.

$$\text{Superoxide anion radical scavenging activity (\%)} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100\%$$

Where, $\text{Abs}_{\text{control}}$ is absorbance of control, $\text{Abs}_{\text{sample}}$ is absorbance of sample.

2.2.4. Hydroxyl radical scavenging activity (HRSA)

The HRSA of MELL was determined using the method of Klein *et al.*[14]. MELL was added to 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%) and 1.0 mL of DMSO (0.85% v/v in 0.1 mol/L phosphate buffer, pH 7.4) were added and the reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%). The reaction mixture was incubated at 80–90 °C for 15 min in a water bath, and the reaction was terminated by the addition of 1 mL of ice-cold trichloroacetic acid (TCA) (17.5% w/v) and 3 mL of Nash reagent (75 g of ammonium acetate, 3 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and made up to 1 L with distilled water) and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. The percentage of hydroxyl radical scavenging activity was calculated by the following formula:

$$\%HRSA = \frac{(A_0 - A_1)}{A_0} \times 100\%$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

2.2.5. Metal chelating activity

The chelating of ferrous ions by MELL was estimated by

the method of Dinis *et al*[15]. Briefly, the leaf extract (250 μ L) was added to a solution of 2 mmol/L FeCl_2 (0.05 mL) and the reaction was initiated by the addition of 5 mmol/L ferrozine (0.2 mL). The reaction mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance was measured at 562 nm. The result was expressed as percentage of inhibition:

$$\% \text{Metal chelating activity} = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} \times 100\%$$

Where, $\text{Abs}_{\text{control}}$ is absorbance of control, $\text{Abs}_{\text{sample}}$ is absorbance of sample.

2.2.6 Ferric thio cyanate (FTC) assay

FTC assay was performed using the method followed by Osawa and Namiki[16]. MELL (4 mg in 99.5% ethanol) was mixed with 4.1 mL of linoleic acid (2.51% in 99.5% ethanol), 8 mL of phosphate buffer (0.05 mol/L, pH 7.0) and 3.9 mL distilled water and kept in tightly closed vials under dark at 40 °C. To 0.1 mL of this solution, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added. After 3 min, 0.1 mL of 2 mol/L ferrous chloride in 3.5% HCl was added to the reaction mixture and the absorbance of the red color was measured at 500 nm each 24 h until one week when the absorbance of the control reached maximum.

2.2.7. Thio barbituric acid (TBA) assay

The same samples prepared for the FTC method were used. To 1 mL of sample solution, 2 mL of 20% aqueous TCA was added and incubated in boiling water bath for 10 min. After cooling, it was centrifuged at 3000 r/min for 20 min and the absorbance of supernatant was measured at 532 nm. Antioxidative activity was recorded based on absorbance on the eighth day[17].

2.2.8. Ferric reducing antioxidant potential (FRAP) assay

The reducing power of plant extract was determined according to the method of Oyaizu[18]. MELL of different concentrations (0.2 to 1 mg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min and 2.5 mL of TCA (10%) was added, the mixture was centrifuged at 3000 r/min for 10 min. Supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of ferric chloride (0.1%) and the absorbance was measured at 700 nm.

2.3. Investigation of phytochemicals

Various qualitative tests were performed on MELL for establishing the phytochemical profile by following standard methods as suggested by Harborne[19]. Further, the predominant phytochemicals preset in the extract such as phenols and flavonoids were quantified by following the methods of Mc Donald *et al.* and Meda *et al.* respectively[20,21].

2.4. Thin layer chromatography (TLC)

The MELL was loaded on pre-coated silica plates which were then developed using the solvents methanol and chloroform in the ratio of 0.75:9.25. The spots were identified under short UV, far UV and in an iodine chamber. The R_f values of the separated compounds were calculated as the ratio of distance travelled by the solute to the distance travelled by the solvent[11].

2.5. Bioautography

The MELL was examined by TLC bioautography. The plant extract was applied to pre-coated silica sheet and run with the developing solvent mixture and allowed to dry. After drying, the plates were dipped in 0.2% DPPH reagent in methanol or ethanol and were left for 30 min at room temperature. The plates were observed under white light. Antioxidant activity was confirmed when the DPPH purple color changed to yellow[22].

2.6. Assessment of antiproliferative activity

2.6.1. Cytotoxicity assay on cancer cell lines

For the assessment of the antiproliferative activity of MELL, MTT assay was performed on, MCF-7 (Breast cancer cell line) and Hep-2 (Liver cancer cell line) cells. The cell lines were collected from National centre for cell sciences Pune (NCCS) and were grown in RPMI-1640 medium at 37 °C under incubated for 6–7 h at 5% CO_2 in a humidified incubator. The cytotoxicity was determined by following the method of Mosmann[23]. To confirm the cytotoxic effect of MELL on cancer cell lines, DNA fragmentation assay was performed. Where, the cancer cell DNA was isolated following standard technique and the isolated DNA was subjected to agarose gel electrophoresis. The resulting bands were compared with a standard marker.

3. Results

3.1. RSA of *L. nepetifolia* extracts

From the dose dependent response curve of DPPH radical scavenging activity of different leaf extracts of (Figure 1).

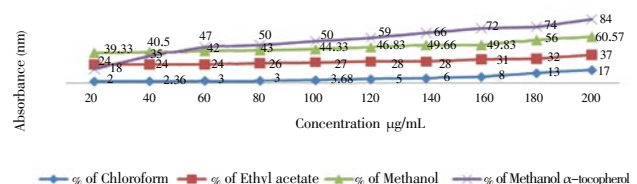


Figure 1. RSA of extracts of *L. nepetifolia*.

L. nepetifolia was observed that MELL had higher scavenging activity than ethyl acetate and chloroform. At a concentration

of 40 $\mu\text{g/mL}$, the scavenging activity of MELL reached 61%, which was comparable to that of standard chemical. In considering this, the methanol leaf extract was chosen for further study.

3.2. Phosphomolybdenum assay

The MELL showed reduction in phosphomolybdenum complex which was observed as reduction in green phosphomolybdenum complex. The results indicate that the methanol extract was significant in the reduction of phosphomolybdenum complex which was evident by the note decrease in absorbance values as shown in Figure 2.

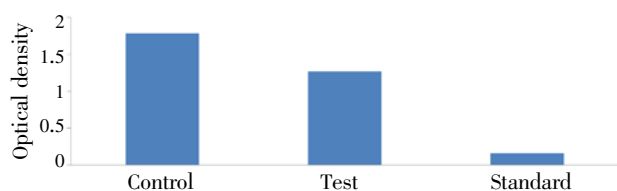


Figure 2. Phosphomolybdenum reducing potential of methanol extract of *L. nepetifolia*.

3.3. Superoxide anion radical scavenging assay

The decrease in the absorbance at 560 nm indicated the consumption of superoxide anion in the reaction mixture. The MELL showed strong superoxide radical scavenging activity which is evident from the decrease in optical density value when compared to control, as shown in Figure 3.

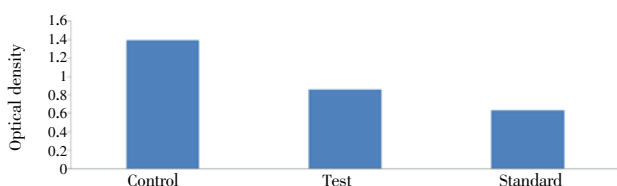


Figure 3. Superoxide scavenging effect of *L. nepetifolia*.

3.4. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of MELL and standard is presented in Figure 4. It was evident that the decrease in absorbance values by the *L. nepetifolia* extract was comparable with the standard used.

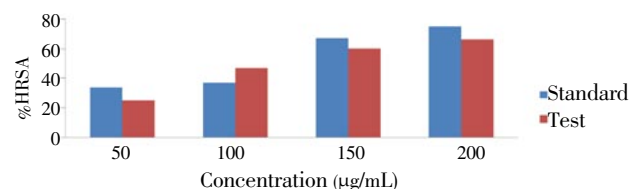


Figure 4. Hydroxyl radical scavenging activity of *L. nepetifolia*.

3.5. Metal chelating activity

In this assay, the presence of chelating agents in the MELL disrupts the ferrozine- Fe^{2+} complex formation, thus

decreasing the red colour and hence the absorbance decreases (Figure 5).

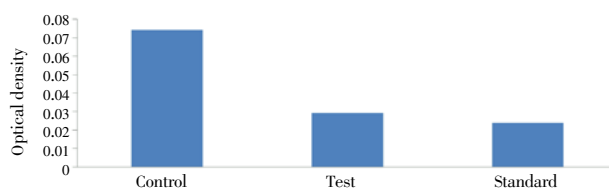


Figure 5. Metal chelating activity of *L. nepetifolia*.

3.6. Ferric thiocyanate (FTC) method

Figure 6 details the absorbance values of MELL. It was interesting to note that the methanol extract exhibited higher antioxidant activity which was obvious from the decrease in the optical density. Also, the FTC reducing potential of sample was comparable to that of the standard used.

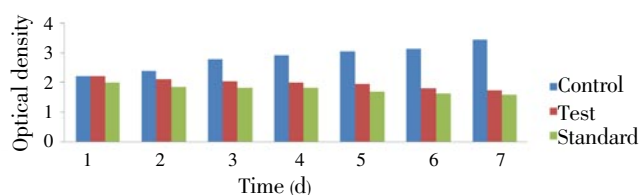


Figure 6. FTC reducing activity of *L. nepetifolia*.

3.7. Thio barbituric acid (TBA) assay

The antioxidative activity of MELL was high on 7th day of incubation (Figure 7). The reduction in TBA by the methanol extract was comparable with that of the standard.

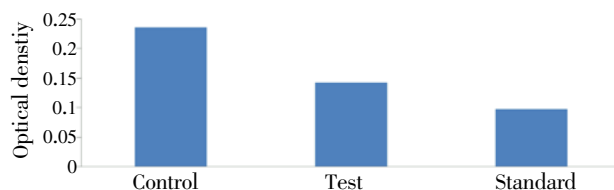


Figure 7. TBA reducing potential of *L. nepetifolia*.

3.8. Reducing power assay

Figure 8 shows the reducing power of the MELL and BHT, as a function of their concentrations. It was evident that the reduction in the absorbance was a function of the sample concentration.

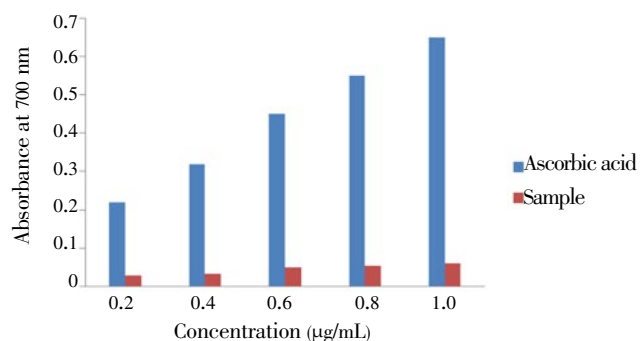


Figure 8. Ferric reducing power of *L. nepetifolia*.

3.9. Qualitative Phytochemical screening of methanol extract of *L. nepetifolia*

The preliminary phytochemical screening of MELL has revealed the presence of phenolics, flavonoids and reducing sugar in high amounts, whereas, terpenoids and tannins were present in moderate amount. Alkaloids, glycosides and saponins were present in trace amount. Proteins were completely absent (Table 1).

Table 1

Qualitative phytochemical screening of *L. nepetifolia*.

Phytochemicals	Results
Alkaloids	+
Phenolic compounds	+++
Glycosides	+
Terpenoids	++
Flavonoids	+++
Tannins	++
Reducing sugars	+++
Saponins	+
Proteins	–

+++; in higher amount; ++; in moderate amount; +; in trace amount; –; not detectable.

3.10. Determination of total phenols and flavonoids by spectrophotometric method

Based upon the preliminary phytochemical test, quantitative determination of phytoconstituents was carried out for the extracts of *L. nepetifolia*. From the standard methods used and found that total phenol 0.107% and flavonoids 0.089% was present in this plant methanolic extract.

3.11. Thin layer chromatography

The chromatogram developed with 10% methanol in chloroform revealed the presence of 10 major compounds at R_f value of 0.21, 0.29, 0.36, 0.42, 0.50, 0.58, 0.68, 0.74, 0.81 and 0.89 as visualized under iodine vapour and UV illumination (Figure 9).

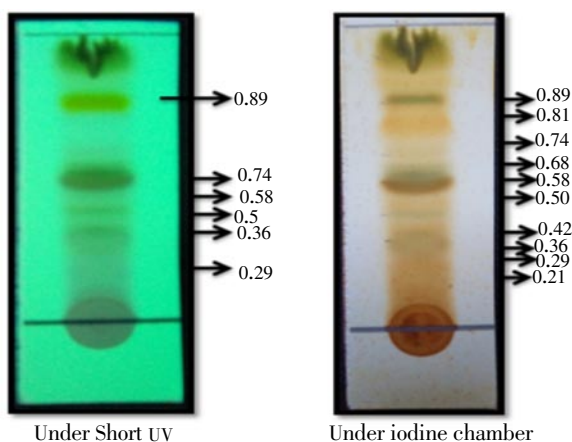


Figure 9. TLC chromatogram of *L. nepetifolia*.

3.12. Bioautography

The specific compound (band) which has anti oxidative properties showed in the R_f value of 0.44, which was chosen as effective compound for obtaining partially purified compound. The purity of the compound was checked by TLC with 20% methanol in chloroform (Figure 10). Antioxidant activity was confirmed when the DPPH purple color changed to yellow.

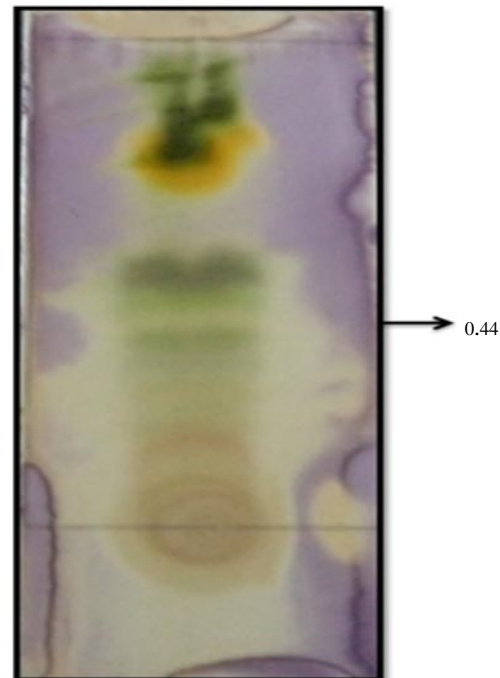


Figure 10. Bioautography of *L. nepetifolia*.

3.13. Antiproliferative effect of MCF-7 cell line

The Antiproliferative activity of the MELL was studied by MTT assay (Figure 11). In this assay, cell death and cell viability were estimated. It had 45.2% cell viability in the ratio of 1:4 (1.25 mg/mL) as shown in Figure 12.

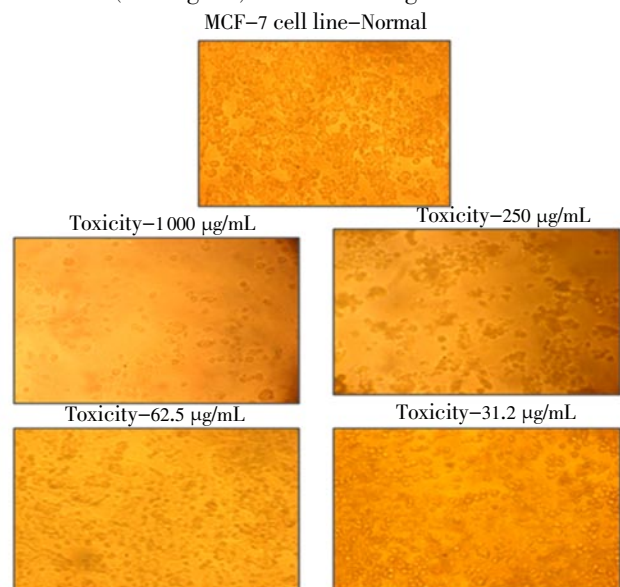


Figure 11. Effect of *L. nepetifolia* on MCF-7 cells (at 400 \times).

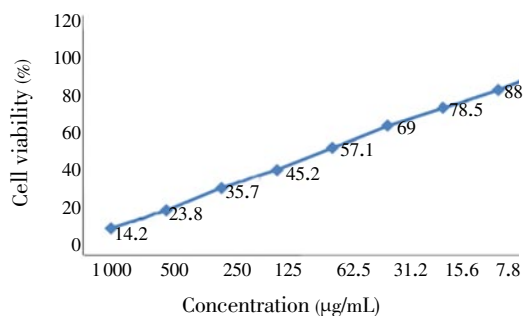


Figure 12. Cytotoxicity of *L. nepetifolia* on MCF-7 cells.

3.14. Antiproliferative effect of Hep 2 cell line

The MELL was subjected for MTT assay (Figure 13). In this assay cell death and cell viability was estimated. It had 56.25% cell viability in the ratio of 1:4 (1.25 mg/mL) as shown in Figure 14.

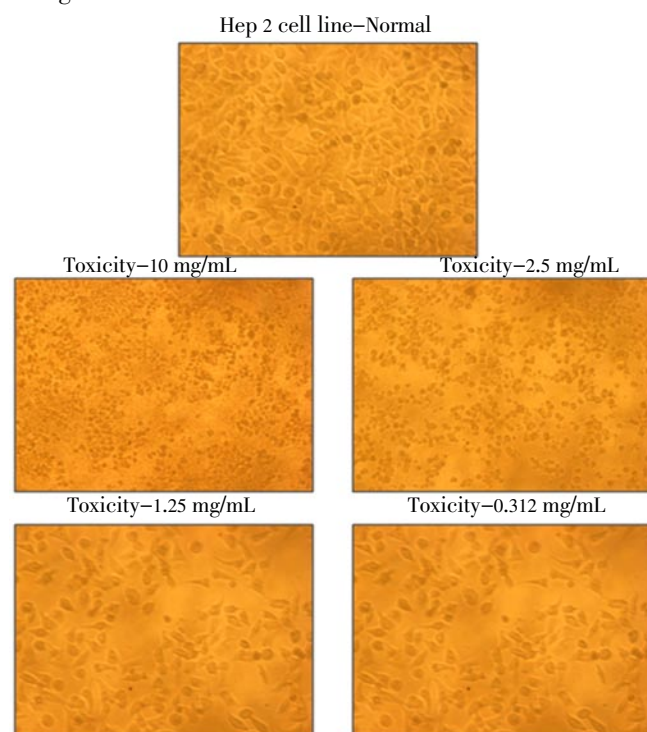


Figure 13. Effect of *L. nepetifolia* on Hep-2 cells (at 400×).

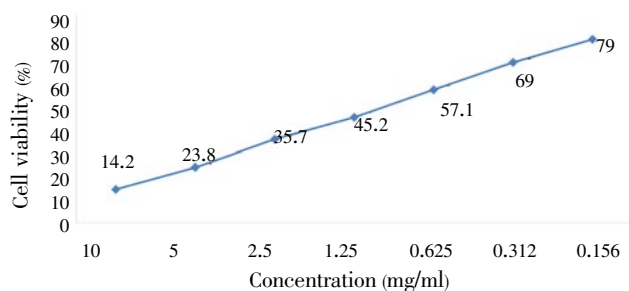


Figure 14. Cytotoxicity of *L. nepetifolia* on Hep-2 cells.

3.15. DNA fragmentation

Further, the DNA fragmentation assay was performed in order to confirm the mechanism of action of the

crude extract. There was prominent fragmentation at the concentrations of 2.5 mg/mL and 1.25 mg/mL which revealed that the MELL inhibits the growth of the Hep2 cells through DNA fragmentations. In case of MCF 7 cells, significant DNA fragmentation was observed at a concentration of 125 µg/mL. The results of DNA fragmentation analysis on MCF7 and Hep2 cells are depicted in Figures 15 and 16.

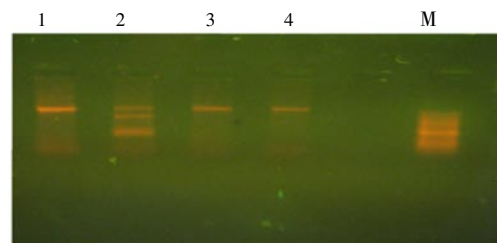


Figure 15. Effect of *L. nepetifolia* on MCF-7 cell DNA fragmentation. Lane 1: Concentration: 62.5µg/mL; Lane 2: Concentration: 125 µg/mL; Lane 3: Concentration: 250 µg/mL; Lane 4: Control; Lane M: Marker 1000 bp.

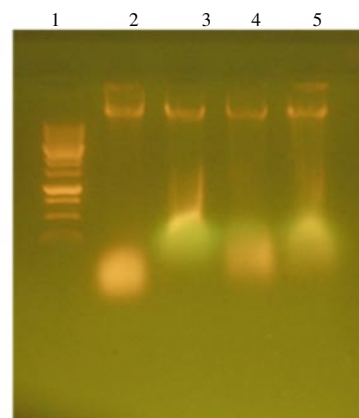


Figure 16. Effect of *L. nepetifolia* on Hep-2 cell DNA fragmentation. Lane 1: Marker 1000 bp ladder; Lane 2: Control; Lane 3: Con 5 mg/mL; Lane 4: Con 2.5 mg/mL; Lane M: Con 1.25 mg/mL.

4. Discussion

Imbalance in pro-oxidant and antioxidant homoeostasis occasioned by excessive free radicals generation or insufficient antioxidants has been implicated in the development of several human disease conditions, such as atherosclerosis, hypertension ischaemic diseases, Alzheimers' disease, Parkinsonism and cancer^[24]. Different extracts from traditional medicinal plants have been tested to identify the source of the therapeutic effects. As a result, some natural products have been approved as new antimicrobial drugs, but there is still an urgent need to identify novel substances that are active towards pathogens with high resistance^[25].

It is well known that from the three extracts, chloroform, ethyl acetate, and methanol, the methanol extract is suitable for the extraction of antioxidant compound^[16]. The DPPH radical scavenging ability of the methanol extract of *L. nepetifolia* was significantly higher than those of α -tocopherol which revealed that the ability of proton donating capacity of the methanol extract of *L. nepetifolia*

was very high. Superoxide radical can be converted into hydrogen peroxide by the action of superoxide dismutase, and the H_2O_2 can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions (e.g. iron and copper), or by UV photolysis. Hydroxyl radicals can attack DNA molecules to cause strand scission^[27]. Metal chelating capacity is important since it has reduced the concentration of the catalysing transition metal in lipid peroxidation^[28]. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron^[29]. The reducing capacity of the plant extract components may serve as a significant indicator of its potential antioxidant activity^[30,31].

Phenolic compounds are dietary constituents widely existing in plants and have been considered to have high antioxidant capacity and free radical scavenging capacity^[32,33]. Flavonoids are well-known antioxidant constituents of plants and possess a broad spectrum of chemical and biological activity, including radical scavenging properties^[9]. Phytochemical screening of the crude extracts revealed the presence of secondary compounds such as alkaloids, flavonoids, steroids, tannins, and phenols^[34]. Presence of these phytochemicals in methanol extract of *L. nepetifolia* attributed to their excellent antioxidant activity.

Data suggests that the crude methanol extract of *L. nepetifolia* reduced the cell viability upto 50% at very low concentration, and the inhibition was time and dose dependent manner. Further, there was a prominent inhibition in the total intra cellular protein content as analyzed by Bradford^[35], followed by the DNA fragmentation, as analyzed by agarose gel electrophoresis compared to control cells which didn't received the crude extract.

TLC of the methanol extract of *L. nepetifolia* followed by bioautography revealed that methanol extract of *L. nepetifolia* have a compound in the region of low polar, based on the zone of inhibition that are capable to scavenge free radicals as analysed by DPPH assay. In the present study, the TLC analysis of methanol extract of *L. nepetifolia* showed 8 compounds with varying R_f values. This signifies that the *in vitro* antioxidant properties and the anti-cancer property of *L. nepetifolia* could be attributed to the presence of these compounds either solely or in combination. Further mechanistic studies are needed for better understanding of their mechanism of action and to elucidate the specific phytochemical that is responsible for the studied property.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

The paper has concentrated on the isolation,

characterization and evaluation of anticancer potentials of *L. nepetifolia* leaf extracts. It also concentrated on some preliminary studies such as phytochemical screening and antioxidant potential of the extracts.

Research frontiers

The paper has information on the following research frontiers:

1. Extraction of crude metabolites from the leaves of *L. nepetifolia*;
2. Antioxidant potential of the above extracts;
3. Antiproliferative activities of the above extracts on breast and laryngeal cancer cell lines;
4. DNA fragmentation assay for confirming the cell death.

Related reports

Free radical-induced oxidative damage is involved in the pathogenesis of many chronic and degenerative diseases, such as cardiovascular disease, cancer, diabetes, neurodegenerative disease and ageing (Azizova 2002, Nagler 2006, Barnham *et al.*, 2004). In recent years, much attention has been devoted to natural antioxidant and their association with health benefits (Arnous *et al.*, 2001). The authors seem to have used adequate standard methodologies to obtain their results.

Innovations & breakthroughs

It has been demonstrated successfully that the leaves of *L. nepetifolia* were significant in scavenging free radicals and causing damage to proliferative cells there by acting against breast and laryngeal cancer cell lines. Thus this study paves avenue to do further research on anticancer activity of the important medicinal plant *L. nepetifolia*.

Applications

This study scientifically suggests that leaves of *L. nepetifolia* were significant in scavenging free radicals and causing damage to proliferative cells there by acting against breast and laryngeal cancer cell lines. This study also has scope to do further research on anticancer activity.

Peer review

This is a good study and considered for publication in the journal. Because, it is a very basic study, it has very good scope to further continue this work with more advanced tools to prove their anticancer activity.

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