Research Article

In vitro evaluation of antioxidant activity of d-limonene

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Abstract

Objective: Reactive oxygen species (ROS) and free radicals are involved in pathogenesis of cancer. D-Limonene, a monoterpene present in citrus fruit has been reported for its potential anticancer activities. The aim of present study was to evaluate the *in vitro* antioxidant effect of D-Limonene. **Material and methods:** Test solutions with different concentration of D-Limonene and Trolox (standard) were prepared. Six different *In vitro* antioxidant assays such as DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging and superoxide radical scavenging assay were performed for evaluation of antioxidant activity of D-Limonene. **Results:** D-Limonene has shown appreciable antioxidant activity in comparison with Trolox. The IC₅₀ of D-Limonene in DPPH (384.73 µM), ABTS (603.23 µM), FRAP (-589.85 µM), iron chelating (-18475.5 µM), hydroxyl radical scavenging (442.75 µM) and superoxide radical scavenging assay (692.89 µM) was comparable with Trolox the IC₅₀ value of Trolox DPPH (153.30 µM), ABTS (203.37 µM), FRAP (-171.73 µM), iron chelating (225.96 µM), hydroxyl radical scavenging (146.37 µM) and superoxide radical scavenging assay (105.25 µM) respectively. **Conclusion:** In vitro antioxidant assays has shown concentration dependent reduction in free radical formation by D-Limonene in comparison with Trolox in all assays except iron chelating assay which suggests its promising role for cancer treatment.

Keywords: Antioxidant; D-Limonene; Reactive oxygen species (ROS)

Introduction

Cancer is one of the dreadful diseases which is associated with the key characteristics of uncontrolled proliferation and metastasis (Fidler, 2003). Cancer is the second leading cause of mortality, responsible for 8.8 million deaths representing nearly 1 in 6 deaths globally in 2015. According to WHO, In India nearly 1 to 1.4 million new patients is diagnosed with incidence of cancer every year, among them 0.54 million dies because of the delayed diagnosis of the disease (WHO, 2018).

Free radicals and reactive oxygen species (ROS) are the molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. It causes tissue injury by reacting with cellular components. Overproduction of ROS is involved in the pathogenesis of several diseases including diabetes, atherosclerosis, premature aging,

neurodegenerative diseases, inflammation and cancer (Collins, 1999). Reactive oxygen species encompasses singlet oxygen (¹O₂), superoxide radical (O₂⁻), hydroxyl radical (OH) and hydrogen peroxide (H₂O₂). ROS produces base pair modification, rearrangement of DNA sequence, miscoding of DNA lesion, gene duplication and the activation of oncogenes (Waris and Ahsan, 2006). Elevated levels of ROS and down regulation of endogenous antioxidant enzymes and ROS scavengers are associated with cellular proliferation, apoptosis, senescence which are implicated in the development of cancer (Agosteinelli and seiler, 2006).

Antioxidants are the chemical trap that inhibits the oxidation, especially one used to counteract the deterioration of stored food products. Numerous antioxidants are commercially available for its use in foods. However, natural antioxidants are preferred over synthetic antioxidants because of its safety and functional and sensory properties. Monoterpenes are the major components of essential oils of many plants, known for its natural antioxidants potential. It was demonstrated that reactive oxygen species can influence the growth and death of cancer cells (Miguel, 2010; Baratta et al., 1998).

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D-Limonene, a monocyclic monoterpene present in citrus fruits is colorless oil sparingly soluble in water with a sweet orange smell. Commercially, it is mostly obtained from waste orange peel and has been reported for its chemopreventive and chemotherapeutic activity against various types of cancers (Kaji et al., 2001; Lu X-G et al., 2004; Miller et al., 2013; Nakaizumi et al., 1997; Uedo N, 1999). D-Limonene is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) for a flavoring agent and is used as an additive in foods, soaps and perfumes (Ciriminna et al., 2014).

In our previous studies, we have reported that D-Limonene has inhibited the growth of K562, chronic myeloid leukemia cells *in vitro* without producing any toxicity on primary hepatocytes isolated from the mouse (Shah et al., 2018).

Since antioxidants can act through various mechanisms, the detection of such activity must be evaluated using various antioxidant assays. The objective of the present study was to evaluate the *in vitro* antioxidant effect of D-Limonene by DPPH, ABTS, FRAP, Iron chelating, Hydroxyl radical scavenging and Superoxide scavenging assay.

Materials and methods

Materials

D-limonene (MP Biomedicals Solon, OH, USA), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), dimethyl sulfoxide (DMSO), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate, Griess reagent, TPTZ (2,4,6-tripyridyl-s-triazine), ferric chloride hexahydrate, sodium acetate trihydrate, hydrochloric acid, sulphuric acid, hydrogen peroxide, were purchased from Sigma Aldrich (St. Louis, MO, USA).

Instrument

Thermo Scientific Multiskan GO, Waltham, Massachusetts, United States.

Test solution preparation

Test solutions of D-Limonene at concentration of 25, 50, 100, 200 and 400 μ M were prepared by adding 0.1% Tween 80. Trolox was used as a standard at concentration of 10, 25, 50, 100 and 250 μ M. The stock solution of trolox 1 mM was prepared in absolute ethanol and working solution of trolox (10, 25, 50, 100 and 250 μ M) were made from the stock solution by dilution with required amount of double distilled water.

Diphenylpicrylhydrazyl (DPPH) assay

DPPH assay of D-Limonene and Trolox at different concentrations was performed with some modifications. 10 ml of stock solution was mixed with 45 ml of methanol. The methanolic solution of DPPH was mixed with the test solutions and allowed to react for 24 h in the dark. Blank solutions were

prepared in the similar way and the absorbance was measured at 517 nm (Brand-Williams et al., 1995; Thaipong et al., 2006). Scavenging activity was expressed as the percentage inhibition calculated by using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

Azobis-ethylbenzthiazoline sulfonic acid (ABTS) assay

The stock solutions of ABTS and potassium persulfate were prepared according to standard assay method. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 mL methanol. Test solutions were allowed to react with ABTS solution for 2 h in a dark. The absorbance was taken at 734 nm. Results were expressed as % inhibition using equation described in DPPH method (Arnao et al., 2001; Thaipong et al., 2006).

Ferric reducing anti-oxidant power (FRAP) assay

The stock solutions of acetate buffer of pH 3.6, TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in HCl, and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution were prepared. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and then warmed at 37°C before using. Test solutions were allowed to react with 2850 μ l of the FRAP solution for 30 min in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were taken at 593 nm. Results were expressed as % inhibition using equation described in DPPH method (Benzie and Strain 1996; Thaipong et al., 2006).

Iron chelating assay

The stock solutions of ferric chloride were prepared based on standard assay method. Test solutions were mixed with 1200 μ l of ferric chloride and 1200 μ l of phenanthroline solution. The absorbance was taken at 510 nm using the spectrophotometer. Results were expressed as % inhibition using equation described in DPPH method (Alam et al., 2013; Dinis et al., 1994).

Hydroxyl radical scavenging assay

The stock solutions of hydrogen peroxide, ascorbic acid, EDTA, ferric chloride, trichloroacetic acid, thiobarbituric acid and deoxyribose were prepared. The working solutions were prepared by mixing hydrogen peroxide (100 μ l), ascorbic acid (100 μ l), EDTA (200 μ l), ferric chloride (500 μ l) and deoxyribose (100 μ l). Test solutions (500 μ l) were allowed to react with 1 ml of working solution for 60 min in the dark. Later, they were incubated with 100 μ l of

trichloroacetic acid and 100 μ l of thiobarbituric acid for 20 min at 100°C. Readings of the colored product were then taken at 546 nm. Results were expressed as % inhibition using equation described in DPPH method (Alam et al., 2013; Ottolenghi, 1959).

Superoxide radical scavenging assay

The stock solutions of alkaline DMSO and nitro blue tetrazolium were prepared according to standard procedures. Test solutions were mixed with 2 ml of alkaline DMSO and 200 μ l nitro blue tetrazolium. Then the absorbance was taken at 560 nm using the spectrophotometer. Results were expressed as % inhibition using equation described in DPPH method (Alam et al., 2013; Meyer and Isaksen, 1995).

Statistical analysis

In order to evaluate the data, a linear regression ($p < 0.05$) was used. Data were expressed as the mean $\pm$ SD of three independent experiments carried out in triplicate.

Results

Effect of D-Limonene on DPPH assay

DPPH assay of D-Limonene ($IC_{50} = 384.73 \mu M$) has shown appreciable concentration dependent reduction in free radical formation in comparison with Trolox ($IC_{50} = 153.30 \mu M$) as a standard. The percentage inhibitions for DPPH assay are given in (Figure 1).

Figure 1. Antioxidant activity of D-Limonene and Trolox by DPPH assay

Figure 2. Antioxidant activity of D-Limonene and Trolox by ABTS assay

Effect of D-Limonene on ABTS assay

D-Limonene ($IC_{50} = 603.23 \mu M$) has shown appreciable concentration dependent reduction in free radical formation by ABTS assay in comparison with Trolox ($IC_{50} = 203.37 \mu M$) as a standard. The percentage inhibitions for ABTS assay are given in (Figure 2).

Effect of D-Limonene on FRAP assay

D-Limonene ($IC_{50} = -589.85 \mu M$) has showed appreciable concentration dependent reduction in free radical formation by FRAP assay in comparison with Trolox ($IC_{50} = -171.73 \mu M$) as a standard. The percentage inhibitions for FRAP assay are given in (Figure 3).

Effect of D-Limonene on Iron chelating assay

D-Limonene ($IC_{50} = -18475.5 \mu M$) did not show any % inhibition in iron chelating assay in comparison with Trolox ($IC_{50} = 225.96 \mu M$) as a standard. The percentage inhibitions for iron chelating assay are given in (Figure 4).

Effect of D-Limonene on Hydroxyl radical scavenging assay

D-Limonene ($IC_{50} = 442.75 \mu M$) has showed appreciable concentration dependent reduction in free radical formation by hydroxyl radical scavenging assay in comparison with Trolox ($IC_{50} = 146.37 \mu M$) as a standard. The percentage inhibitions for hydroxyl radical scavenging assay are given in (Figure 5).

Figure 3. Antioxidant activity of D-Limonene and Trolox by FRAP assay

Figure 4. Antioxidant activity of D-Limonene and Trolox by iron chelating assay

Figure 5. Antioxidant activity of D-Limonene and Trolox by Hydroxyl radical scavenging assay

Figure 6. Antioxidant activity of D-Limonene and Trolox by Superoxide radical scavenging assay

Effect of D-Limonene on Superoxide radical scavenging assay

D-Limonene ($IC_{50} = 692.89 \mu M$) has showed appreciable concentration dependent reduction in free radical formation in by superoxide radical scavenging assay comparison with Trolox ($IC_{50} = 105.25 \mu M$) as a standard. The percentage inhibitions for superoxide radical scavenging assay are given in (Figure 6).

Discussion

In humans, about 1–3% of the O_2 consumed by the body is converted into superoxide and other ROS which may also damage DNA, proteins, or lipids. These deleterious effects are found to be responsible for the development of diseases like CVD and cancer (Halliwell, 1996). Because of their safety natural antioxidants are the only alternative to synthetic antioxidants in counteracting the free radicals. Monoterpenes from essential oils promote health partly via their antioxidant and free radical scavenging effects. It protects the cellular components against free radical induced damage. But due to their diverse chemical structures, they are likely to possess different antioxidant capacities (Dorman et al., 2000; Grassmann, 2005).

D-Limonene is a major component of oils obtained from citrus plants such as orange, lemon and grapefruit. It is commonly used

as an additive in foods, soaps and perfumes (Whysner and Williams, 1996). It has been used to prevent gastric diseases, such as to dissolve gallstones and is also suggested to exert antiproliferative effects in various cancer cell types (Kaji et al., 2001; Lu X-G et al., 2004; Miller et al., 2013; Nakaizumi et al., 1997; Uedo, 1999).

In our study, we have evaluated the *in vitro* antioxidant activity of D-Limonene by six different antioxidant assays such as DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging and superoxide radical scavenging assay. Except iron chelating assay in all antioxidant assay, D-Limonene has appreciably reduced the ROS formation by different mechanisms. In these assays, D-Limonene has reduced the level of free radicals in test solutions in concentration dependent manner; however the IC_{50} of D-Limonene was higher than that of standard Trolox in all assays.

In DPPH assay of D-Limonene, DPPH radical was converted to stable form by donation of hydrogen atom. D-Limonene donated hydrogen atom to DPPH radical and the antioxidant effect was determined by the disappearance of DPPH radical in test samples which was directly proportional to antioxidant activity of D-Limonene. In ABTS assay, D-Limonene reacted with ABTS radical cation formed by mixture of ABTS and sodium persulfate. D-Limonene has shown appreciable antioxidant activity by reducing the level of ABTS radical in concentration dependent manner. In FRAP assay, reduction of Fe^{3+} to Fe^{2+} was occur with D-Limonene treatment which has increased by conversion of Ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion. Coloured ferrous-probe complex was formed from a colourless ferric-probe complex which was determined by increased absorbance of the test solution. D-Limonene has also shown reducing effect and donated electrons to free radicals to stabilize them which was indicated in hydroxyl radical scavenging assay of D-Limonene. Superoxide scavenging assay involves conversion of nitroblue tetrazolium to diformazan by superoxide radical. D-Limonene has stabilized the superoxide anion formation in concentration dependent manner. However, in iron chelating assay, no change in % inhibition was observed with increased concentration of D-Limonene which suggests its reduced chelating efficacy (Brand-Williams et al., 1995; Thaipong et al., 2006).

Conclusion

We confirmed the concentration dependent antioxidant activity of D-Limonene by *in vitro* antioxidant assays. Besides the anticancer activity, D-Limonene has shown appreciable concentration dependent antioxidant activity

by reducing the free radical formation in all assays except in iron chelating assay which makes it a promising molecule for treatment of cancer.

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Declaration of conflict of interest

No conflict of interest.

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