FREE RADICALS SCAVENGING ACTIVITY OF SPEARMINT METHANOLIC EXTRACT

Hassan, R. A.*; H. B. Hamed*; M. I. Sanad and K. A. Said Ahmed**

*** Agri. Chemistry Dept., Faculty of Agricultural, Mansoura University, Egypt.**

**** Agri. Chemistry Dept., Faculty of Agricultural, Damietta University, Egypt.**

ABSTRACT

Plant kingdom contains about 620 families. One of them is family lamiaceae which has spearmint plant. This plant has a pungent taste with digestive effects. Phytochemical screening, chemical composition and antioxidant activity of spearmint aerial parts methanolic extract were investigated. Phytochemical analysis indicated the presence of terpenes, tanins, flavonoids, saponins, glycosides, alkaloids and phenolic glycosides. Total phenolic and flavonoids contents of the methanolic extract were found to be 35.65mg gallic acid equivalent (GAE)/g and 27.47mg quercetin equivalent (QE)/g respectively. Different antioxidant procedures were used to determine the activity of spearmint as antioxidant such as: DPPH, antioxidant capacity, nitric oxide radical scavenging, superoxide dismutase activity, hydroxyl radical scavenging and reducing power assay.

Keywords: Spearmint, Phytochemical screening, chemical composition, ROS, antioxidant activity.

INTRODUCTION

Mint species have been exploited by man for more than 2000 years. The genus Mentha (*Lamiaceae*) is composed of 19 geographically wide spread species and 13 hybrids. Spearmint belongs to the genus Mentha in the family *labiateae*. This family is strong antioxidant properities. Three mentha species, *M.x piperita L*. (Peppermint), *M. aravensis L*. (cornmint) and *M. spicata* (spearmint) are commonly cultivated in the world and used extensively in the liquor and confectionary industries, flavoring, perfume production and medicinal purposes (Moreno *et al*., 2002). Naidu *et al* (2012) studied the chemical composition and the antioxidant activity of *mentha spicata* methanolic crude extract. The chemical constituents and antioxidant activity of the crude extracts of *mentha spicata* were investigated. Phytochemical analysis indicated the prescence of sugar, flavonoids and alkaloids in the crude extracts of *mentha spicata.* GC analysis indicated the presence of terpenoids, terpenoid alcohol. Caryophyllene and glycosides. Total phenolic components of the crude extracts were found to be 27.26 mg/g gallic acid equivalent which was determined by folin-ciocalteu method. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was found to increase with increasing concentrations and was found to be 54.84 %. They indicated that the antioxidant activity may be due to the presence of flavonoids and fatty acid methyl esters which has the scavenging potential by reducing the free radicals.

Choudhury *et al*. (2006) mentioned that mint belongs to the genus *Mentha* in the family *Labiatae* (*Lamiaceae*) and is pungent in taste with post digestive effects and hot potency. They studied ten samples of mint leaves, collected from four different locations in North-West parts of India. Further, studies on DPPH free radical scavenging activity of diethyl ether extract showed 100% activity.

Spearmint is a potential resource for natural antioxidants. This property may be due to some compounds such as phenolic terpenes, flavonoids and phenolic acids (Bimakr *et al.,* 2011).

The objective of this study was to evaluate the composition and the antioxidant activity of methanolic spearmint extract.

MATERIALS AND METHODS

Preparation of spearmint extract:

Spearmint was obtained from the farm of agriculture college, Mansoura University and identified by a botanical taxonomist at Department of Botany, Agriculture College Mansoura University. The aerial parts were cleaned and dried at room temperature. The dried plant samples were grinded to powder, then 60 g were mixed with methyl alcohol (1.0 L) in a closed flask and kept for 24 hours. Methanolic extract (ME) was concentrated to dryness in a rotary evaporator. The dried extract was stored in a refrigerator at 4^{o}C till further use.

Qualitative analysis:

Terpenes, tannins, flavonoids, saponins, resins and alkaloids were detected in ME according to the method described by (Harborne, 1998).

Total polyphenols:

Total polyphenols were determined using Folin-Ciocalteu method according to (Li *et al.,* 2007). The absorbance was measured at 750 nm. Total phenol contents were expressed as milligram gallic acid equivalent (GAE)/g extract.

Total flavonoid content (TF):

Aluminum chloride colorimetric method was used for quantitative flavonoids determination (Chang *et al.,* 2002). The absorbance was measured at 415 nm . Total flavonoid content was expressed as mg of quercetin equivalent (QE) / g extract.

HPLC for polyphenols:

Phenolic compounds were extracted according to the method outlined by (Ben-Hammouda *et al.,* 1995). Identification of individual phenolic compounds were performed on a Hewlett-Packard HPLC (Model 1100), in national research center, Doki, Giza, Egypt. Seven standard phenolic compounds were used namely gallic acid, protocatechuic, catechin, caffeic acid, salicylic acid, ellagic acid and cinnamic acid were obtained from sigma (St. Louis, USA) and Merck-Schuchardt (Munich, Germany) chemical companies.

Spearmint antioxidant activity:

Study on spearmint (ME) as antioxidant includes various methods (i.e) DPPH radical, total antioxidant capacity, scavenging of Nitric Oxide Radical,

Superoxide Dismutase Activity, OH radical Scavenging and reducing power assay.

DPPH radical scavenging assay:

The measurement of the DPPH radical scavenging activity was performed according to methodology described by (Rekha *et al.,* 2012). The changes in color (from deep violet to light yellow) were read at 517 nm.

Calculation

% Scavenging activity = $(A_0 - A_1/A_0) \times 100$

Where,

A_0: is the absorbance without extract.

A_1: is the absorbance in the presence of the extract or standard sample.

Total antioxidant capacity:

The total antioxidant capacity was evaluated by the phosphomolybdenum method according to the procedure described by (Prieto *et al.,* 1999). The absorbance was read at 695 nm against blank. Ascorbic acid was used as reference standard.

Scavenging of nitric oxide radical:

The nitric oxide radical scavenging capacity of the extract was measured by Griess reaction (Sangameswaran *et al.,* 2009). The absorbance was measured at 540 nm. Quercitin was used as reference standard. Sodium nitroprusside in phosphate buffer saline (PBS) was used as control.

Calculation

% Nitric oxide radicals scavenging = $[(A_o - A_1) / A_o] \times 100$

$A_{0:}$ is the absorbance without sample or quercitin (control sample).

$A_{1:}$ is the absorbance in the presence of the sample or quercitin.

Superoxide dismutase activity assay:

Superoxide dismutase activity was assayed by the method of Dechatelet *et al.* **(1974).** The increase in absorbance was measured at 560 nm during 5 min. The reading was taken in each minute and the change in the mean absorbance/min was then calculated.

Nitro blue dye inhibition% = $[(\Delta A_c - \Delta A_s) / \Delta A_c] X$ l00

$A_{c:}$ is the rate of absorbance change of control (without sample).

$A_{s:}$ is the rate of absorbance change of sample.

Scavenging of hydroxyl radical:

Hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton (1984). The absorbance was measured at 520 nm and he percent of scavenging activity was calculated as follows:

Hydroxyl radical inhibition% $= [(A_i - A_0) / (A_c - A_0)] X 100$

A_i: Absorbance in the presence of the tested compound.

A_0: Absorbance in the absence of the tested compound.

A_c: Absorbance in the absence of the tested compound plus EDTA-Fe(II) and H_2O_2.

Reducing power assay

Reducing power assay was determined as described in the method of (Ferreira *et al*., 2007). Ascorbic acid was used as reference standard. Absorbance was measured at 700 nm against the corresponding blank solution.

RESULTS AND DISCUSSION

Phytochemical screening of spearmint:

Phytochemical analysis of spearmint methanolic extract (ME) indicated the presence of terpenes, tanins, flavonoids, saponins, glycosides, alkaloids and phenolic glycosides, as shown in Table 1.

Table1: Qualitative analysis of phytochemical constituents of spearmint methanolic extract.

Constituent	Level*
Terpenes	+++
Tannin	+
Flavanoid	++
Saponins	+
Glycosides	+++
Alkaloids	+
Phenolic glycosides	++
Resins	—

*** + = Low concentration, ++ = High concentration, — = Not detectable.**

Total phenolic content:

Phenolic compounds are considered as the major class of natural antioxidants present in plants and are usually measured using the Folin–Ciocalteu method. They calculated as gallic acid equivalents (GAE) per gram of dry extract(Table 2). Phenolic compounds have considerable free-radical scavenging activity and their content in plants is an important parameter as antioxidant components (Subramanian *et al*., 2011).

Total flavonoid content:

Polyphenols consist of flavonoids which occupied the largest amount and other compounds with the smallest amount (Lotito and Frei, 2006). It is well known that flavonoids which are polyphenolic secondary metabolites are responsible for the radical scavenging effects of most plants. The total flavonoid content is presented in Table 2 and determined as mg of quercetin equivalent (QE) per gram of dry extract. The mechanisms of the flavonoids action are through scavenging or chelating processes. The number of hydroxyl groups and substitution with electron-donating alkyl or methoxy groups of flavonoids cause an increase in their antioxidant activity (Awaad *et al*, 2012).

Table 2: Total phenolic and total flavonoid contents of spearmint aerial part.

Total phenolic, (mg GAE / g)[a]	Total flavonoid, (mg QE / g)[b]
35.65	27.47

[a](mg GAE/ g): mg of gallic acid equivalent per g of dry plant extract.
[b](mg QE/ g): mg of quercetin equivalent per g of dry plant extract.

HPLC For Polyphenols Extracted From Spearmint

Figure 1, shows HPLC chromatogram for polyphenols with different retention times and peak area for main components found in spearmint. Comparing results with standard compounds, it could be noticed that Caffeic acid was the predominant identified compound with an average percentage value of 3.406% as shown in Table 4. Other six components could be detected and collected in Table 3, which are Gallic acid, Protocatechuic acid, Catechin, Salicylic acid, Ellagic acid and Cinnamic acid. The chromatogram also shows a highest peak area % percent of 29.067 % for unknown compound.

Fig. 1: HPLC chromatogram of spearmint. (1)Gallic acid, (2)Protocatechuic acid, (3) Catechin, (4) Caffeic acid, (5)Unknown, (6)Salicylic acid, (7)Ellagic acid, (8) Cinnamic acid

Table 3: HPLC for polyphenol components of spearmint aerial parts methanolic extract.

No.	Components	Rt	Area %
1	Gallic acid	6.957	0.469
2	Protocatechuic acid	8.286	1.0369
3	Catechin	8.473	0.654
4	Caffeic acid	10.002	3.406
5	Unknown	11.799	29.067
6	Salicylic acid	12.319	0.926
7	Ellagic acid	13.445	0.770
8	Cinnamic acid	14.815	0.304

DPPH scavenging ability:

DPPH, is an organic radical used to evaluate the antioxidant activities of many plant extracts (Brand-Williams *et al.,* 1995). Methanolic spearmint extract was assessed for its ability to scavenge DPPH free radical along with vitamin C as a positive control. The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter to measure the antioxidant activity. A lower IC_{50} value indicates a higher scavenging power. Different concentrations from 20 to 500 µg/ml of the extract were used in comparsion with vitamin C as control. It is clear that DPPH has maximum absorption at 517 nm and the extract as antioxidant reduces it to yellow colored diphenyl-picrylhydrazine. Free radical scavenging capacity of the extract was found to be increased with increasing concentrations. Results revealed that free radical scavenging capacity values IC_{50} of methanolic extract and vitamin C are 439 µg/ml and 27.4 µg/ml respectively.

Total antioxidant capacity:

Phosphomolybdenum method is based on the reduction of Mo^{+6} to Mo^{+5} by the antioxidant compounds and the formation of a green Mo^{+5} complex with a maximal absorption at 695 nm (Prieto *et al.,* 1999). The antioxidant capacity of methanolic extract with absorbance value was compared with vitamin C as control (Table 4 and Fig. 2). The study revealed that the antioxidant capacity of the extract increased by increasing concentration of the methanolic extract concentration. Results declear also that methanolic extract antioxidant capacity absorbance value was significantly lower than that of vitamin C at 100 µg/ml. Average values of 0.31 and 1.31 were detected for their absorbance values respectively.

Table 4: Total antioxidant capacity of different concentrations of spearmint methanolic extract comparing with vit.c.

Concentration (µg/ml)	ME absorbance	Vit.C absorbance
25	0.14	0.55
50	0.19	0.98
75	0.25	1.21
100	0.31	1.31

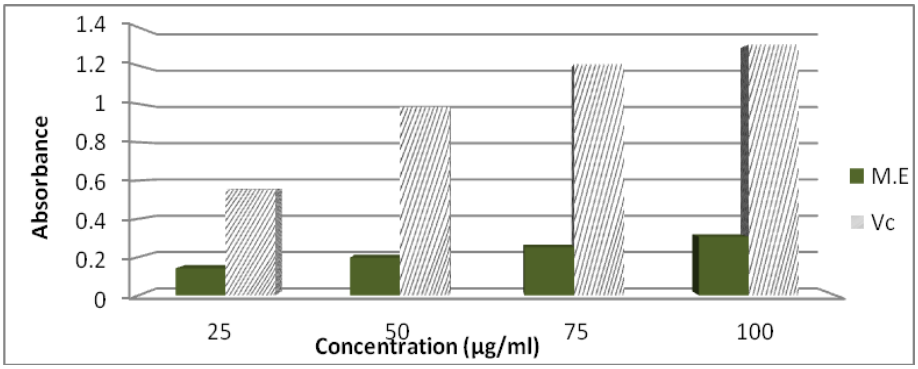

Fig. 2: Antioxidant capacity of different concentrations of spearmint methanolic extract in comparison with vit. c.

Scavenging of nitric oxide radical:

Nitric oxide can act as a free radical electrophile and an oxidising agent. It reacts with oxygen to form nitrogen dioxide which causes membrane damage by auto oxidation of fatty acids due to its ability to abstract hydrogen from unsaturated fatty acids (Steinert *et al*., 2010). The measure of NO-radical scavenging activity depends on the principle that sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions which can be evaluated using a Griess reagent (Sangameswaran *et al.,* 2009). Scavengers of nitric oxide decrease oxygen levels and, thus, reduce production of nitrite ions which can be monitored at 540 nm. Table 5 and Fig. 3 illustrates the effect of different concentrations of methanolic spearmint extract and the standard quercetin on the inhibition percent (I%) of nitric oxide radical. The inhibition percent was increased with increasing concentration of extract. As shown Table 5, This extract has better scavenging $NO^{\bullet}$ radical activity than quercetin at the four using levels.

Table 5: Nitric oxide scavenging activity of spearmint methanolic extract using quercetin (Qu) as reference.

Concentration (µg/ml)	**ME I%**	Quercetin **I%**
50	45.45	34.11
100	46.8	36.21
200	49.5	40.41
300	52.2	44.61

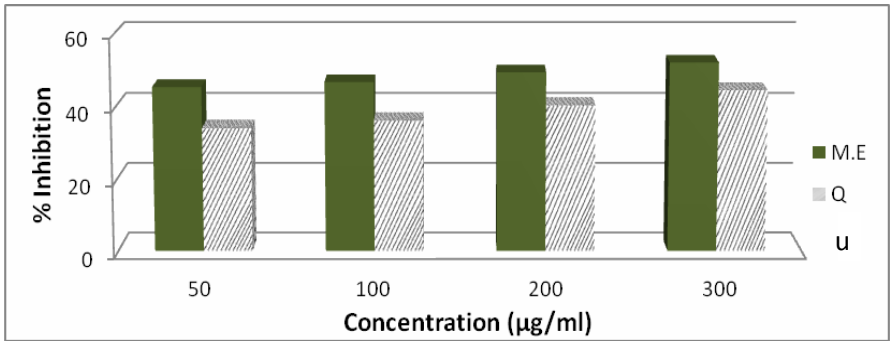

Fig. 3: Nitric oxide scavenging activity comparing with quercetin (Qu).

Superoxide dismutase activity assay:

Superoxide anion is an oxygen-centered radical with selective reactivity and precursor to active free radicals as hydrogen peroxide, hydroxyl radical and singlet oxygen that have ability to react with biological macromolecules and thereby inducing tissue damage (Wickens, 2001). The inhibition percent of superoxide anion radical generated at 400 µg/mL concentration of methanolic extract (Table 6 and Fig. 4) was found as 58.6 %.

Table 6: Superoxide dismutase activity for different spearmint methanolic extract concentration.

Concentration (µg/ml)	ME Inhibition %
100	30.7
200	40.07
300	51.0
400	58.6

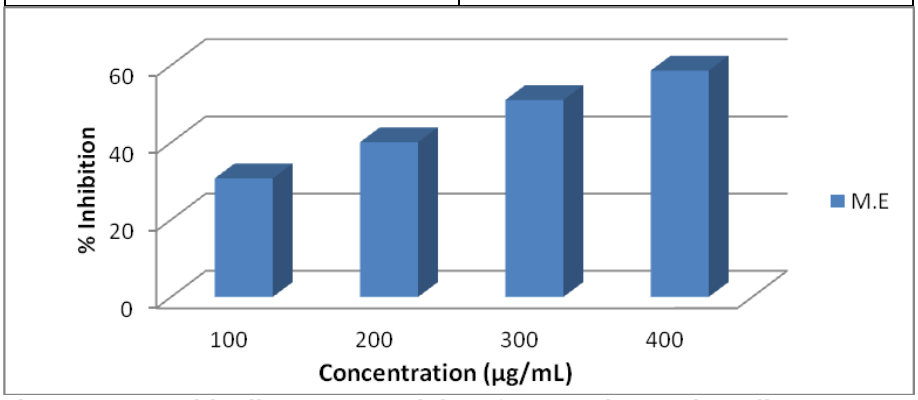

Fig. 4: Superoxide dismutase activity of spearmint methanolic extract.

Hydroxyl radical scavenging activity assay:

Hydroxyl Radical $HO^{\cdot}$ is the most reactive and interacts with the purine and pyrimidine bases of DNA. It can also abstract hydrogen atoms from biologic molecules, including thiols, leading to the formation of sulfur

radicals capable to combine with oxygen to generate oxysulfur radicals, able to damage biologic molecules (Halliwell, 1991). The hydroxyl radicals ($HO^{\bullet}$) in aqueous media were generated through the fenton (Fe^{++}/EDTA/H_2O_2) reaction. The hydroxyl radical bleached the safranin, so decreased the absorbance of the reaction mixture indicating a decrease in hydroxyl radical scavenging ability (Winterbourn and Sutton, 1984). The obtained results (Table 7 and Fig. 5) showed that the scavenging of hydroxyl radical increased by increasing methanolic extract concentrations. It reached 68.84 % at the concentration 480 µg/mL.

Table 7: Hydroxyl radical scavenging activity of different concentrations of methanolic extract (ME).

Methanolic extract inhibition %	Concentration (µg/ml)
19.3	160
30.7	240
42.2	320
56.3	400

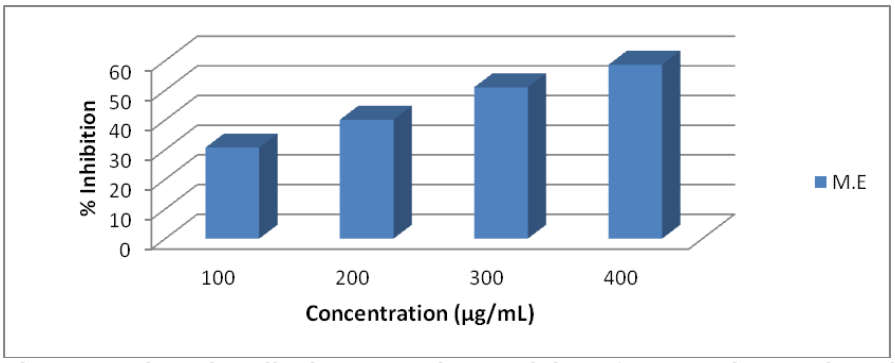

Fig. 5: Hydroxyl radical scavenging activity of spearmint methanolic extract.

Reducing power assay:

Reducing power assay measures the total reducing capability of antioxidants on the basis of the methanolic extract or vitamin C ability to reduce Fe^{+3} to Fe^{+2} ion. This assay treats the antioxidants in the samples as reductants in a redox-linked colorimetric reaction and is relatively simple and easy procedure to be standardized. One possible disadvantage with this assay is the fact that this assay does not react fast with some antioxidants, such as glutathione. However, It is still suitable for assessment of antioxidant activity of extract because only limited amounts of plant glutathione are absorbed by humans (Schafer and Buettner, 2001). The reducing power capacity of methanolic extract as absorbance value of (0.24), was significantly lower than that of vitamin C (2.4) at 25 µg/ml (Table 8 and Fig. 6).

Table 8: Reducing Power of various concentrations of methanolic extract.

Vit.C absorbance	ME absorbance	Concentration (µg/ml)
1.09	0.10	10
1.53	0.15	15
1.96	0.20	20
2.4	0.24	25

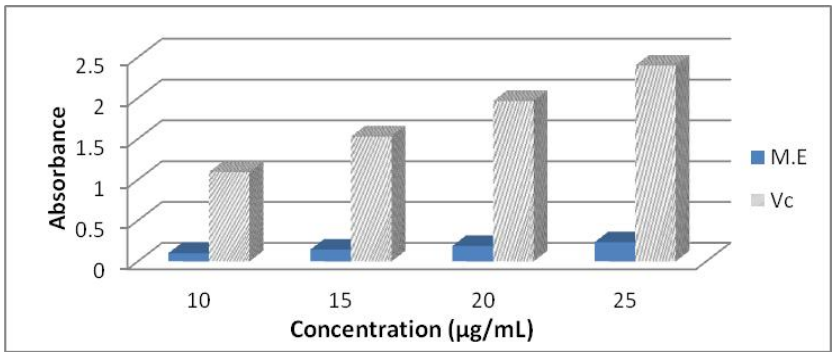

Fig. 6. Reducing Power of spearmint methanolic extract.

Finally from the above mentioned results it could be concluded that spearmint methanolic extract showed to contain phenolic compounds that have considerable free-radical scavenging activity. The presence of flavonoids that contain anumber of hydroxyl groups beside methoxy groups cause an increase in antioxidant activity of spearmint extract. HPLC technique for spearmint polyphenols revealed the presence of 7 components with higher antioxidant capacity. Spearmint methanolic extract showed also a higher scavenging $NO^{\cdot}$ radical activity of 52.2% than that of quercetin (44.61%). Superoxide dismutase and hydroxyl radical scavenging activities of Spearmint extract revealed the inhibition of 58.6 and 56.3% under the same concentration of 400 µg/ml, respectively.

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التخلص من الشوارد الحره بواسطة المستخلص الميثانولي لنبات النعناع

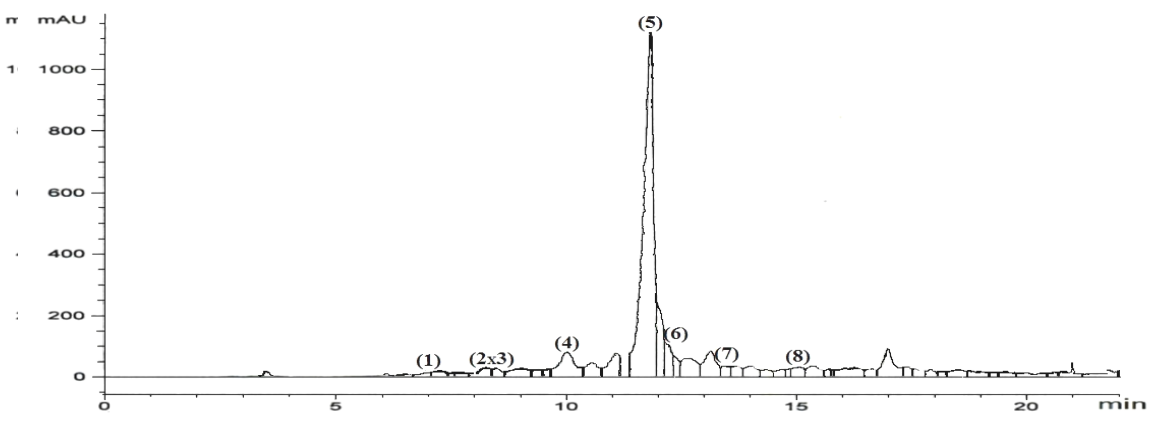
رمضـــان أحمـــد حســـن*، حســـان بركـــات حامـــد*، مصـــطفى إبـــراهيم ســـند* و خالد عبد الفتاح سيد أحمد**

*** قسم الكيمياء الزراعية – كلية الزراعة – جامعة المنصورة – مصر .**

**** قسم الكيمياء الزراعية – كلية الزراعة- جامعة دمياط - مصر.**

في الدراسة الحالية تم استخدام المستخلص الميثانولي للأجزاء الهوائية لنبات النعناع كمضاد اكسدة طبيعية لكبح جماح الشوارد الحرة. وقد ُقدرت كمية الفلافونات والفينولات الكلية في المستخلص الميثانولي وكان تركيز كل منهما ٢٧.٤٧ و ٣٥.٦٥ ملجرام/جرام مستخلص على التوالي. ثم اجري تفريد للمركبات الفينولية الموجودة في نفس النبات باستخدام تكنيك الكروماتوغرافيا السائلة عالية الضغط وتبين وجود مركبات فينولية متنوعة بتركيزات مختلفة. كذلك اجريت تجربة لتوضيح القوة الاختزالية للمستخلص.

اجريت اختبارات معملية لاثبات قدرة المستخلص على مسك انواع مختلفة من الشوارد الحرة مثل DPPH، السوبر اكسيد، النيترواكسيد والهيدرواكسيد. حيث اعطى المستخلص الميثانولي تثبيط الجذور الحرة بكمية قدرة ٤٦٤.٣٠ ميكروجرام/مل من المستخلص الاثيري اللازم لاكسدة ٥٠ % من مركب DPPH. وكذلك كان له قدرة عالية في مسك كل من السوبراكسيد و النيترواكسيد كما أظهر قدرة عالية على مسك الشوادر الحره الهيدروكسيدية.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