ABSTRACT: The present study aimed to determine and compare total phenolic, flavonoid contents and antioxidant activity of Nigella sativa L. seeds crude extract and various fractions obtained through macroporous resin. Antioxidant activity were evaluated by DPPH assay. Total phenolic and flavonoid contents were determined as pyrocatechol and rutin equivalents, respectively, and correlated with antioxidant activities. Results showed that fraction with 40% ethanol exhibited the highest content of total phenolic (138.85 ± 0.53 mg PE/g extract). Besides, the highest concentration of flavonoids (140.11±5.47mg RU/g extract) and the highest antioxidant activity DPPH assay (IC50 = 1.26±0.21 µg /ml) were observed for fraction with 20% ethanol. Total phenolic content and total flavonoid highly correlated each other and well correlated with antioxidant activity. To conclude, macroporous resin could be a very good low cost and nontoxic technical for natural sources of antioxidant substances with high value for fractionation of N. sativa L. seeds with 20% ethanol.

KEY WORDS: Antioxidant activity, DPPH assay, Macroporous Resin, Total flavonoid, Total phenolic.

INTRODUCTION
Oxidation process is considered as one of the most important routes for generating free radicals in food, drugs and even living systems (Pourmorad et al., 2006). Free radicals mostly refer to reactive oxygen species (ROS) and reactive nitrogen (RNS) species, as the most common pro-oxidants. These originate either from normal metabolism or as a result of UV radiation and environmental pollutants. These reactive species cause severe oxidative damage to proteins, lipids, enzymes and DNA by covalent binding and lipid peroxidation, with subsequent tissue injury. Increase in free radicals has been associated with many human diseases like cancer, Alzheimer’s disease, cardiac reperfusion abnormalities, kidney and liver disease, fibrosis, atherosclerosis, arthritis, neurodegenerative disorders and aging (Sarma et al., 2010).

Harmful effects of disturbed antioxidant-prooxidant balance can be largely prevented by increasing intake of antioxidant substances (Ghosh et al., 2008; Ognjanovic et al., 2008). Antioxidants are natural compounds occurring in plants that may help counter the detrimental effects of free radicals. The main characteristic of an antioxidant is ability to scavenging these free radicals. Currently, the interest in natural antioxidants has increased considerably because of their beneficial effects of prevention and risk reduction in several diseases (Siger et al., 2012). While synthetic antioxidants that have shown genotoxicity, plant-derived antioxidants exhibit high degree of safety (Thitilertdecha et al., 2008; Valentao et al., 2002).

The presence of antioxidants has been widely reported in fruits, seeds, vegetables, herbs and cereals extracts. (Majhenič et al., 2007; Al-Farsi et al., 2008; Subhasree et al., 2009). Phenolic compounds such as flavonoids, phenolic acids and tannins in plants possess strong antioxidant activity.
Plants Material

*Nigella Sativa* L. seeds were obtained from Assiut governorate, Egypt. The seeds were collected during summer 2016 and identified by a botanist in the department of medicinal and aromatic plants researches, Horticulture research institute, Agricultural research center. The current study was conducted during 2016 and 2017 years at Institute of Chinese Materia Medica, Pharmaceutical College of Henan University, Kaifeng, Henan, China.

**Extraction and Fractionation of crude extract**

Dried seeds of *N. Sativa* L. were crushed and the oil was extracted, then the residue was extracted 3 times with 70% ethanol at 40°C for 9 hours. The extract was filtered under Buchner funnel and concentrated under reduced pressure at 40°C using a rotary evaporator. The extract kept at −40°C overnight then dried in a freeze dryer. The crude extract subjected to D101 Macroporous resin adsorption column, eluted with 20% ethanol, 40% ethanol, 60% ethanol and 95% ethanol to obtain 4 fractions, Fr1, Fr2, Fr3 and Fr4, respectively.

**Total phenolic content determination**

The content of total phenolic compounds was determined spectrophotometrically according to the Folin-Ciocalteu method (Singleton et al., 1999) with slight modification. The diluted extract (1.25 mg/ml methanol) was used in the analysis. A 0.2 ml aliquot of the diluted extract was mixed with 2.5 ml of 10% Folin-Ciocalteu’s reagent in water. The mixture was covered and incubated for 2 mints in dark place then 2 ml of 7.5% Na2CO3 dissolved in water was added. The mixture was incubated for one hour at room temperature and absorbance was measured at 765 nm against blank. The blank had the same constituents except that the extract was exchanged by distilled water. Pyrocatechol was used as standard for preparing the calibration curve. The total phenolic content was expressed as mg pyrocatechol equivalents (PE) per g of extract.

**Total flavonoid content determination**

The total flavonoid content of crude extract and fractions was determined by a colorimetric method (Zielen’ski et al., 2007). Briefly, 0.5 ml of the extract (1.25 mg/ml methanol) was diluted with 2.5 mL of distilled water. Then 0.15mL of a 5% NaNO2 solution was added, and the mixture kept at room temperature. After 6 mints, 0.3 mL of a 10% AlCl3·6H2O solution was added and the mixture was allowed to stand for a further 5 mints. After that, 1.0 ml of 1 M NaOH was added. Finally, 0.55 ml distilled water was added to the mixed solution. The final solution was well mixed, and absorbance was immediately measured against the prepared blank at 510 nm. The blank had the same constituents except that the extract was exchanged by distilled water. Rutin was used as a standard to construct the standard curve. Total flavonoid content was expressed as mg rutin equivalent (RU) per g of extract.

**Scavenging activity against DPPH radical with micro plate Assay**

The stable free radical DPPH was dissolved in MeOH to give a 200 µM solution; 10 µL of a test compound in methanol (or methanol itself as blank control) was added to 175 µL of the methanol DPPH solution. For each test compound, different concentrations were tested. After further mixing, the decrease in absorbance was measured at 515 nm after 20 min. The actual

**MATERIALS AND METHODS**

**Plants Material**

*Nigella Sativa* L. seeds using macroporous resins. No study reporting isolation and fractionation of *N. Sativa* L. seeds, as nutritional and medicinal plant, have traditionally been used for thousands of years as folk medicine and some of its active compounds were reported against many ailments (Toncer and Kizil, 2004).

Several pharmacological effects have been attributed to this medicinal plant such as gastric ulcer healing (Javed et al., 2010), anti-microbial effect (Mariam and Al-Basal, 2009), anti-cancer activity (Shafi et al., 2009), cardiovascular disorders (Sultan et al., 2009), gastroprotective and antioxidant activity (El-Abhar et al., 2003), immunomodulatory, anti-inflammatory and anti-tumor effects (Majdalawieha et al., 2010), antitussive effect (Hosseinzadeh et al., 2008), anti-anxiety effect (Boskabady et al., 2010), anti-asthmatic effect (Chehl et al., 2009), anti-inflammatory effects in pancreatic cancer cells (Salem, 2005), anti-helicobacter activity (Tingfang et al., 2008), tumor growth suppression (Eugene et al., 2011), anti-viral activity against cytomegalovirus (Salem and Hassain, 2000), hepatoprotective activity (Khan, 1999). Although there are multiple studies reporting level of total Phenolic compounds and their antioxidant activity for many medicinal plants such as Kenaf seeds (Yusri et al., 2012), corn tassel (Mohsen and Ammar, 2009), *Melissa officinalis* L. (Canadanovic-Brunet et al., 2008), *Salvia* species (Tosun et al., 2009) and *Mitragyna rotundifolia* (Kang et al., 2010), few reports have been published on the total Phenolic compound content and antioxidant activity for *N. Sativa* L. seeds.

In this study, macroporous resins has been used for separation and purification natural products because it’s unique adsorption properties associated with its pore structure and surface functional groups (Gao et al., 2007; Yang et al., 2016). In addition, macroporous resins are relatively low-cost and easily regenerated, which is especially important for industrial applications. However, to our knowledge, there is no study reporting isolation and fractionation of *N. Sativa* L. seeds using macroporous resins.

**Extraction and Fractionation of crude extract**

N. sativa L. also known as black cumin is an annual herbaceous plant belonging to the Ranunculaceae family. The plant is indigenous to Mediterranean areas, through it is grown in other parts of the world as well. The plant commonly grows in the Middle East, Eastern Europe and Western and Central Asia. *N. Sativa* L. seeds, as nutritional and medicinal plant, have traditionally been used for thousands of years as folk medicine and some of its active compounds were reported against many ailments (Toncer and Kizil, 2004).

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Nigella sativa L. seeds as an antioxidant

The decrease in absorption induced by the test compound was calculated by subtracting that of the control. The antioxidant activity of each test sample was expressed as an IC50 value, i.e. the concentration in µg/mL that inhibits DPPH absorption by 50%, and was calculated from the concentration-effect linear regression curve (Wang et al., 2008; Zhang et al., 2009). PG and BHA were used for positive control. The DPPH radical scavenging activity of each plant extract was calculated as the percentage inhibition. % Inhibition of DPPH radical activity = \[(A0-A1)/A0\] × 100%

Where: A0 is the absorbance of the DPPH itself, A1 is the absorbance of sample and the positive control.

Statistical analysis

Data were analyzed statistically using Statistix Version 8.1 software. Differences between means were determined using the least significant difference test at P < 0.05. The data are presented as mean ± SD.

RESULTS AND DISCUSSION

Total phenolic content (TPC)

Total phenolic content (TPC) for crude extract and different fractions of N. sativa L. seeds using Folin-Ciocalteu method are reported in Table 1. The TPC was determined using Pyrocatechol as calibration standard as shown in Fig. 1.

The TPC values varied over a wide range of concentrations from 18.45 to 138.85 mg PE/g extract. The highest content of total phenolic was observed for fraction with 40% ethanol followed by fraction with 20% ethanol and fraction with 60% ethanol. Meanwhile fraction with 95% ethanol had the lowest concentration of TPC, lower than crude extract.

The solvent polarity plays a major role for extraction of TPC (Lee et al., 2007). The total phenolic content in extracts of the N. sativa depends on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction. (Zhou and Yu, 2004; Mohsen and Ammar, 2008). Moreover, the findings agree with Yusri et al. (2012) who found that the phenolic compounds in kenaf seeds are highly polar in nature, and thus more efficiently extractable by polar solvents. Also, Matthaus (2002) who reported that the high efficiency of polar solvents, i.e., water and methanol, in extracting phenolic compounds from several oilseeds.

Total flavonoid content (TFC)

The concentration of flavonoids in various fractions and crude extract of N. sativa L. seeds are presented in Table 2. The total flavonoid content was determined using rutin as standard as shown in Fig. 2. The concentrations of flavonoids ranged from 31.47 to 140.11mgRU/g extract. The highest flavonoid

| TABLE 1. Total phenolic content (TPC) of Nigella sativa L. seed crude extract and fractions. CE, crude extract; Fr1, fraction with 20% ethanol; Fr2, fraction with 40% ethanol; Fr3, fraction with 60% ethanol; Fr4, fraction with 95% ethanol. Data are mean ± SD (n = 3). Values followed by the same letters within a column are not significantly different (P< 0.05) according to the least significant difference test. |
|-----------------|------------------|
| Crude extract /fractions | TPC (mg PE/g extract) |
| CE              | 41.94 ±1.29 c    |
| Fr1             | 123.00 ± 0.65 b  |
| Fr2             | 138.85 ± 0.53 a  |
| Fr3             | 42.91 ± 0.84 c   |
| Fr4             | 18.45 ± 0.19 d   |

| TABLE 2. Total flavonoid content (TFC) of Nigella sativa L. seed crude extract and fractions. CE, crude extract; Fr1, fraction with 20% ethanol; Fr2, fraction with 40% ethanol; Fr3, fraction with 60% ethanol; Fr4, fraction with 95% ethanol. Data are mean ± SD (n = 3). Values followed by the same letters within a column are not significantly different (P < 0.05) according to the least significant difference test. |
|-----------------|------------------|
| Crude extract /fractions | TFC (mg RU)/g extract) |
| CE              | 31.47±1.85 d     |
| Fr1             | 140.11±5.47 a    |
| Fr2             | 135.44±2.14 a    |
| Fr3             | 46.19±1.40 c     |
| Fr4             | 67.22±0.70 b     |
content was found for fraction with 20% ethanol followed by fraction with 40% ethanol, fraction with 95% ethanol and fraction with 60% ethanol. Furthermore, the lowest content of flavonoid was recorded for crude extract. However, there were no significant differences in total flavonoid content between fraction with 20% ethanol and fraction with 40% ethanol.

The concentration of flavonoids in plant extracts depends on the polarity of solvents used in extract preparation (Gao and Liu, 2005). The highest content of flavonoids was obtained by using high polarity solvent. These results are in a similar pattern with those of Goga et al. (2012) they found that the highest total Phenolic content and flavonoid content of *N. sativa* L. seeds obtained from Soxhlet extraction using a polar solvent. Total flavonoid content is well correlated with TPC with \( r = 0.902 \) as illustrated in (Fig. 3), indicating that flavonoids might be the major contributors towards the phenolic compounds count for *N. sativa* L. seeds.

**FIGURE 3. Correlation between the total phenolic content of extracts and the total flavonoid content.** Correlation coefficient \( r = 0.902 \).

Antioxidant activity assay

The antioxidant activity of different fractions and crude extract of *N. sativa* L. seeds was determined using the DPPH radical-scavenging assay. DPPH is a very stable free radical. The effect of an antioxidant on DPPH radical scavenging is due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form diphenylpicrylhydrazine with the loss of its violet color (Molyneux, 2004). The DPPH radical scavenging activity was determined as percent inhibition of DPPH radical and expressed in terms of IC\(_{50}\) (µg/ml) values, the results are shown in Table 3. The obtained values for antioxidant activity examined by DPPH radical scavenging activity ranged from 1.26 to 15.25 µg/ml. A lower IC\(_{50}\) value indicates higher antioxidant activity. Thus, the fraction with 20% ethanol had the highest antioxidant activity followed by fraction with 40% ethanol, crude extract, fraction with 60% ethanol. While the lowest antioxidant activity was obtained from fraction with 95% ethanol. However, there were no significant differences in antioxidant activity between fraction with 20% ethanol and fraction with 40% ethanol. A high correlation was found between the values for the concentration of phenolic compounds and the antioxidant activity data from DPPH assay (Fig. 4) of different fractions and crude extract of *N. sativa* L. in addition, total flavonoid content was well correlated (Fig. 5) with the antioxidant activity (\( r = 0.718 \)).

**FIGURE 4. Correlation between the DPPH radical scavenging assays of extracts and the total phenolic content.** Correlation coefficient \( r = 0.925 \).

**FIGURE 5. Correlation between the DPPH radical scavenging assays of extracts and the total flavonoid content.** Correlation coefficient \( r = 0.718 \).

<table>
<thead>
<tr>
<th>Table 3. Antioxidant activity DPPH assay of <em>Nigella sativa</em> L. seed crude and fractions.</th>
<th>CE, crude extract; Fr1, fraction with 20% ethanol; Fr2, fraction with 40% ethanol; Fr3, fraction with 60% ethanol; Fr4, fraction with 95% ethanol. Data are mean ± SD (( n = 3 )).Values followed by the same letters within a column are not significantly different (( P &lt; 0.05 )) according to the least significant difference test. PG and BHA used as a positive control.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crude extract/fractions</strong></td>
<td><strong>IC(_{50}) (µg/ml)</strong></td>
</tr>
<tr>
<td>CE</td>
<td>8.54±0.64 (^{c})</td>
</tr>
<tr>
<td>Fr1</td>
<td>1.26±0.21 (^{d})</td>
</tr>
<tr>
<td>Fr2</td>
<td>2.30±0.14 (^{d})</td>
</tr>
<tr>
<td>Fr3</td>
<td>10.02±0.48 (^{b})</td>
</tr>
<tr>
<td>Fr4</td>
<td>15.25±1.04 (^{a})</td>
</tr>
<tr>
<td>PG</td>
<td>0.069±0.04</td>
</tr>
<tr>
<td>BHA</td>
<td>0.227±0.002</td>
</tr>
</tbody>
</table>
The extraction of antioxidant substances with different chemical structure was achieved using solvents of different polarity. Numerous investigations of qualitative composition of plant extracts revealed the presence of high concentrations of phenols in the extracts obtained using polar solvents (Canadanovic-Brunet et al., 2008). Based on results of this study, the extracts with the highest antioxidant activity had the highest concentration of phenols. These compounds are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may contribute directly to their antioxidant action (Tosun et al., 2009). Flavonoids are class of secondary plant metabolites with significant antioxidant and chelating properties. Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups (Sharififar et al., 2008). Extracts from N. sativa have high concentration of total phenols and flavonoids which is in correlation with intensive antioxidant activity of these extracts. Numerous investigations of the antioxidant activity of plant extracts have confirmed a high linear correlation between the values of phenol concentration and Flavonoids and antioxidant activity; (Kang et al., 2010; Meziti et al., 2012; Goga et al., 2012).

CONCLUSIONS

To the best of our knowledge this is the first report of use of macroporous resin for fractionation of N. sativa L. Our results study showed that the seeds of N. sativa L. had phenolic compounds and these compounds correlates with antioxidant activity in vitro. The highest content of total phenolic was observed for fraction with 40% ethanol. However, the highest concentration of flavonoids was found for fraction with 20% ethanol, moreover the highest antioxidant activity DPPH assay was noticed for fraction with 20% ethanol. Total flavonoid content gave high correlation with total phenolic indicating that flavonoids might be the major contributors towards the phenolic compounds count for N. sativa L. seeds. Also, total flavonoid content had well correlation with antioxidant activity. So, our suggestion, using macroporous resin for fractionation of N. sativa L. with 20% ethanol could be a very good low cost and nontoxic technical for natural sources of antioxidant substances with high value.

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Nigella sativa L. seeds as an antioxidant