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In vitro antioxidant properties of Scopoletin

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ABSTRACT

Scopoletin is a coumarin and a major component of Aegle Marmelos. In the present study, we determined the antioxidant activity of Scopoletin by employing various in vitro antioxidant assay such as 1, 1-diphenyl-2-picrul-hydrazil free radical (DPPH$^{\bullet}$) scavenging, Hydrogen peroxide scavenging, superoxide radical scavenging, Hydroxyl radical scavenging activity and ferrous ion chelating activity by using α-Tocopherol as a reference antioxidant at 45 µg/ml concentration level. All data obtained were of significant at concentration level 45 µg/ml, Scopoletin showed 63.79%, 70.21%, 68.98%, 39.97% and 38.61% respectively free radical scavenging activity. On the other hand reference α-Tocopherol showed 84.54, 92.51, 74.98, 49.76 and 32.17% respectively. Scopoletin may play an important role in regulating free radical generated via various body metabolic activities such as mitochondrial transport of long chain free fatty acids and cytochrome-p450 transport chain. These data suggest that Scopoletin has the propensity to modulate endogenous oxidative stress and may be effective nutraceutical to abrogate oxidative stress in the body.

Key Words: Nutraceutical, Scopoletin, α-Tocopherol, free radical scavenging activity.

INTRODUCTION

Oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species. They are continuously produced by the body's normal use of oxygen such as respiration. Oxidative stress due to increase free radical generation or impaired endogenous

antioxidant mechanism is an important factor that has been implicated in various diseases. The reactive oxygen species include such as superoxide anion radicals, hydroxyl radicals and non free radicals species such as hydrogen peroxide[1]. Reactive oxygen species are continuously produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, crucial bimolecular such as nucleic acids, proteins and carbohydrate [2-3]. Antioxidant can protect the human body from free radical and reactive oxygen species effect. They retard the progress of many chronic diseases. Hence, a need for identifying alternative natural and safe source of food nutraceutical antioxidant has been created[4].

Natural coumarin exerts varied and pronounced effects on living organism. Coumarins exhibit numerous pharmacological and physiological activities such as antibacterial, vasodilator and diuretic effects. Macrophages play an important role in last defense against infection and tumor development and this activity is regulated through the production of several mediates. The production of Nitric oxide by macrophage mediates killing or growth inhibitor of tumor cells, bacteria, fungi and parasites [5-6]. Scopoletin is widely distributed is plant kingdom and is isolated from the *Aegle Marmelos* Linn. *Aegle Marmelos* Linn. has been used for hundreds of years used to cure dysentery, diarrheal, hepatitis, tuberculosis, dyspepsia and is good for heart and brain. Scopoletin is a nutraceutical compound reported to possess therapeutic properties against diabetes mellitus, diarrhea and cancer. Recently it has attracted much attention due to its significant medicinal potential. The aim of this study was to investigate 1, 1-diphenyl-2-picrul-hydrazil free radical ($DPPH^{\bullet}$) scavenging, Hydrogen peroxide scavenging, superoxide radical scavenging, hydroxyl radical scavenging activity and ferrous ion chelating activity of Scopoletin. In addition, an important main role of this study was to clarify the radical scavenging and metal chelating mechanism of Scopoletin.

EXPERMENTAL SECTION

Chemicals

Scopoletin, nitroblue tetrazolium (NBT), DPPH•, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), α -tocopherol, polyoxyethylenesorbitan monolaurate (Tween-20) and trichloroacetic acid (TCA) were obtained from Sigma (Sigma–Aldrich, India).. All other chemicals used were analytical grade and obtained from either Sigma–Aldrich or Merck.

1, 1-diphenyl-2-picrul-hydrazil free radical ($DPPH^{\bullet}$) scavenging activity

The total radical scavenging capacity of the tested compound was determined and compared to the α-Tocopherol. The hydrogen atom or electron donation ability of pure Scopoletin was measured by the bleaching of a purple colored methanol solution of stable $DPPH^{\bullet}$ radical. This spectrophotometric assay use the stable radical 1, 1-diphenyl-2-picrul-hydrazil free radical ($DPPH^{\bullet}$) as a reagent. The method of blois was used with slight modification in order to access the $DPPH^{\bullet}$ free radical scavenging capacity of Scopoletin. The $DPPH^{\bullet}$ radical absorb at 517 nm, but upon reduction by antioxidant or a radical species its absorption decreased. When a hydrogen atom or electron was transferred to the odd electron in $DPPH^{\bullet}$ radical, the absorbance at 517nm decrease proportionally to the increase of non radical form of DPPH. 0.1 mM solution of DPPH in ethanol was prepared and one ml solution of this solution was added 3 ml of Scopoletin solution in water at different concentrations (10-45 µg/ml). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity[7].

The free radical scavenging activity of Scopoletin was determined at λ_{max} 517nm by using formula-

$$\%\ (DPPH\bullet)\ \text{radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

OH- radical-scavenging activity

The reaction mixture consisted of 200 µl of $FeSO_4.7H_2O$ (10mM), EDTA (10 mM) and 2-deoxyribose (10mM) H_2O_2 was added and the reaction mixture was incubated at 37°C for 4 h. After incubation 1 ml of 1% TBA and 1 ml of 2.8% TCA were mixed and placed in a boiling water bath for 10 min. After cooling, the mixture was centrifuged (5 min, 400g) and the absorbance was measured at 532 nm[8].

$$\text{Formula} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Superoxide anion radical scavenging activity

Superoxide radicals were generated by the method described by Zhishen et al. with slight modification. All solutions were prepared in 0.05M phosphate buffer (pH 7.8). The photo induced reactions were performed using fluorescent lamps (20 W). The concentration of scopoletin in the reaction mixture was 30 µg/mL. The total volume of the reaction mixture was 3mL and the concentrations of the riboflavin, methionine and NBT were 1.35×10^{-5}, 4.45×10^{-5} and 8.15×10^{-8} M, respectively. The reaction mixture was illuminated at 25 °C for 40 min. The photochemically reduced riboflavin generated $O2^{\bullet-}$ which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. Scopoletin was added to the reaction mixture, in which $O2^{\bullet-}$ was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage of superoxide anion scavenged was calculated by using the following formula:

$$O2^{\bullet-}\text{scavenging}\,(\%) = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch et al. The principle of this method is that there is a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2. A solution of 45mM H_2O_2 was prepared in 0.1Mphosphate buffer (pH 7.4). Scopoletin at 30µg/mL concentration in 3.4mL phosphate buffer was added to 0.6mL of H_2O_2 solution (45mM) and absorbance of the reaction mixture was recorded at 230 nm [1,8]. A blank solution contained the sodium phosphate buffer without H_2O_2. The concentration of hydrogen peroxide (mM) in the assay medium was determined using a standard curve (r^2: 0.9939): absorbance = $0.029[H_2O_2] + 0.3971$

The percentage of H_2O_2 scavenging by Scopoletin and standard compounds was calculated using the following formula:

$$H_2O_2 \text{ scavenging effect (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \text{ X } 100$$

Fe2+ chelating activity

Scopoletin 10-30mcg/ml in .04 mL was added to a solution of 2mM $FeCl_2$ (0.05 mL). the reaction was initiated by addition of 5 mM ferrozine 0.2 ml and total volume was adjusted to 4 ml of ethanol. Then the mixture was shaken vigorously and left at room temperature for ten minutes. Absorbance of the solution was the measured at 562 nm[4,8,9,10,11].

RESULTS AND DISCUSSION

$$Formula = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow-colored diphenyl-picrylhydrazine. The method is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Fig. 2A illustrates a significant decrease ($p < 0.01$) in the concentration of DPPH radical due to the scavenging ability of scopoletin and the reference compounds. α-tocopherol was used as reference for radical scavenger activity. The scavenging effect of scopoletin and standard on the DPPH radical decreased in the order of α-tocopherol (51mM), and scopoletin (60mM) (84.54%, and 63.79%, respectively) at the concentration of 45µg/mL. DPPH free radical scavenging activity of scopoletin also increased with increasing concentrations ($r2$: 0.9947). EC50 for scopoletin was 34.86µg/mL. Lower EC50 value indicates a higher DPPH free radical scavenging activity. DPPH• radical-scavenging methods is common spectrophotometric procedures for determining antioxidant capacities of components. When an antioxidant is added to the radicals, there is a degree of decolorization owing to the presence of the antioxidants, which reverses the formation of the DPPH• radical cation:

DPPH• + AH → DPPH2 +A•

DPPH radical scavenging is easy to use, have a high sensitivity, and allow for rapid analysis of the antioxidant activity of a large number of samples.

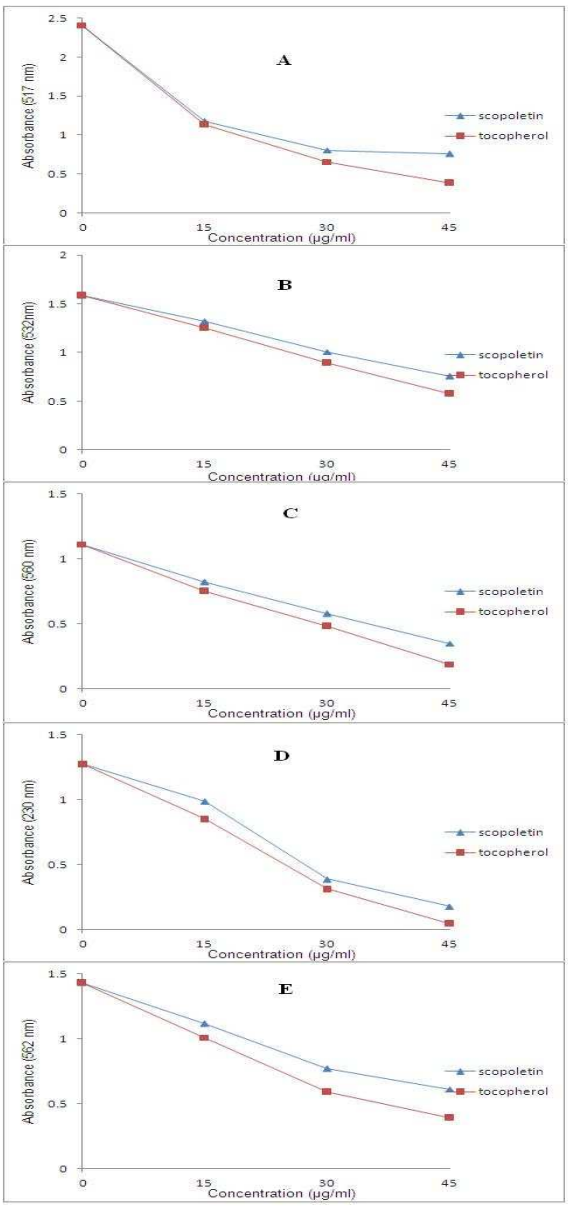

Figure 1-Radical scavenging activity of different concentrations (15–45_g/mL) of scopoletin and compared with α-tocopherol.

(A) DPPH free radical scavenging activity (1-diphenyl-2-picryl-hydrazylfree radical). (B) OH- radical-scavenging activity. (C) Superoxide anion radical scavenging activity. (D) Hydrogen peroxide scavenging activity. (E) Fe2+ chelating activity.

Scopoletin had effective reducing power using the potassium ferricyanide reduction method when compared to the standards. For the measurements of the reductive ability of scopoletin, the Fe^{3+}–Fe^{2+} transformation was investigated using the method of Oyaizu. At different concentrations (15–45g/mL), scopoletin demonstrated powerful reducing ability ($r2$: 0.9937) and these differences were statistically very significant ($p < 0.01$). The reducing power of scopoletin and α-tocopherol increased steadily with increasing concentrations of samples. Reducing power

of scopoletin and standard compound exhibited the following order: scopoletin > α-tocopherol. The results demonstrate the electron donor properties of scopoletin for neutralizing free radicals by forming stable products. In vivo, the outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging. Scopoletin had effective ferrous ions (Fe^{2+}) chelating capacity. The difference between the 45µg/mL concentration of scopoletin and the control values was statistically significant ($p < 0.01$, Table 1). In addition, at 15µg/mL concentration, scopoletin (20mM) exhibited 38.61±4.2% chelation of ferrous ion. On the other hand, the ferrous ion chelating capacities of the same concentrations of α-tocopherol (17mM) was found to be 32.17±6.2%. These results show that the ferrous ion chelating effect of scopoletin was statistically higher than, α-tocopherol. The ability of scopoletin to scavenge hydrogen peroxide is shown in Table 1 and compared with that of α-tocopherol as reference compound.

Table 1: Comparison of hydrogen peroxide (H_2O_2) scavenging activity, ferrous ion (Fe2+) chelating activity, superoxide anion radical (O2•−), OH-radical scavenging and DPPH scavenging activity of scopoletin and standard antioxidant compound α-tocopherol at the concentration of 15 (µg/mL)

s. no.	Activity	Scopoletin* %scavenging	α-Tocopherol* %scavenging
1	DPPH scavenging	63.79	84.54
2	H_2O_2 scavenging	70.21	92.51
3	Ferrous ion chelating	38.61	32.17
4	Superoxide scavenging	68.98	74.98
5	OH-radical scavenging	39.97	49.76

** All the values are in triplicate*

Hydrogen peroxide scavenging activity of scopoletin at 15µg/mL (20mM) was found to be 70.21±3.9%. On the other hand α-tocopherol exhibited 92.51±3.5, hydrogen peroxide scavenging activity, respectively, at the same concentration. These results show that scopoletin has an effective hydrogen peroxide scavenging activity. At the above concentration, the hydrogen peroxide scavenging effect of scopoletin and standard compound decreased in the order of scopoletin < α-tocopherol.

The inhibition by scopoletin of superoxide radical generation is lower than that by for α-tocopherol. As seen in Table 1, the inhibition of superoxide anion radical generation at the concentration of Scopoletin was 68.98±7.4%. On the other hand, at the same concentration, α-tocopherol 74.98±5.1% superoxide anion radical scavenging activity. According to these results, scopoletin had higher superoxide anion radical scavenging activity. As shown in Fig. 2C, scopoletin was an effective OH-radical scavenger in a concentration-dependent manner (15–45µg/mL, *r*2: 0.9958). There was a significant decrease ($p < 0.05$) in the concentration of OH-radical due to the scavenging capacity at all scopoletin concentrations. The scavenging effect of scopoletin and standards decreased in the order: scopoletin< α-tocopherol, which was at the concentration of 45µg/mL, respectively.

CONCLUSION

Scopoletin had higher superoxide anion radical scavenging activity. As shown in Fig. 2C, scopoletin was an effective OH-radical scavenger in a concentration-dependent manner (15–

45μg/mL, *r*2: 0.9958). There was a significant decrease ($p < 0.05$) in the concentration of OH-radical due to the scavenging capacity at all scopoletin concentrations. The scavenging effect of scopoletin and standards decreased in the order: scopoletin< α-tocopherol, which was at the concentration of 45μg/mL, respectively.

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