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Antioxidant and Antimicrobial Activities of *Echinacea purpurea L.* Extracts.

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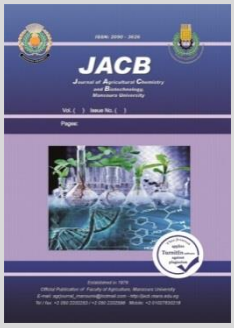

ABSTRACT

Echinacea purpurea L. (*E. purpurea*) is one of the most important medical herbs belongs to *Asteraceae* family. This plant contains many chemical compounds. It has also demonstrated numerous biological activities as both antioxidant and antibacterial. Methanol was used to extract green plant sample and consider as crud extract. This last one was partitioned into four solvents i.e. petrolium ether, CH_2Cl_2, $C_2H_5COOCH_3$ and C_4H_9OH . The obtained fractions extracts were tested for their total phenolic content (TPC), total flavonoid content (TFC). Antioxidant activities were carried out using three methods I.e phosphomolybdenum, reducing power and radical assay DPPH. Crude methanolic extract was subjected to six microbial strains included fungi, yeast and bacteria i.e: *Aspergillus oryzae* , *Candida albicans*, *Saccharomyces cerevisiae*, *Alternaia solani* and *Escherichia coli* (G^-), *Streptococcus faecalis* (G^+). *E. purpurea* extracts had the highest effect on *yeast (Saccharomyces cerevisiae*) with average value of 15.0 mm 100 ppm extract. Results showed that the highest concentration of TPC for ethyl acetate extract was 90.6 mg GAE/g dw wheras pet. ether gave the lowest value of 33.83 mg GAE/g d.w. TFC was ranged from 146.53 to 322.23 mg QE/g dw for ethyl acetate and methanol extracts, respectively. Butanol extract showed the highest antioxidant activity with average value of 70.22 µg/ml.

Keywords: *Asteraceae*; DPPH; total polyphenols, Flavonoids; Antimicrobial; antioxidant.

INTRODUCTION

Echinacea purpurea L., which is commonly known as purple coneflower, is an herbaceous perennial and a member of *Asteraceae* family with a long tradition of medicinal use in North America, Europe and Australia (Speroni, *et al.* ,, 2002).

In modern cultures, *E. purpurea* is used for medicinal purposes, in treating acute upper respiratory infections, urinary tract infections, burns and disorders such as viral infections, cutaneous affections and chronic disease due to deficiency of immunological responses (Luo *et al.* ,, 2003).

E. purpurea stimulates various immune cells including macrophages and natural killer cells and has anti-inflammatory effects (Barrett, 2003).

E. purpurea showed good immunoregulation, anti-inflammation and antioxidant capacity and with no hypersensitivity or other side effects (Zhai *et al.* , 2007). Also, kinds of E. *purpurea* showed to contain common principal ingredients including alkamides, caffeic acid derivatives, polyacetylene, flavonoids and essential oils (Thygesen *et al.*, 2007). However, caffeic acid derivatives and alkamides have been showed to be ingredients with immunoregulation effects (Matthias *et al.*, 2008).

Microbial pathogens may contribute to foodborne disease incidence and the emergence of some drugs resistant bacteria - such as *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) - caused an increase in mortality and morbidity (Miladi *et a l.*, 2016).

This work aimed to study the TPC and TFC of the plant samples. The research contained also antioxidant activity study. Plus fungi, yeast and bacteria which were subjected to plant extract.

MATERIAL AND METHODS

Plant material :

In March 2018, *E. purpurea* fresh leaves were Purchased from Agric. farm Fac. Of Agric. Mansoura University and left in the shade to remove moisture, then grinded to powder and kept in closed refrigerator containers until use.

Plant extracts :

powdered plant samples were steeped in a sufficient amount of methanol and left overnight at lab. condition (25 °C). Then the extract was purified and the residue was re-extracted two times by wetness in methanol. The total extracts were dried by rotary evaporator. The extracts were preserved under cold conditions before further use.

Fractionation of Methanolic Extract :

Pet ether, (CH2Cl2), (C2H5COOCH3) and (C4H9OH) were used to fractionate Methanolic extract obtained from plant leaves under study. The extraction process was performed three times, after which the solvents were evaporated. The fractions obtained were kept in a refrigerator before use.

Total phenolic content:

Estimation of whole phenolic extracts was done as described by (Singleton *et al* . , 1999). Gallic acid was selected with levels ranging from 0.025 to 0.5 mg / mL as a reference. Each extract(1.0ml) containing (0.3 mg / mL)

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was added to 1 mL reagent Folin-Ciocalteu, 1 mL (7% wt / v) Na2CO3 and 9 mL of distilled water . The solution was left at room temperature for 90 minutes., then, the absorbance was determined at 765 nm and the phenolics content were expressed as mg GAE / g dw..

Total flavonoids content:

Flavonoids content of *E. purpurea* extracts were determined using ($ALCL_3$) method, as mentioned by Lin and Tang (2007). Each extract (1.o ml) was applied to a 0.1 mL of (1 M potassium acetate), 0.1 mL of (10% w /v) $ALCL_3$ and 2.8 mL of distilled water. After 40 minutes the mixture was left in Lab. temperature and at 415 nm the absorbance was estimated. between 0.005 to 0.1 mg/mL Quercetin (Q) was utilized as a reference and flavonoids were determined as mg QE/ g dw extract.

Assay for antioxidant Activity.

1- Free-radical scavenging activity (DPPH) :

using 2,2 diphenyl-1-picrylhydrazyl (DPPH), as defined by Moon and Terao (1998). Free-radical scavenging activity was measured with some modification.

Separately different volumes of each extract (0.3 mg / mL) was added to form a total volume of 2.0 mL. The absorbance was measured at 517 nm using UV/ Vis spectrophotometer, after standing at lab. condition for 15 min. Low free radical scavenging activity was indicated in the reaction mixture with high absorbance . However, butylated hydroxyl toluene (BHT) has been used as a favourable device. Inhibition of free radical was studied as follows:

$$\textbf{Antiradical activity (\%)} = (A_{control} - A_{sample}) / A_{control} \times 100.$$

Where : $A_{control}$ **= Control absorbance**

A_{sample} **= Sample absorbance**

The IC_{50} valueis defined as the amount of antioxidant required to reduce the initial concentration of DPPH by 50 percent. the percentage antiradical activity against the concentration of the compounds being tested was determined based on linear regression of plots (Nahak and Sahu,2010). The experiment was performed in three stages and the findings are recorded as mean values.

2- Phosphomolybdenum (PM) method

The antioxidant activity of the plant extracts was evaluated by the phosphomolybdenum method defined by Prieto *et al.* (1999). 0.4 mL of each plant extract was combined with 4 mL of reagent solution [0.6 M sulfuric acid (H_2SO_4) , 28 mM sodium phosphate(Na_3PO_4), and 4 mM ammonium molybdate [$(NH_4)_2MoO_4$)]. In the case of the blank, 0.4 mL of the solvent was used instead of sample. The tubes were capped and incubated in water bath at 95 ºC for 90 min. Samples were cooled to room temperature, the absorbance of each solution was measured at 695 nm. Ascorbic acid was used as standard. The antioxidant capacity was expressed as an equivalent of ascorbic acid (mg ascorbic acid/g extract) .

3- Reducing power assay (RP)

Reducing power (RP) of the plant extracts was determined according to the method of Oyaizu (1986) with a slight modifications. The reaction mixture contained 1 mL of each plant extract was combined with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [K_3 $Fe(CN)_6$]. After incubation at 50ºC for 20 min, 2.5 mL of 10% trichloroacetic acid (TCA) were added to the mixture. Finally, 2.5 mL of previous mixture were combined with 1 mL of 0.1% FeCl3. Absorbance of these mixtures were measured at 700 nm using a UV spectrophotometer after incubation at room temperature for 10 min. The RP of samples were expressed as µmol of ascorbic acid equivalents (AAE) / g dry weight.

4- Antimicrobial activity

1. Microbial media and plant extracts Sterilization:

Potato Dextrose Agar (PDA) and nutrient Agar (NA) media were prepared for fungal and bacterial growth, respectively, as indicated by (Oxoid, 2006). These ready-made media were purchased and sterilized for 15 min at 121°C in autoclave. Using micro filter and the plant extract was also sterilized. (Flowpore D 0.2µm, Made in Germany).

2. Microbial strains and maintenance :

Six microbe species have been collected from Agric. of Dept. Microbiology, Fac. of Agric., Damietta University, Damietta, Egypt. The following bacteria, yeast and fungi were part of these microorganisms : *Escherichia coli* (G^-), *Streptococcus faecalis* (G^+),*Candida albicans*, *Saccharomyces cerevisiae, Alternaia solani* and *Aspergillus oryzae*. The fungal or bacterial strains were maintained on PDA or NA media at 5°C, befor use, respectively. Microbial strains were sub-cultured on new NA or PDA media slants (bacteria, the first four strains or yeast and fungi, the last two strains) and kept at 25°C for 5 days, respectively.

3. Methods of cultivation and antimicrobial determination of activity:

All the microbial strains were cultivated for one day according to the microbes on NA or PDA slants at 37 °C and 25°C, respectively. Sterilized saline solution 5 ml (0.09% sodium chlorid) was applied to every slant. Activity of antimicrobial has been evaluated by methods of well diffusion on Petri dishes that contain around 10 ml of NA or PDA media (El-Kadi *et al*., 2018). Plates were injected using a sterile cotton swab with suitable microbial strains. A sterilized cork borer was then used to make 6.3 mm for three small wells in diameter. Each well had been loaded with 30, 50, 100 ppm of *E. purpurea* extract. At 37 and 25°C, all plates were incubated according to the microbes. carefully measured Inhibition zones that appeared around the well using a digital Vernier caliper after one or 6 days according to the microbes (El-Fadaly *et al*., 2018). They determined the mean value of three replicates.

Antimicrobial agents evaluation = A-B (mm).

Where :

A: full clear zone (mm) diameter .

B: cork borer diameter (6.35) (Azzaz *et al*., 2017).

RESULTS AND DISCUSSION

1. Total phenolic content (TPC):

A secondary metabolite of plants and an essential part of both human and animal diets are considered the entire phenolic material.

Table (1) showed TPC (as mg GAE/ g d.w) leaves extract of *E. purpurea*. The occurrence of natural phenolic antioxidant compounds in all extracts was indicated the findings.

In comparison with other extracts, a high total phenolic content (90.6 mg GAE/g dw) was observed for ethyl acetate fraction. In the meantime, pet. ether fraction displayed the lowest overall phenolic value (33.83 mg GAE/g dw) .

On the other hand, the medium values of total polyphenols are methylene chloride fraction , methanol extract and butanol fraction , which are 64.03, 58.33 and 47.9 mg GAE/g d.w, respectively.

results presented from Table (1) showed that the average value of TPC for methanol extract was 58.33 mg GAE/ g dw. These findings were higher than results of Lee *et al*. (2009) which gave an average value of 11.0 mg GAE/ g dw of *E. purpurea* ethanol extract. Also, the same author (2010) reported that *E. purpurea* ethanol extract gave a mean amount of 22.3 mg GAE/ g dw

On the other hand, our results are in agreement with those of Stanisavljevic *et al.* (2009), who have a mean amount of 60.2 mg GAE /g dw for TPC of *E. purpurea* ethanolic extract.

Some parameters such as solution kind, dry process, species of plant and ripening period can affect the amount of polyphenols (Negro *et al.*, 2003)

Table 1. Total phenolic content of *E. purpurea* extracts

Methanol fractions	TPC as mg GAE/g dw
Pet. Ether	33.83
Methylene chloride	64.03
Ethyl acetate	90.6
Methanol	58.33
Butanol	47.9

2. Total flavonoid Content (TFC) :

Flavonoids has a broad collection of flavonols, flavanols and anthocyanin compounds Data from Table (2) showed T FC as mg QE/g dw of *E. purpurea* leaves extracts.

The highest concentration of TFC for methanol extract 322.23 mg QE/g dw, while the lowest value was for ethyl acetate fraction with average value of 146.53 mg QE/g dw. On the other hand, pet. ether, methylene chloride and butanol fractions have medium values of 205.36, 156 .00 and 149.00 mg QE/g dw for TFC, respectively.

Data in Table (2) showed that TFC for methanol extract was recorded 322.23 mg QE/g dw. This value was 10 fold these reported by Stanisavljevic *et al.* (2009) who gave low a mean amount of 32.3 mg RE/g dw for *E. purpurea* leaves ethanolic extract. However, These results were higher than those of Lee *et al.* (2010) who found average amount of 86.0 mg QE/ g dw of *E. purpurea* ethanol extract.

Antibacterial, anticarcinogenic, anti-virus, immune-stimulating, and the anti-inflammatory properties of flavonoids have positive effects on human health, (Havsteen, 2002.). The advantage of the intake of fruits and vegetables are largely due to the beneficial impact of flavonoids (Howard *et al.*, 1997).

Table 2. Total flavonoids of *E. purpurea* extracts

Methanol fractions	TFC(mg QE/g dw extract)
Pet. Ether	205.36
methylene chloride	156.00
Ethyl acetate	146.53
Methanol	322.23
Butanol	149.00

3- Scavenging activity of DPPH radical in *E. purpurea* extracts :

The antioxidant ability was described quantitatively and defined by the antioxidant concentration needed to scavenge 50 percent of DPPH• (IC_{50}). The lower IC_{50} point referred to the higher antioxidant activity.

The antioxidant activity of *E. purpurea* extracts showed in Table (3).

Data in Table (3) indicated that butanol fraction was the most active compared to other extracts (70.22 µg/ml), while, pet. ether fraction has the least active value with 1818 µg/ml. However, ethyl acetate , methylene chloride fractions and methanol extract have medium activity of 245.94 , 337.60 and 523 µg/ml for IC_{50}, respectively.

Table 3. Antioxidant activity of *E. purpurea* extracts:

Extracts	IC_{50} (µg/ml)
Pet. Ether	1818
methylene chloride	337.60
Ethyl acetate	245.94
Methanol	523.00
Butanol	70.22

The DPPH IC_{50} value of methanol extract was obtained as 523 µg/ml in our study. These findings are higher than those reported by Jukić *et al.* (2015) who gave DPPH IC50 for *E. purpurea* (15.67 µg/ml) in ethanolic extract .

In another study, Stanisavljevic *et al.* (2009) found that IC_{50} values for *E. purpurea* ethanol extract was 65.48 mg/ml , this data is not agreed with our findings (523 µg/ml).

4- Antioxidant activity Inhibition % (AOA) :

The data from Table (4) showed that extracts of *E. purpurea* leaves had varying antioxidant activity(AOA) levels.

The inhibition percentage was determined as follow:

inhibition percentage =$(A_{control} - A_{sample})/A_{control} \times 100$.

The data gained appear that using of 200 µg/ml condensation of various extraction of *E. purpurea* was more successful than the others when calculating the AOA percentage . Ethyl acetate extract had the highest inhibition proportion value followed by methylene chloride, methanol**,** butanol and pet. ether extract, in a mean amount of 34.07, 27.63, 16.88, 12.56 and 5.02 %, respectively.

Table 4. Antioxidant activity (AOA) inhibition percentage caused by extracts of *E. purpurea* leaves .

Concentrations (µg/ml)	Pet. ether extract	methylene chloride extract	Ethyl acetate extract	Methanol extract	Butanol extract
50	1.75	12.46	24.52	9.69	7.58
100	3.51	16.28	26.33	11.60	8.04
200	5.02	27.63	34.07	16.88	12.56

5-Phosphomolybdenum (PM) assay

The total antioxidant activities of *E. purpurea* extracts were investigated using phosphomolybdenum assay and expressed as Ascorbic Acid Equivalent (mg AAE/g extract).

The antioxidant activities of this plant extracts were shown in the Table 5.

From this Table, there is a variation in PM of the plant extracts. The values varied from 21.92 to 38.46, from 23.07 to 43.07 and from 25.00 to 51.15 mg AAE/g extract at 50, 100 and 200 ppm, respectively.

The highest antioxidant activity was detected in pet. ether extract followed by ethyl acetate, methylene chloride, methanol and butanol extract for concentrations 50 and 100

ppm. While, at 200 ppm the highest antioxidant activity was found in methylene chloride followed by pet. ether, ethyl acetate, methanol and butanol extract.

In this study, the biggest PM values at a dose 200 ppm were 51.15, 49.23 and 40.76 mg AAE/g extract for methylene chloride, pet. ether and ethyl acetate extracts, respectively. While the highest values at 100 and 50 ppm were found at pet. ether in average values of 43.07 and 38.46 mg AAE/g extract, respectively.

In contrast, the lowest PM value was found in butanol extract in average values of 21.92, 23.07 and 25.00 mg AAE/g extract at 50, 100 and 200 ppm, respectively.

These results are higher than those of Tusevski *et al.* (2014) who award medium amount of 20.36 mg AAE/g dw for methanol extract of *Achillea holosericea* (family Asteraceae)

Table 5.Total antioxidant activities (mg AAE/g extract) of *E. purpurea* extracts tested by phosphomolybdenum assay .(PM)

extracts	Total antioxidant activitiy (mg AAE/g extract)				
Cons.	Pet. Ether	Methylene chloride	Ethyl acetate	Methanol	Butanol
50 ppm	38.46	25.38	32.69	25.00	21.92
100 ppm	43.07	31.15	33.46	27.69	23.07
200 ppm	49.23	51.15	40.76	35.38	25.00

6. Reducing power (RP) assay

Antioxidant activities of *E. purpurea* extracts were estimated using RP assay and expressed as Ascorbic Acid Equivalents (µmol AAE/g dw extract) as shown in Table 6.

The obtained data showed that the increase in ferric reducing power accompanied with the raise in concentration.

The data in Table (6) indicated that using concentration of 200 ppm of different extracts during the evaluation of RP was more effective than the others.

As shown in the same Table , the highest RP are detected in ethyl acetate extract followed by pet. ether , methylene chloride, butanol and methanol extract at 200 ppm. While, at a dose 100 ppm the highest RP were estimated in pet. ether extract followed by ethyl acetate , methylene chloride, butanol and methanol extracts.

On the other hand, the reducing power antioxidants consequent in descending rates as follows: pet. ether, ethyl acetate, butanol, methylene chloride and methanol , at 50 ppm (µg/mL) test sample.

These results are lower than those reported by Tusevski *et al*. (2014) who gave average value of 156 µmol AAE/g dw for antioxidant activity using RP assay for methanol extract of *Achillea holosericea* (family Asteraceae).

Malik *et al*. (2017) mentioned that the reducing power of methanolic leaves extract of wild chicory(*Cichorium intybus)(* family Asteraceae) had a strong reducing activity of 0.674 µg/ml

Table 6. Antioxidant activity of *E. purpurea* extracts using reducing power trail. (RP assay)

extracts	Total antioxidant activitiy (µmol AAE/g dw)				
Cons.	Pet. Ether	Methylene chloride	Ethyl acetate	Methanol	Butanol
50 ppm	0.104	0.092	0.103	0.087	0.093
100 ppm	0.122	0.105	0.115	0.088	0.096
200 ppm	0.143	0.127	0.148	0.089	0.098

7. Antimicrobial activity

The antimicrobial activities of the methanolic extracts of *E. purpurea* were evaluated against six microorganisms. The results illustrated that methanolic extract of *E. purpurea* at 100 ppm was most effective. Data in Table (7) showed that *Streptococcus faecalis was less resistant than E.coli* .

Table 7. Antimicrobial activities of *E. purpurea* methanolic extract .

Strains of	The inhibition zone Diameter (mm)		
microorganism	30ppm	50 ppm	100 ppm
Escherichia. Coli	5	7	12
Streptococcus faecalis	9	10	10
Candida albicans	0	6	8
Saccharomyces cerevisiae	8	10	15
Aspergillus oryzae	0	5	8
Alternaria solani	0	10	11

Methanolic extract of *E. purpurea* did not offer any antimicrobial activities against *candida albicans, Aspergillus oryzae and Alternaria solani* at concentration 30 ppm. Although, the same extract had an antimicrobial activity against *Escherichia coli*, *Streptococcus faecalis* and *Saccharomyces cerevisiae* at the same concentrations.

On the other hand, methanolic extract *of E. purpurea* gave the highest antimicrobial activity against *E. coli* and *Saccharomyces cerevisiae* at concentration 100 ppm, (12 and 15 mm), respectively.

The effect of *E. purpurea* methanol extract on *Aspergillus oryzae* was ranged between 5.0 to 8 mm.

Data obtained showed that the highest effect of *E. purpurea* extracts on *Saccharomyces cerevisiae* was 15.0 mm with a 100 ppm level . while moderate activity was 10 mm recorded for *Streptococcus faecalis, Saccharomyces cerevisiae* and *Alternaria solani* at 50 ppm condensation , respectively . *Aspergillus oryzae* (8 mm) displayed the lowest antimicrobial activity at 100 ppm .

In another study, Rehman *et al.* 2012, stated that the antibacterial effects of *E. purpurea* methanolic extract against some microbes concentration ranged from 400 to 500 µg/ml were for *S. aureus* (6.0, 11.0, and 16.0 mm) and *E. coli* (8.0, 11.0, and 16.0 mm). Our findings were in contrast with these.

Similar to our findings the Methanolic extract of E. *purpurea* was shown by Korcan *et al.* (2018) who demonstrated antimicrobial activity against *Escherichia coli* at a concentration of 50 µI with a zone inhibition of 10 mm.

On comparison, they found that same extract at 100 µI on *Bacillus cereus* and *Escherichia coli* showed inhibition zone of 19 and 14 mm, respectively.

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الانشطة المضادة للأكسدة والميكروبات في مستخلصات نبات القنفدية الارجوانية

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نبات القنفدية الارجوانية هو واحد من أهم الأعشاب الطبية والذى ينتمى الى العائلة المركبة. يحتوي هذا النبات على العديد من المركبات الكيميائية. كما ان له بعض الأنشطة البيولوجية المختلفة مثل مضادات الأكسدة ومضادات للميكروبات. تم استخلاص المركبات الفعالة من الاوراق الجافة لهذا النبات بالميثانول للحصول على مستخلص الميثانول كمستخلص خام. وعن طريق الاستخلاص التجزيئي لمستخلص الميثانول بمذيبات متدرجة القطبية وهى الاثير البترولي وكلوريد الميثيلين وخلات الايثايل والبيوتانول وبذلك امكن الحصول على اربعة مستخلصات تجزيئية لكل من هذه المذيبات. تم تقدير المحتوى من الفينولات الكلية (TPC) والفلافونيدات الكلية (TFC) ودراسة نشاط مضادات الأكسدة باستخدام ثلاث طرق وهى : طريقة المولبيدات و طريقة القوة الاختزالية وطريقة ال DPPH في جميع المستخلصات. وكذلك تم تقدير النشاط المضاد للميكروبات فى المستخلص الميثانولي ضد البكتيريا الموجبة لجرام *Streptococcus faecalis (G+)*, والسالبة لجرام *Escherichia coli (G-)* واثنين من كل من الخمائر *Candida albicans* و *Saccharomyces cerevisiae* واثنين من الفطريات *Alternaia solani* و *Aspergillus oryzae* وكانت افضل تأثير للمستخلص الميثانولى بتركيز 100ppm على *Saccharomyces cerevisiae* حيث بلغ قطر التثبيط 15 مم . أظهرت النتائج أن أعلى تركيز لـ TPC كان للمستخلص التجزيئي خلات الإيثيل بقيم 6.90 ملجم GAE / جم وزن جاف بينما لوحظ أن أقل قيمة كانت للمستخلص التجزيئي الإيثر البترولى 83. 33 ملجم GAE / جم وزن جاف . من ناحية أخرى تراوح المحتوى من الفلافونويدات الكلية (TFC) بين 53.146 و23.322 ملجم QE / جم وزن جاف لمستخلصات خلات الإيثايل و الميثانول على التوالي. و كان أعلى نشاط مضاد للأكسدة لمستخلص البيوتانول بنسبة 22.70 ميكروجرام / مل.