Original Research Article

Antioxidant activity of polyphenolic leaf extract from *Santolina chamaecyparissus* and the isolated luteolin-7-O-glucoside

ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Aim: The present study aimed to investigate the chemical constituents and antioxidant potential of *Santolina chamaecyparissus* polyphenolic extract (SCPE). **Materials and Methods:** The extract containing phenolic compounds, was extracted with ethyl acetate and luteolin-7-O-glucoside was separated and purified using flash chromatography on slica gel column. This isolated compound was identified according to its physicochemical properties and spectral data (UV, LC–TOF-MS, 1H NMR, 13C NMR and 2D NMR). Antioxidant activity of the isolated compound and SCPE was investigated using DPPH•, ABTS+, reducing power, superoxide anion radical scavenging and β-carotene bleaching assays. **Results:** Results showed that SCPE and luteolin-7-O-glucoside exhibited significant antioxidant activity. The antioxidant activity of SCPE was comparable to that of luteolin-7-O-glucoside. Luteolin-7-O-glucoside was isolated for the first time from *Santolina chamaecyparissus* polyphenolic extract. **Conclusion:** Results of the present investigation clearly indicate that SCPE and isolated compound (luteolin-7-*O*-glucoside) have powerful antioxidant capacity against various antioxidant systems *in vitro.*

Keywords: Antioxidant activity, flavonoids, luteolin-7-O-glucoside, Santolina chamaecyparissus.

1. INTRODUCTION (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

The human body produces reactive oxygen species (ROS), such as superoxide anion radical, hydroxyl radical and hydrogen peroxide, by many enzymatic systems through oxygen consumption. In small amounts, these ROS can be beneficial as signal transducers and growth regulators [1]. However, during oxidative stress, large amounts of these ROS can be produced and may be dangerous because of their ability to attack proteins, lipids and DNA [2]. The cell can reduce the impact of ROS either by an endogenous system implicating enzymes such as catalase and superoxide dismutase or by an exogenous system using antioxidants, vitamin C, α-tocopherol and polyphenols [1].

Nowadays, natural antioxidants, especially those of plant origin, have become a major area of scientific research. Many different plant materials have recently become a major interest of scientific research as a result of naturally occurring anti-oxidant, which may protect cell constituents against oxidative damage and therefore, limit the risk of various degenerative diseases associated to oxidative stress [3]. Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants [4]. It is known that these secondary metabolites display a remarkable array of biochemical interactions, probably due to antioxidant properties. These substances may act as potent metal chelators and/or free radical scavengers [5].

Santolina chamaecyparissus L. (Asteraceae) is an aromatic plant wide spread in Mediterranean region. The plant is used in folk medicine for their analgesic, anti-inflammatory, antiseptic, antispasmodic, bactericidal, digestive and vulnerary properties [6, 7]. Recently, it has been reported that the polyphenolic and aqueous extracts of *Santolina chamaecyparissus* leaves modulate human

neutrophil functions such as chemotaxis, degranulation and phagocytosis [8]. Phytochemical studies of *Santolina chamaecyparissus* yielding a number of secondary metabolites such as essential oils, [9] flavonoids, [10] coumarins, [11] and polyacetilenic compounds [12]. In this study, luteolin-7-O-glucoside was isolated, characterized and the antioxidant activity of this compound and of SCPE was examined, for a better characterization and exploitation of these natural products.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

2.1. General experimental procedures

All NMR spectra were recorded on a Bruker-400 MHz spectrometer with TMS as an internal standard. UV spectra were recorded on a Shimadzu UV-260 UV–Vis spectrometer. GF254 for TLC was produced by Qingdao Ocean Chemical Group Co. of China. LC-TOF MS analysis was performed on an Agilent 6210 TOF LC-MS instrument.

2.1. Plant material

Santolina chamaecyparissus was collected in mid-May 2013 from Hammam Essoukhna, Setif, in eastern region of Algeria. The plant was identified, authenticated taxonomically by Pr. H. Laouer (Laboratory of Botany, University of Setif 1, Algeria) and a voucher specimen (No. S.c. 2009-1) was preserved at the local Herbarium of Botany, Department of Botany, University of Setif, for future reference. Leaves were air-dried at room temperature and then reduced to powder.

2.3. Extraction and isolation

Polyphenolic extract was prepared by maceration of 100 g of powdered leaves with 80% methanol at room temperature for 24 h with frequent agitation. After filtration, the filtrate was concentrated under reduced pressure at 40ºC and the residue was dissolved in boiled water and then treated with ethyl acetate for 24 h at room temperature. The combined ethyl acetate layer was concentrated under reduced pressure and then the residue was lyophilized. The yield obtained was 1.75% [13]. The extract (10 g) was subjected to Flash chromatography, using silica gel (40 g, Redisep) as the stationary phase and eluting with hexane and a gradients of ethyl acetate and methanol to give 140 fractions (250 ml each). According to TLC basis, similar fractions were combined. 62-67 fractions were combined to Luteolin-7-O-glucoside.

2.4. DPPH free radical scavenging activity

The free radical scavenging activity of extract, isolated compound and standards was measured by 1,1-diphenyl-2-picryl-hydrazil DPPH• [14]. Briefly, 0.1 mM solution of DPPH• in ethanol was prepared and 1ml of this solution was added to 3 ml of tested compounds at different concentrations (2.5 to 30 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

2.5. Determination of reducing power

The reducing power of *Santolina chamaecyparissus* extract, isolated compound and standards was determined [15]. Various concentrations of tested compounds (2.5 - 30 µg/ml) in 1 ml of methyl alcohol were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K3Fe(CN)6]. The mixture was incubated at 50°C for 20 min and 2.5 ml of trichloroacetic acid (10%) was added to the mixture, and then centrifuged for10 min at 1000 g. The upper layer of solution (2.5 ml) was mixed with the same volume of distilled water and 0.5 ml of FeCl3 (0.1%), and the absorbance was measured at 700 nm. A higher absorbance indicates a higher reductive capability.

2.6. Superoxide anion radical scavenging activity

Measurement of superoxide anion scavenging activity of extract, isolated compound and standards was tested [16]. Superoxide radical is generated in PMS–NADH systems by oxidation of NADH and

assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radical was generated in3ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1ml of NBT (50 M), 1ml NADH (78 M) and tested compounds (5-40 µg/ml) were mixed. There action was started by adding 1 ml of phenazine methosulphate (10M) (PMS) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

2.7. Beta Carotene Bleaching Assay

The ability of the extract and isolated compound to prevent the bleaching of β-carotene was determined [17]. Briefly, 0.5 mg of β-carotene in 1 ml chloroform was mixed with 25 mg of linoleic acid and 200 mg of Tween-40. The chloroform was evaporated under vacuum at 40°C, then 100 ml distilled water was added and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. An aliquot (2.5 ml) of the β-carotene-linoleic acid emulsion was transferred to tubes containing 0.5 ml of each sample (100µg/ml). A control consisted of 0 .5 ml of distilled water instead of the sample solution was prepared. The tubes were immediately placed at 50°C in water bath. The absorbance of samples at 470 nm were taken at zero time (t = 0). Measurement of absorbance was continued until the color of β-carotene disappeared in the control reaction (120 min) at 15 min intervals. A mixture prepared as previously mentioned without β-carotene served as blank. BHT was used as standard antioxidant for comparative purpose.

2.8. ABTS radical cation decolorization assay

The ABTS+ radical scavenging activity of extract, isolated compound and standards was evaluated [18]. ABTS+ is blue–green in color with a characteristic absorbance at 734 nm. ABTS+ cation radical was produced by reacting ABTS (2 mM) in H2O and potassium persulphate (2.45 mM) at room temperature for 4 hours. The ABTS+ solution was diluted with phosphate buffer (0.1 M, pH 7.4) to achieve an absorbance of 0.750 ± 0.025 a t734 nm. Then, 1 ml of ABTS+ solution was added to 3 ml of tested compounds at different concentrations (2.5 - 30 µg/ml). These samples were vortexed and incubated in the dark for 30 min, and then the absorbance at 734 nm was measured for each concentration relative to a blank. Decreased absorbance of the samples indicates ABTS+ cation radical scavenging activity.

2.9. LC-TOF-MS analysis

The apparatus used was an Agilent 6210 LC-TOF-MS instrument with a Poroshell 120EC-C18 column (3.0 × 50 mm, 2.7 µm; Agilent Technologies, Palo Alto, CA, USA) with an injection volume of 10 µl. The mobile phase consisted of the eluent A- water with 0.1% formic acid and 5 mM ammonium formate and B- acetonitrile. The flow rate was 0.7 ml/min at 35°C.The gradient program was fixed as follows: 0-1 min, 10% B ; 1-8 min, 10% B ; 8-11.1 min, 95% B ; 11.1-13 min, 10% B ; 13-14 min, 10% B. Total time of evaluation was 14 min. TOF analyses were carried out in negative ion mode; gas temperature, 325°C and column temperature, 35°C; drying gas flow, 0.7 ml/min ; fragment or voltage,175 V.

Statistical analysis

Data were represented as mean ± standard error of the mean and analyzed by one-way analysis of variance followed by Tukey's multiple comparison tests. The results were considered statistically significant when $*P = .05$, $**P = .01$, $***P = .001$.

3. RESULTS

3.1. Phytochemical content

Structural elucidations of the isolated active compound were carried out using various spectral techniques, 1HNMR, 13C NMR, and LC–TOF/MS. Accordingly, isolated compound was obtained as yellowish solid. By analysis of the NMR data and the comparison of the physical and spectral data with compounds in the literature, the isolated compound was identified as luteolin-7-O-glucoside (Figure 1). 1H NMR, 13C NMR spectral data and LC-TOF-MS chromatogram of isolated compound were given in Figure 2, 3 and 4, respectively.

Luteolin-7-O-glucoside: yellowish solid;1H-NMR (400 MHz, DMSO-d6) δ 6.76 (s, H-3), 6.45 (d, J = 1.7 Hz, H-6), 6.79 (d, J = 1.7 Hz, H-8), 7.45 (d, J = 8.4 Hz, H-2'), 6.90 (d, J = 8.4 Hz, H-3'), 7.43 (brs, H-6'), 5.08 (d, J = 7,3 Hz, H-1''), 3.26 (m, H-2''), 3.45 (m, H-3''), 3.17 (m, H-4''), 3.30 (m, H-5''), 3.48 (m, H-6a), 3.72 (d, J = 9.9 Hz, H-6b); 13C-NMR (100 MHz, DMSO-d6) δ 164.9 (C-2), 103.5 (C-3), 182.3 (C-4), 161.6 (C-5), 99.9 (C-6), 163.4 (C-7), 95.1 (C-8), 157.4 (C-9), 105.8 (C-10), 121.6 (C-1'), 119.6 (C-2'), 116.4 (C-3'), 150.7 (C-4'), 146.4 (C-5'), 113.9 (C-6'), 100.3 (C-1''), 73.6 (C-2''), 77.6 (C-3''), 70.0 (C-4''), 76.9 (C-5''), 61.1 (C-6); LC-TOF-MS m/z 447.1035 [M-H]- (clcd for C21H19O11, 447.0927). Isolated luteolin-7-O-β-glucoside is a known compound, 1H-NMR and 13C-NMR chemical shift values are agreement with the literature [19].

3.2. DPPH radical scavenging activity

The scavenging activity of the extract and reference antioxidants (BHA, BHT and trolox) against DPPH• was investigated. As shown in figure 5, all tested compounds displayed concentration-dependently DPPH• scavenging effect at the tested concentrations of 5-100 µg/ml. The IC50 value of luteolin-7-O-glucoside (4.28 µg/ml) is better than of SCPE (8.02 µg/ml) and similar to that of trolox.

3.3. Determination of reducing power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [20]. For the measurements of the reductive ability, we investigated the Fe3+-Fe2+ transformation in the presence of SCPE, isolated compound and standard. The reducing power of SCPE and trolox were excellent and were 1.25 and 1.18 at 30 µg/ml, respectively (figure 6). Similar effects were observed with BHT and Luteolin-7-O-glucoside at the same concentration. The reducing powers of all tested compounds increased with increasing concentration.

3.4. Superoxide radical scavenging activity

Superoxide radicals are generated in a NADH-PMS system by oxidation of NADH and are determined by the reduction of NBT [21]. SCPE was found to be an efficient scavenger of superoxide radical and was comparable to that of luteolin-7-O-glucoside. Figure 7 shows that both the extract and luteolin-7-O-glucoside exhibited dose dependent inhibition on superoxide radical. This inhibition was higher than that obtained with BHA and trolox.

3.5. Bleaching of β-Carotene

The β-carotene bleaching assay is based on the radical adducts of carotenoid with free radicals from linoleic acid. The reductions in the absorbance of the β-carotene-linoleate emulsion in the presence of SCPE, luteolin-7-O-glucoside and the positive control, BHT, at a concentration of 100 µg/ml are shown in figure 8. All the samples and the standard inhibited bleaching of β-carotene in comparison with the negative control. The oxidation of linoleic acid was effectively inhibited by luteolin-7-O-glucoside (93.66 %), followed by SCPE (68.33 %), respectively. The antioxidant activity of synthetic BHT was 100%.

3.6. ABTS radical cation decolorization assay

The result clearly indicates that SCPE and luteolin-7-O-glucoside have an interesting ABTS radical cation scavenging activity, compared to BHA and trolox (Figure 9). The antioxidant ability of SCPE, luteolin-7-O-glucoside to scavenge the blue-green-colored ABTS radical cation was measured relative to the radical scavenging ability of BHA, BHT and trolox. luteolin-7-O-glucoside exhibited potent scavenging effects against ABTS with an IC50 value of 2.97 µg/ml almost equivalent to that of standard BHT and BHA (IC50 values 2.64 µg/ml and 2.66 µg/ml, respectively). Similar effects were observed with SCPE and trolox.

4. DISCUSSION

Plants are important source of potential compounds for the development of new therapeutic agents. Plant phenolics are widely distributed in the tissues of plants as well as play a vital role in the highly effective freeradical scavengers and antioxidant activity. It has been reported that the antioxidant

activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers [22]. Typical phenolics that possess antioxidant activity are known to be mainly phenolic acid and flavonoids. Flavonoids are very effective antioxidants and they protect the cardiovascular and oxidative disease. They have the ability to modulate the activity of various enzymes and interactions with specific receptors [23]. The presence of hydrogen of hydroxyl groups in flavonoids, able to reduce free radicals and delocalization of unpaired electron leads to the formation of a stable phenoxyl radical. Phenols are very important plant constituents because of scavenging of radicals and containing hydroxyl groups [21]. It was reported that *Santolina chamaecyparissus* polyphenolic leaf extract contained polyphenols and flavonoids (213.2 mg GAE/g of extract and 49.8mg QE/g of extract, respectively) [8]. The phenolic compounds can contribute directly to evaluate antioxidative action [24].

The natural antioxidants contained in a plant may contribute to the antioxidant activity and thus towards the total or partial alleviation of some clinical disorders. A medicinal plant removing ROS is the most effective defense of a living body against diseases. In this study, the antioxidant activities of the polyphenolic extract of *Santolina chamaecyparissus* and luteolin-7-O-glucoside isolated from this extract were analyzed and compared to BHA, BHT and trolox as positive controls.

There are several antioxidant assays to evaluate the antioxidant potential of natural plant extracts. It is imperative to evaluate more than one antioxidant assay when screening the antioxidant efficacy of plant extracts because different extract possess different phytoconstituents in different concentrations and it will scavenge which radicals is not known. The various antioxidant assays generally used are DPPH radical scavenging activity, Super oxide anion radical scavenging activity, ABTS cation radical scavenging activity, FRAP activity, Reducing capacity assessment, Hydroxyl radical scavenging activity, Hydrogen peroxide scavenging activity, Nitric oxide radical scavenging activity, Oxygen radical absorbance capacity, α-amylase inhibitory activity, β-carotene bleaching by linoleic acid activity, Metal chelating activity, Linoleic acid peroxidation, FTC (Ferric thiocyanate) activity, Ferrous ions chelating activity, etc [25]. Results of the present investigation clearly indicate that SCPE and isolated compound (luteolin-7-O-glucoside) have powerful antioxidant capacity against various antioxidant systems in vitro. The scavenging effect of antioxidants on DPPH radical is thought to be due to their hydrogen donating ability [14]. SCPE and luteolin-7-O-glucoside exhibited a very significant free radical scavenging activity. This antiradical activity is probably due to polyphenols components known for their antioxidant activity [26].There is a close relationship and positive correlation between the phenolic content and antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals by their hydrogen donating ability [25].

In this study, the reducing capacity of *Santolina chamaecyparissus* extract, isolated compound and standard was estimated. The reducing power of SCPE and trolox were excellent. Similar effects were observed with BHT and Luteolin-7-O-glucoside. The capacity of reducing observed for SCPE due to the presence of reductones and may serve as an indicator of its potent antioxidant activity. The antioxidant effect of reductones is based on the destruction of the free radical chain by donating a hydrogen atom [27]. Polyphenols which may act in a similar way as reductones react with free radicals to turns them into more stable products and abort free radical chain reactions [28].

Many enzymatic and autoxidation reactions within the body produce the superoxide anion radical, by addition of an electron to molecular oxygen. Superoxide is poorly reactive but can take part in further reactions leading to the formation of more reactive oxygen species, contributing to tissue damage and various diseases [21]. In this system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Results showed that both the extract and luteolin-7-O-glucoside exhibited dose dependent inhibition on superoxide radical. This inhibition was higher than that obtained with BHA and trolox. The effect of SCPE appears to be related to its richness in polyphenols and flavonoids.

β-carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As a result, β-carotene will be oxidized and broken down in part. Subsequently, the system loses its chromophore and characteristic orange color,

which can be monitored spectrophotometrically. Obtained results showed that SCPE and luteolin-7-O-glucoside were capable of inhibiting the bleaching of b-carotene. The presence of different antioxidants can hinder β-carotene bleaching by neutralizing the linoleic-free radical and other free radicals formed in the system [28]. Therefore *Santolina chamaecyparissus* can be used for preventing lipid oxidation in pharmaceutical products, maintaining nutritional quality and prolonging the shelf-life of pharmaceuticals and foods.

The ABTS assay is based on the inhibition of the absorbance of radical cation, ABTS+, which has a characteristic wavelength at 734 nm, by antioxidants. The principle behind the technique involves the reaction between ABTS and potassium per sulphate to produce the ABTS radical cation (ABTS+) which is a blue green chromogen. In the presence of antioxidant reductant, the colored radical is converted back to colorless ABTS [29]. The ABTS+ scavenging method has been used to investigate the antioxidant activity of the compounds because of its simple, rapid and sensitive procedure. In this method, the antioxidant capacities of compounds are measured according to the decreasing color of ABTS+ resulting from its reaction with antioxidant compounds, leading to the formation of ABTS. The result clearly indicates that SCPE and luteolin-7-O-glucoside have an interesting ABTS radical cation scavenging activity .The order of ABTS radical scavenging activity of the tested compounds was almost similar to that observed for DPPH.

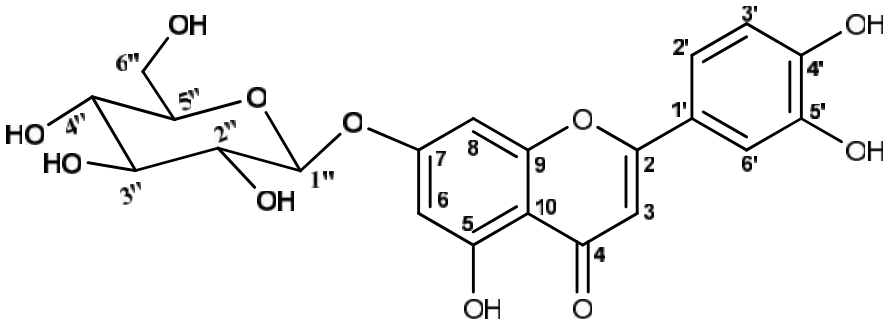


Figure 1: Structure of luteolin-7-*O*-glucoside.

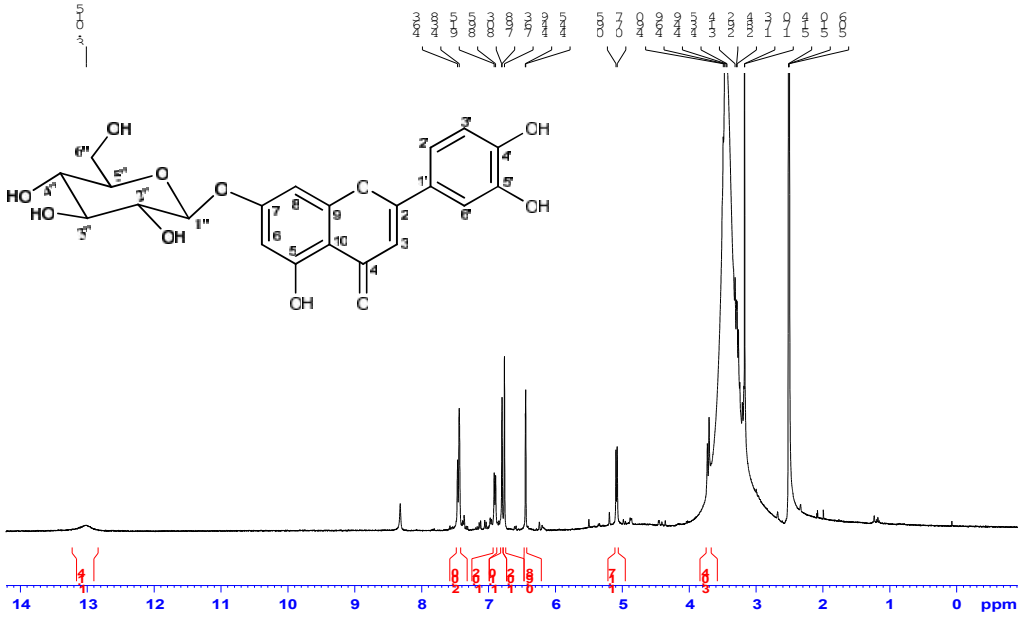


Figure 2: ^{1}H-NMR spectrum of luteolin-7-*O*-glucoside (^{1}H-NMR 400 MHz, DMSO-d_6).

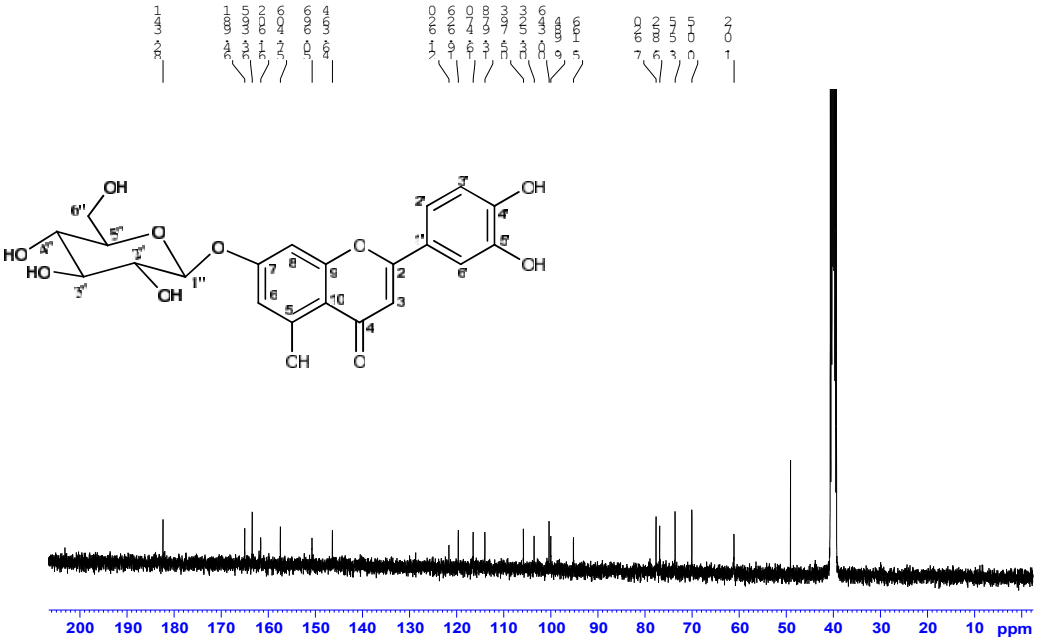


Figure 3: ^{13}C-NMR spectrum of luteolin-7-*O*-glucoside (^{13}C-RMN 100 MHz DMSO-d_6).

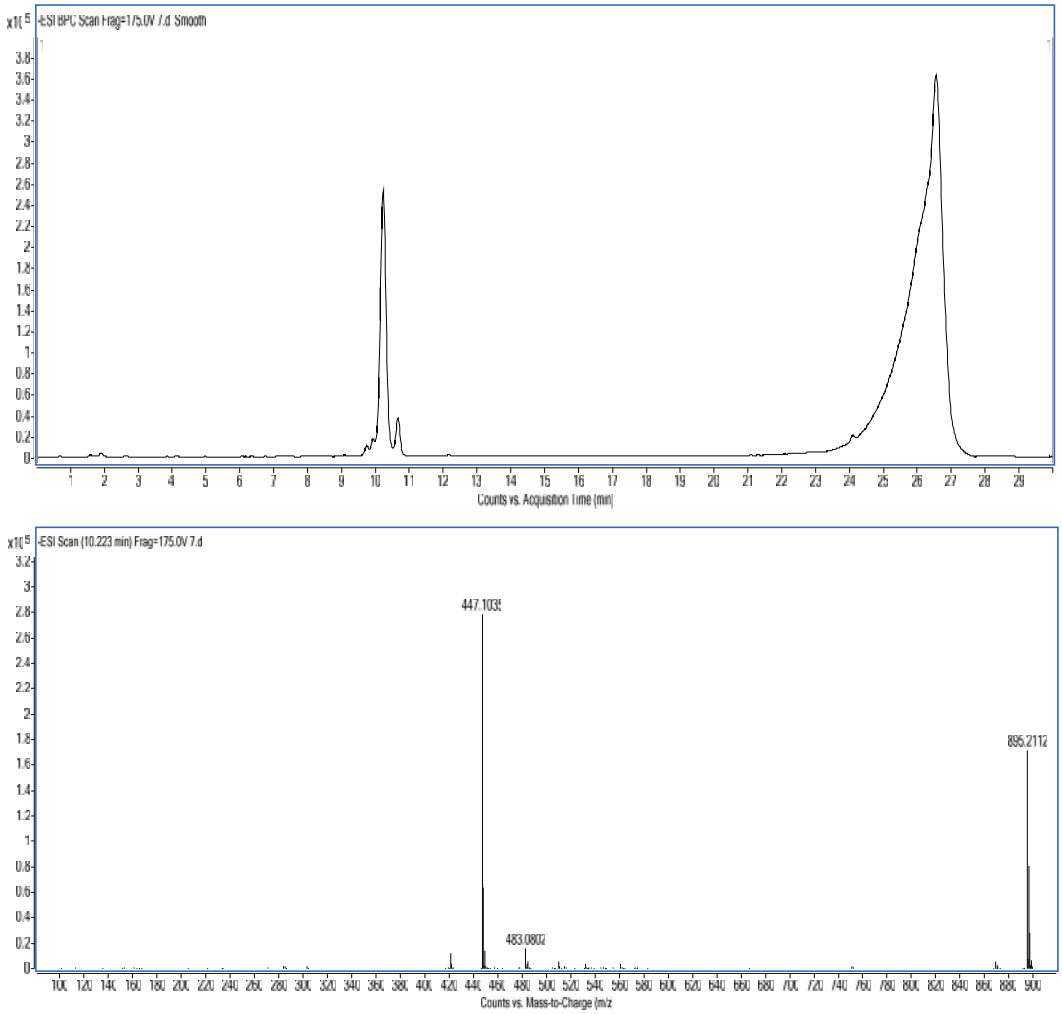


Figure 4: LC-TOF-MS chromatogram of luteolin-*7-O*-glucoside.

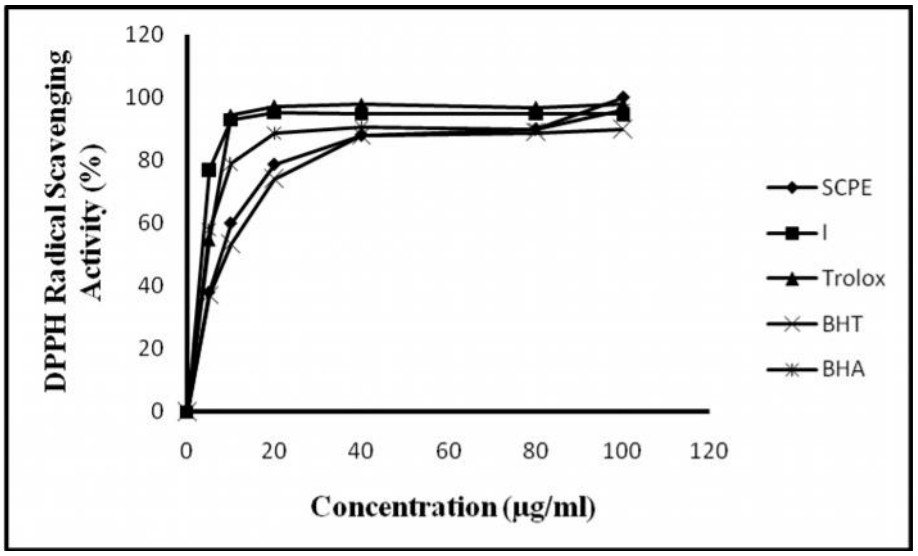


Figure 5: Free radical scavenging activity of different concentrations of SCPE, isolated compound (I), BHA, BHT, and trolox by 1,1-diphenyl-2-picrylhydrazyl radicals.

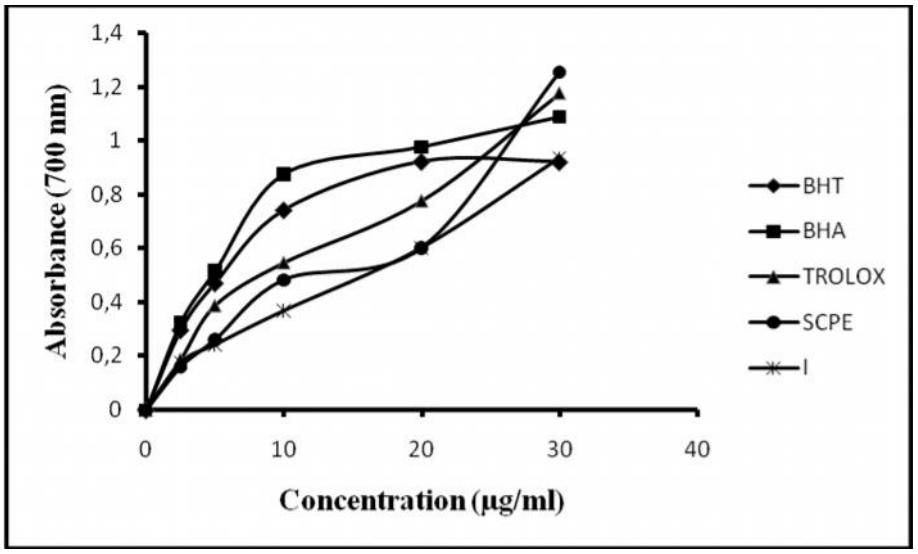
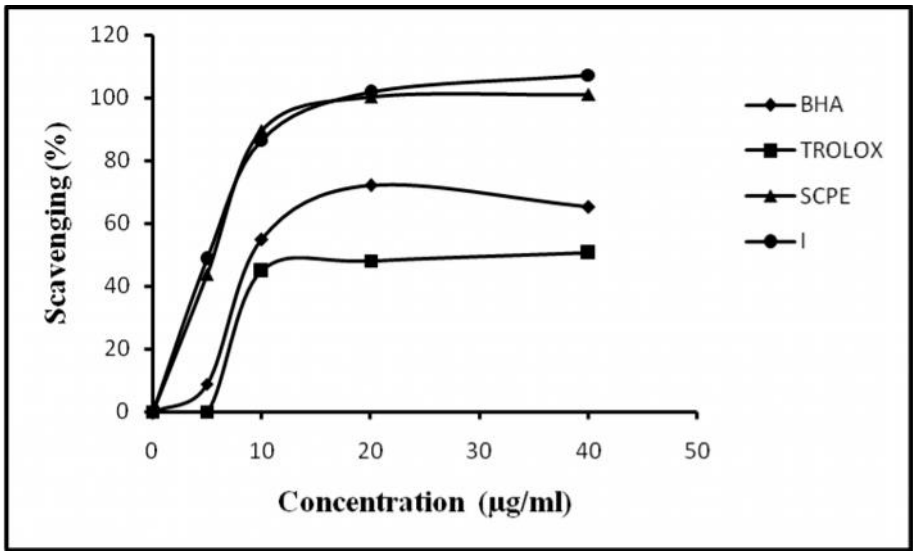


Figure 6: Comparision of reducing power of different concentrations of SCPE, isolated compound (I), BHA, BHT, and trolox by spectrophotometric detection of the Fe^{+3}–Fe^{+2} transformation at 700 nm.



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308 **Figure 7:** Comparison of superoxide anion radical scavenging activity of different concentrations of SCPE,
309 isolated compound (I), BHA and trolox.
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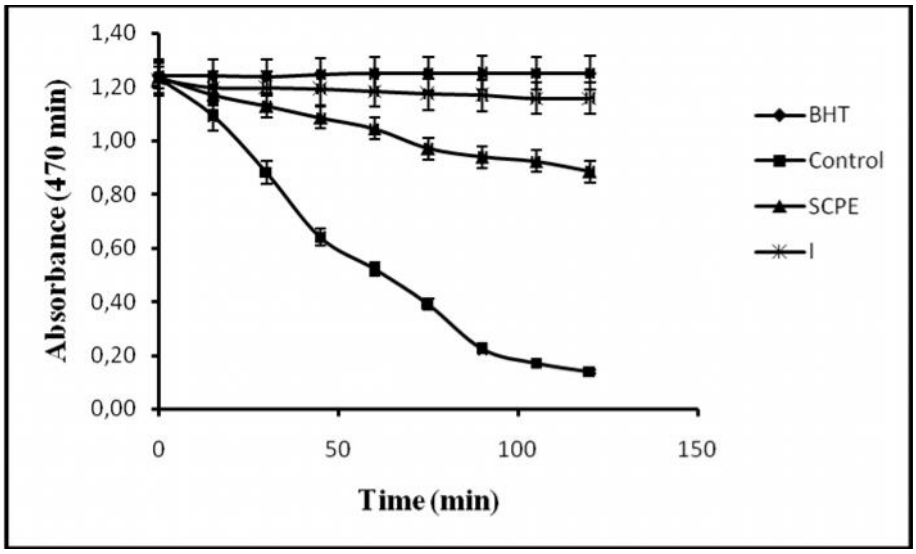

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313 **Figure 8:** β-Carotene bleaching assay of SCPE, isolated compound (I), and BHT.
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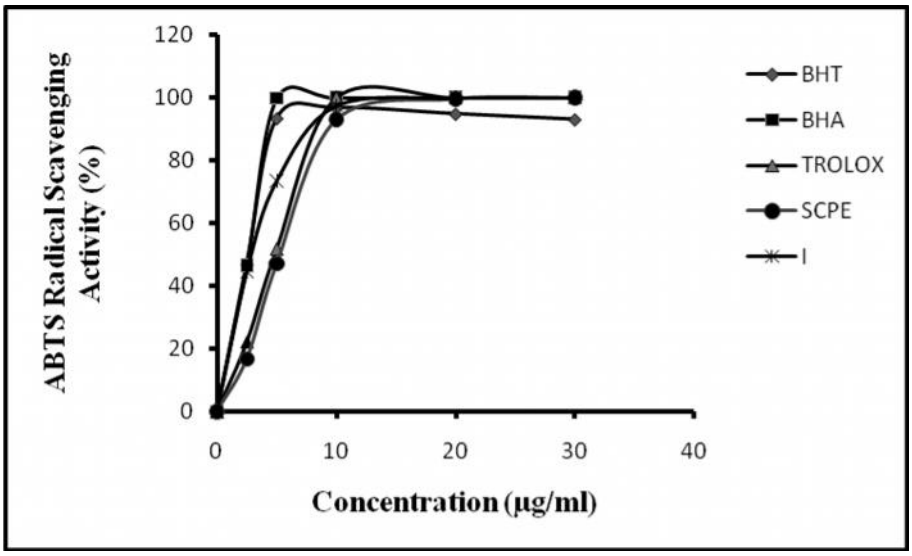

Figure 9: Free radical scavenging activity of different concentrations of SCPE, isolated compound (I), BHA, BHT, and trolox by ABTS radicals.

5. CONCLUSION

Results of the present investigation clearly indicate that SCPE and isolated compound (luteolin-*7-O*-glucoside) have powerful antioxidant capacity against various antioxidant systems *in vitro.* These properties support the use of this plant to treat oxidative stress related diseases.

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