Research Article

In Vitro Evaluation of Antioxidant Activity of Different Extracts of *Myristica fragrans* Houtt

Saranya Sivaraj[1], Vaidehi Patel[1], Alamelu Mangai[1], Gomathi Kannayiram[1], Gayathri Dasararaju[2,*]
[1]Department of Biotechnology, Dr. M.G.R Educational and Research Institute University, Maduravoyal, Chennai, India.
[2]CAS in Crystallography and Biophysics, University of Madras, Guindy campus, Chennai, India.
***Corresponding author's E-mail:** drdgayathri@gmail.com

Received: 30-11-2016; **Revised:** 10-01-2017; **Accepted:** 18-01-2017.

ABSTRACT

In vitro free radical scavenging efficacy of the sequentially extracted (hexane, dichloromethane (DCM), ethyl acetate (EtAc) and ethanol extracts) *Myristica fragrans* (mace) was investigated through DPPH and hydrogen peroxide radical scavenging assays. Thin layer chromatography (TLC) study was performed for all extracts using hexane/ethyl acetate solvent system (6:4). All four extracts scavenged the DPPH radical in the dot blot method. At 500 µg/ml concentration, DCM and ethyl acetate extracts showed the maximum scavenging of 90% and 92%, respectively, when compared with hexane and ethanol extract (76% and 83%). In the case of hydrogen peroxide scavenging assay, hexane and ethyl acetate showed 85% and 96% inhibition at the concentration of 500 µg/ml of extract against the DCM and ethanol, which showed 71% and 76% inhibition respectively. TLC study revealed the presence of maximum phytoconstituents in DCM and ethyl acetate extracts. The results of this study strongly indicate that among four extracts ethyl acetate showed good inhibition of DPPH and hydrogen peroxide radicals indicating its potential antioxidant capability.

Keywords: *Myristica fragrans*, Antioxidant, DPPH, Hydrogen peroxide, TLC.

INTRODUCTION

Reactive oxygen species (ROS) are unstable and highly reactive structures, which may be produced due to partial chemical reductions of oxygen that include superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). ROS and other free radicals are produced inside the human body as a result of normal essential internal metabolic processes or from external sources like X-rays, ozone, smoking, chemicals and pollutants[1]. When there is an imbalance between ROS and antioxidants in the human system, oxidative stress occurs that would damage nucleic acids, proteins, and lipids. Damaging the basic components of the human system results in an occurrence of various diseases such as cancer[2], neurodegeneration[3,4], atherosclerosis, diabetes[5], aging[6] and inflammation[7]. Synthetic antioxidants vitamin C, tocopherols, carotenoids, flavonoids and variety of phenolic compounds have been developed, which would help in creating a disease free environment in the human system. Unfortunately, prolonged usage of synthetic compounds could result in chronic diseases like liver cirrhosis, renal failure and cancer. Therefore, researchers have started focusing on developing antioxidants from natural sources particularly plants.

Plants are known to treat and manage various human ailments since ancient times. This property is due to the presence of a variety of chemical substances that generally occur as secondary metabolites, which include saponins, tannins, flavonoids, phenols, essential oils and alkaloids[8]. A huge number of plants are evaluated for their medicinal properties and reported to play a significant role in the prevention of degenerative diseases[9]. Recently, aromatic spices and herbs have been discovered as the major source of many phytochemicals, which act as powerful antioxidants. One such spice is *Myristica fragrans* (*M.fragrans*), which is widely used as a food flavoring in soups, sweet sauces, fruit juice, curries, and etc[10]. The plant is extensively known for its medicinal properties such as memory enhancer, aphrodisiac, anti diarrheal, anticancer and anti-inflammatory[11]. Further, the essential oil from the plant has been used to treat rheumatism and stomach disorders in many countries like Indonesia, Malaysia, England, and China. With the above scenario, the sequentially extracted (hexane, dichloromethane, ethyl acetate, and ethanol extracts) mace was subjected to various assays in order to evaluate their free radical scavenging capabilities.

MATERIALS AND METHODS

Chemicals and reagents

2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich. Ascorbic acid was purchased from Hi-Media laboratories Pvt. Ltd. Solvents were purchased from Loba Chemicals. 30% hydrogen peroxide was obtained from Merck Millipore.

Plant material and Extraction

The aril part of the *Myristica fragrans* was obtained from traditional Indian spices market and authenticated by Dr. N. Radha krishnan, Assistant Professor, Centre of Advanced Study in Botany, University of Madras, Guindy Campus, Chennai. Mace was shade dried and powdered using electrical grinder and was sequentially extracted with organic solvents based on increasing polarity starting from hexane, DCM, ethyl acetate and ethanol[12]. 100 g of

powdered mace was soaked in 300 ml of 100% hexane for three days with intermittent shaking. Later the fraction was filtered using Whatmann filter paper No. 1 and dried using china dish covered with a perforated aluminum foil. DCM solvent was added to the sediments of mace; incubated, filtered and dried. Similar procedure was performed for ethyl acetate and ethanol samples. The fractions were stored at 4 °C for future investigations.

DPPH radical scavenging Method

Dot blot Method

Diluted plant extract sample were loaded onto a 5 cm × 5 cm TLC layer (silica gel 60 F_{254}; Merck) and allowed to dry (3 min). The sheet bearing the dry spots was placed upside down for 10 s in a 0.4 mM DPPH solution. The excess solution was removed with a tissue paper and the layer was dried. Stained silica layer exposed a purple background with white spots at the location where radical scavengers were present. The intensity of the white color depends on the amount and nature of radical scavenger present in the sample[13].

DPPH assay Method

DPPH assay was performed based on the protocol[14]. The decrease in the absorption at 517 nm is used to measure the ability of the extract to scavenge the stable free radical DPPH. To an ethanol solution of DPPH (0.01 mM), varying concentration of different extracts were added and made up to a final volume of 1 ml with ethanol. After 30 minutes incubation, absorbance was recorded at 517 nm in a UV-visible double beam spectrophotometer. The DPPH radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = $[(A_0 – A_1/A_0) \times 100]$

Where A_0 is the absorbance of the control and A_1 is the absorbance of plant extract.

Hydrogen peroxide radical scavenging assay

The assay was performed to determine the capability of the mace extracts to scavenge hydrogen peroxide (H_2O_2) based on the protocol[15]. 40 mM hydrogen peroxide was prepared using phosphate buffer, *pH* 7.4 and incubated with different concentrations of plant extracts (50-500 µg/ml) for ten minutes at room temperature. The incubated reaction mixture was measured for its hydrogen peroxide activity at 230 nm against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard and the percentage inhibition was calculated using the following formula:

Hydrogen peroxide radical scavenging activity (%) = $[(A_0 – A_1/A_0) \times 100]$

Where A_0 is the absorbance of the control and A_1 is the absorbance of plant extract.

Thin layer chromatographic (TLC) studies

All the extracts of mace were subjected to TLC studies using a silica plate ($60F_{254}$, 24 x 24, Merck). Each extract was diluted with respective solvent and 2 µl of the sample was loaded on to the plate. TLC chamber was pre-saturated with the mobile phase; hexane/ethyl acetate (6:4), later the TLC plate loaded with the sample was kept inside the chamber. After the run, the plates were viewed under the UV and also developed using anisaldehyde mixture. R_f values of the compounds were calculated based on the formula given below.

R_f = Distance traveled by the solute/Distance traveled by the solvent front

RESULTS

Dot blot assay

Fig 1 explains the dot blot method of visual screening of the extracts, whether it has free radical scavenging effect before quantifying the potency spectrophotometrically. Equal concentration of different extracts was placed as a dot on a TLC layer that was later stained with DPPH. The emergence of pale yellow spot around the purple background suggests the antioxidant potential of extracts. This assay works based on the principle that inhibition of free radicals by antioxidants leads to appearance of pale spot. From this method, it was inferred that all the four extracts such as hexane, DCM, ethyl acetate, and ethanol have free radical scavenging effect with ethyl acetate extract showing the intense yellow color indicating the presence of more antioxidants.

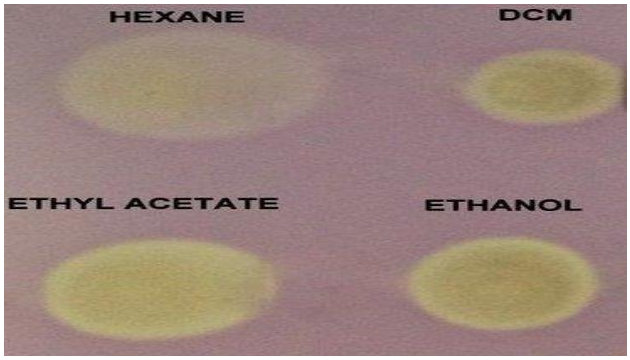

Figure 1: Dot blot DPPH radical screening of different extracts

DPPH scavenging assay

DPPH radical scavenging is widely used to quantitate the antioxidant potential of the extracts. Fig 2 shows the DPPH radical scavenging ability of four different extracts in four concentrations. The results clearly indicated that all the extracts have antioxidant potential in all four concentrations. The scavenging ability of the different extracts increases in a dose dependent manner when compared with the control.Ascorbic acid at a concentration of 100 µg/ml was used as a control for comparison study. The percentage inhibition of DPPH radical by DCM and ethyl acetate extract was better when compared with the other two extracts. At 500 µg/ml

concentration, DCM and ethyl acetate extracts showed the maximum scavenging of 90% and 92%, respectively, when compared with hexane and ethanol extract (76% and 83%).

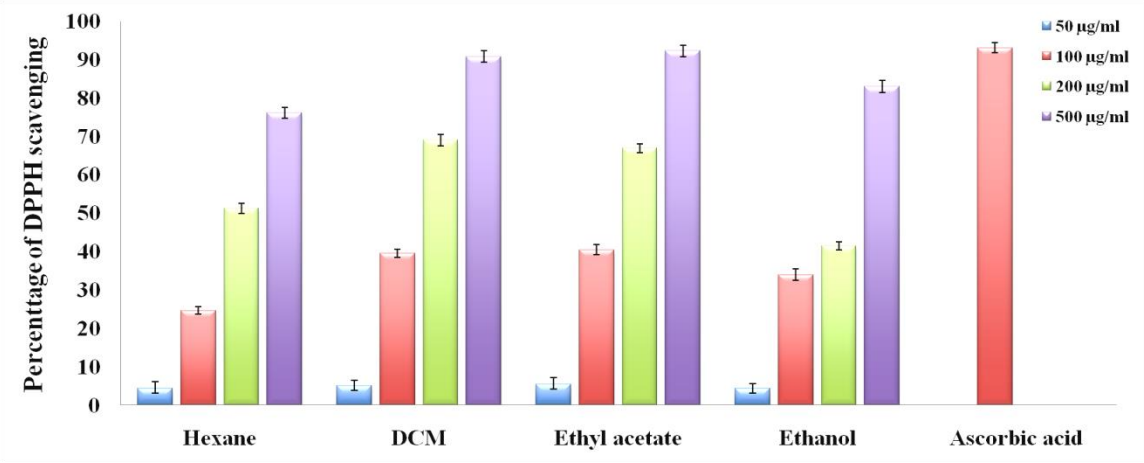

Figure 2: DPPH scavenging activity of different extracts. Values are expressed in mean ± SD (n = 3).

Hydrogen peroxide radical scavenging assay

The quantitative determination of hydrogen peroxide scavenging ability of plant extracts is essential as this free radical is involved in oxidative cellular damages. Fig 3 depicts the hydrogen peroxide scavenging activity of different plant extracts. All the plant extracts and the positive control ascorbic acid demonstrated a concentration-dependent scavenging activity of hydrogen peroxide radicals. The inhibitory activity was minimum in low concentration of plant extract and increases with increase in the concentration. Three extracts namely, hexane, DCM and ethyl acetate showed hydrogen peroxide scavenging of more than 50% at 100 µg/ml except for the ethanol extract. Hexane and ethyl acetate showed 85% and 96% inhibition at the concentration of 500 µg/ml of extract against the DCM and ethanol, which showed71% and 76% inhibition respectively.

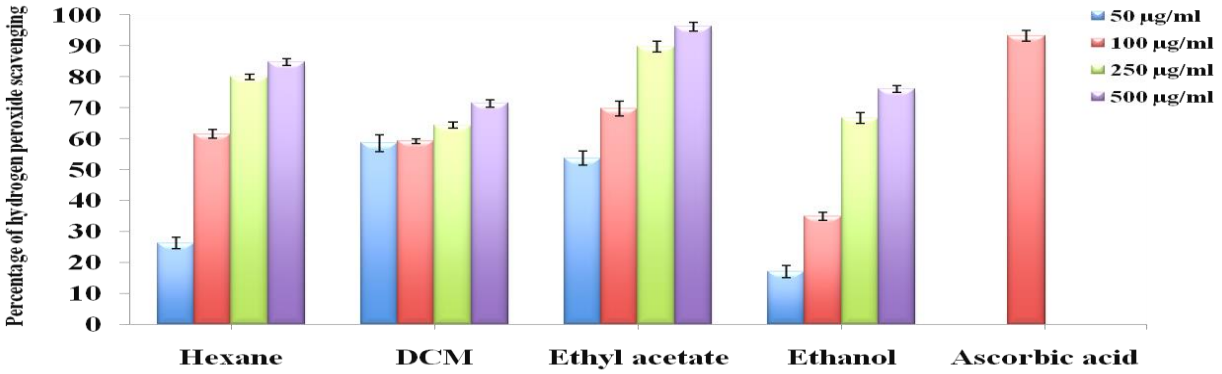

Figure 3: Hydrogen peroxide scavenging activity of different extracts of mace with control ascorbic acid. Values are expressed in mean ± SD (n = 3).

Thin layer chromatography studies

Thin layer chromatography of four extracts of mace was performed to identify the number of compounds and their polarity in the extract. Different ratios of a solvent system were prepared using hexane and ethyl acetate to run the TLC by increasing the polarity of the solvents starting from 9:1 ratio till 6:4. Better separation of the compounds was observed in 6:4 (hexane: ethylacetate) under UV_{254} nm and the plate was stained with anisaldehyde to visualize the separation in visible light (Fig 4). The results showed that there were similar kinds of compounds present in all of the four extracts of mace with the difference in concentrations. Compounds with R_f value of 0.8, 0.7, 0.5, 0.3, 0.2 were very prominent for the ethyl acetate and DCM extract whereas hexane and ethanol extracts of mace contained fewer concentrations of the compound at R_f 0.2.

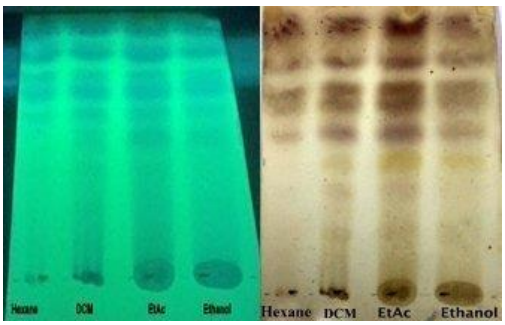

Figure 4: TLC plate with four extracts of mace under UV_{254} nm and anisaldehyde staining showing compounds at different R_f values.

DISCUSSION

Antioxidants from herbs and spices are majorly responsible for inhibiting the harmful consequences of oxidative stress by suppressing the enzymes or by chelating the trace elements. DPPH free radical scavenging assay is widely used due to its simple, rapid, inexpensive, and reproducible procedure. DPPH assay for mace revealed that DCM and EtAc extracts were shown to possess good antioxidant property, which might be due to the presence of flavonoids, tannins, and terpenoids[16,17]. Previous reports suggest that compounds having catechol structure are considered as good antioxidants as it can donate hydrogen easily to scavenge a free radical[18]. The increased DPPH scavenging ability of DCM and EtAc extracts observed in the present study indicates that these extracts may contain some phytochemicals which has the potential of scavenging ability thereby protect the biological system from oxidative attack.

Hydrogen peroxide is formed by two-electron reduction of O_2, which is not a free radical, but an oxidizing agent. In the presence of O_2 and transition metal ions, H_2O_2 can generate OH radical via fenton's reaction. So the removal of H_2O_2 is important for the antioxidant defense mechanism. In the present investigation, all the four plant extracts showed the scavenging effect of hydrogen peroxide radical. According to Lobo et al., the phenolic compounds in the extracts scavenge these free radicals by donating electrons to hydrogen peroxide thereby neutralizing it into water and are evident from the above hydrogen peroxide assay[19]. Ethyl acetate extract showed the maximum scavenging of these radicals probably due the presence high concentrations of phenols, which could convert these free radicals in to water soluble complex. The TLC studies showed the presence of similar kinds of compounds in all of our four extracts. According to Qiu et al., the major chemical constituents present in mace are alkyl benzene derivatives such as myristicin, elemicin, safrole, myristic acid, alpha-piene, terpenes, beta-pinene and trimyristin[20]. Some of these components might have been present in our extracts that were revealed as bands in our TLC studies, which might be responsible for the radical scavenging potential of our plant extract.

CONCLUSION

The results of the above study indicated that four extracts (hexane, DCM, ethyl acetate and ethanol) of aril part of *Myristica fragrans* has concentration dependent radical scavenging property. Among all the extracts, DCM and ethyl acetate extracts shown to possess higher DPPH scavenging ability and ethyl acetate extract also showed good hydrogen peroxide scavenging property when compared with other extracts. The antioxidant property of the ethyl acetate extract is comparatively better and this was proven in TLC studies. Further work is required to identify and isolate the compounds from the ethyl acetate extract that support antioxidant property.

REFERENCES

1. Bagchi K, Puri S, Free radicals and antioxidants in health and disease, East. Mediterr. Health. J, 4(2), 1998, 350–360.
2. Trachootham D, Alexandre J, Huang P, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?, Nat. Rev. Drug. Discov, 8(7), 2009, 579-591.
3. Andersen JK, Oxidative stress in neuro degeneration: cause or consequence?, Nat. Med, 10, 2004, 18-25.
4. Shukla V, Mishra SK, Pant HC, Oxidative stress in neuro degeneration, Adv. Pharmacol. Sci, 2011, 572634.
5. Matough FA, Budin SB, Hamid AZ, Alwahaibi N, Mohamed J, The role of oxidative stress and antioxidants in diabetic complications, Sultan Qaboos Univ. Med. J, 12(1), 2012, 5-18.
6. Haigis MC, Yankner BA, The aging stress response, Mol. Cell, 40(2), 2010, 4 333-344.
7. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB, Oxidative stress, inflammation, and cancer: How are they linked, Free Radic. Biol. Med, 49(11), 2010, 1603-1616.
8. Doctor TR, Manuel JF, Phytochemical screening of selected indigenous medicinal plants of Tublay, Renguet province, Cordillera administrative region, Philippines, Int. J. Sci. Res. Publi, 4(4), 2014, 1-12
9. Sofowora A, Ogunbodede E, Onayade A, The role and place of medicinal plants in the strategies for disease prevention, Afr. J. Tradit. Complement Altern. Med, 10(5), 2013, 210-229.
10. Nagano I, *Myristica fragrans*: an exploration of the narcotic spice, Entheogen. Rev, 16(1), 2008, 15-24.
11. Nagja T, Vimal K, Sanjeev A, *Myristica fragrans*: A comprehensive review, Int. J. Pharm. Pharm. Sci, 8(2), 2016, 27-30.
12. Jeyaseelan EC, Jenothiny S, Pathmanathan MK, Jeyadevan JP, Antibacterial activity of sequentially extracted organic solvent extracts of fruits, flowers and leaves of *Lawsonia inermis* L. from Jaffna, Asian Pac. J. Trop. Biomed, 2(10), 2012, 798-802.
13. Soler-Rivas C, Espin JC, Wichers HJ, An easy and fast test to compare total free radical scavenger capacity of foodstuffs, Phytochem. Anal, 11(5), 2000, 33-338.
14. Tagashira M, Ohtake Y, A new antioxidant 1,3-benzodioxle from Melissa officinalis, Planta. Med, 64, 1998, 555-558.
15. Rajamani kandan S, Sindhu T, Durgapriya D, Sophia D, Ragavendran P, Gopalakrishan VK, Radical scavenging and antioxidant activity of ethanolic extract of *Mollugonudicaulis*by *in vitro* assays, Ind. J. Pharm. Edu. Res, 45(4), 2011, 310-316.
16. Haraguchi H, Inoue J, Tamura Y, Mizutani K, Antioxidative components of *Psoralea corylifolia*, Phytother. Res, 16(6), 2002, 539-544.
17. Bousselessela H, Yahia M, Mahboubi A, Benbia S, Massinissa Y, Antioxydiant and Antibacterial activity of alkaloids and terpenes extracts from *Euphorbia granulata*, Int. J. Bio. Biomol. Agri. Food Biotech. Eng, 7, 2013, 166-169.
18. Kumar S, Pandey AK, Chemistry and Biological activities of Flavonoids: An overview, The Sci. World J, 2013, 1-16.
19. Lobo V, Patil A, Phatak A, Chandra N, Free radicals, antioxidants and functional foods: Impact on human health, Pharmacogn. Rev, 4(8), 2010, 118-126.
20. Qiu Q, Zhang G, Sun X, Liu X, Study on chemical constituents of the essential oil from *Myristica fragrans* Houtt. by supercritical fluid extraction and steam distillation, Zhongyaocai, 27, 2004, 823-826.

Source of Support: Nil, **Conflict of Interest:** None.