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ScienceDirect
Food Chemistry 103 (2007) 1358–1364
Food Chemistry
www.elsevier.com/locate/foodchem

Antioxidant activity of the essential oil and various extracts of *Nepeta flavida* Hub.-Mor. from Turkey

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Received 3 July 2006; received in revised form 23 August 2006; accepted 20 October 2006

Abstract

This study was designed to examine the chemical composition and *in vitro* antioxidant activity of the essential oil and various extracts (hexane, dichloromethane and methanol sub-fractions) of *Nepeta flavida*. GC and GC–MS analyses of the essential oil resulted in the identification of 68 compounds, representing 96.4% of the oil; 1,8-cineole (38.9%) and linalool (25.1%) were the main components, comprising 64.0% of the total oil. The samples were subjected to a screening for their possible antioxidant activities by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. In the first case, the IC_{50} value of the *N. flavida* essential oil was determined to be 42.8 ± 2.19 μg/ml. Among the extracts, the strongest activity was exhibited by the polar sub-fraction of the methanol extract with an IC_{50} value of 63.2 ± 1.75 μg/ml. In the β-carotene-linoleic acid system, *N. flavida* essential oil exhibited 86.3% ± 1.69 inhibition against linoleic acid oxidation. Among the extracts prepared with various solvents, a correlation was observed between the polarity and antioxidant activity. The extracts exhibited the same activity pattern in this system the most active one is the polar sub-fraction, 79.7% ± 0.89. On the other hand, 1,8-cineole, a major compound of the essential oil, exhibited marked antioxidant activity in both systems, whereas the other compound, linalool, did not show any activity. The amount of total phenolics was highest in the polar and non-polar sub-fractions. Particularly, a positive correlation was observed between the total phenolic content and the antioxidant activity of the extracts. As estimated from the results, amounts of phenolic compounds were less in hexane and dichloromethane extracts than in the others. In conclusion, antioxidant potentials of polar and non-polar methanol sub-fractions could be attributed to their high phenolic contents. In both systems, antioxidant capacities of BHT, ascorbic acid, curcumin and α-tocopherol were also determined in parallel experiments.

Keywords: *Nepeta flavida*; Antioxidant activity; Essential oil; Total phenolics; Various extracts

1. Introduction

Free radicals were a major interest for early physicists and radiologists and much later were found to be a product of normal metabolism. Today, we well know that radicals cause molecular transformations and gene mutations in many types of organisms. Oxidative stress is well-known to cause many diseases (Storz & Imlay, 1999), and scientists, in many different disciplines, have become more interested in natural sources which could provide active components to prevent or reduce its impact on cells (Ulubelen et al., 1995; Yan, Murphy, Hammond, Vinson, & Neto, 2002).

Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases (Halliwell, Gutteridge, & Cross, 1992). The number of antioxidant compounds synthesized by plants as secondary products, mainly phenolics, serving in plant defence mechanisms to counteract reactive oxygen species (ROS)

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0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.10.049

in order to survive, is currently estimated to be between 4000 and 6000 (Havsteen, 2002; Peterson & Dwyer, 1998; Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999; Wollgast & Anklam, 2000). The phenolic content and composition of plants and the products produced from them depend on genetic and environmental factors, as well as post-harvest processing conditions (Cowan, 1999; Vaya, Belinky, & Aviram, 1997). The antioxidant activities of phenolics are related to a number of different mechanisms, such as free radical-scavenging, hydrogen-donation, singlet oxygen quenching, metal ion-chelation, and acting as substrates for radicals such as superoxide and hydroxyl. A direct relationship has been found between the phenolic content and antioxidant capacity of plants (Al-Mamary, Al-Meeri, & Al-Habori, 2002; Cowan, 1999; Robards et al., 1999; Wollgast & Anklam, 2000; Vaya et al., 1997).

The genus *Nepeta* (*Lamiaceae*) comprises 280 species that are distributed over a large part of central and southern Europe, and west, central, and southern Asia. About half of the existing species are recorded in Iran. The genus *Nepeta* is represented in Turkey by 33 species and altogether 38 taxa, 17 of these being endemic in Turkey (ca. 45%) (Davis, 1982).

Nepeta species are widely used in folk medicine because of their antispasmodic, diuretic, antiseptic, antitussive, antiasthmatic, and febrifuge activities. The feline attractant properties of several *Nepeta* species have been known for a long time. Nepetalactone and its isomers are considered to be responsible for the feline attractant activity of *Nepeta* species (Bicchi, Mashaly, & Sandra, 1984; De Pooter, Nicolai, De Buyck, Goetghebeur, & Schamp, 1987). As far as our literature survey could ascertain, there are no reports on the antioxidant activity of the essential oil and/or extracts and the amount of total phenolics of *N. flavida*. Thus, this study is the first report on this plant.

The aims of this work are; (i) to evaluate the *in vitro* antioxidant properties of the essential oil and various extracts, obtained by using a Clevenger distillation apparatus and Soxhlet extraction, respectively, (ii) to determine the chemical composition of *N. flavida* essential oil, and (iii) to determine the amount of total phenolics of the extracts. *In vitro* antioxidant activities were determined by using two complementary assays, namely inhibition of DPPH radical and β-carotene-linoleic acid systems. The chemical composition of the essential oil was evaluated by using gas chromatography (GC) and gas chromatography–mass spectroscopy (GC–MS).

2. Materials and methods

2.1. Collection of plant material

N. flavida plants were collected from Cebel plauteu (900 m), Yarpuz, Osmaniye, Turkey, when flowering (07 July, 2004). The voucher specimen was identified by a senior plant taxonomist, Dr. H. Askin Akpulat at the Department of Biology, Cumhuriyet University, Sivas-Turkey and has been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (CUFH-Voucher No: AA 3414).

2.2. Isolation of the essential oil

A portion (100 g) of dried and finally ground aerial parts of *N. flavida* was submitted, for 3 h, to water-distillation using a Clevenger-type apparatus (British type) (yield 1.34% v/w). The obtained essential oil (EO) was dried over anhydrous sodium sulphate and, after filtration, was stored at +4 °C until tested and analyzed.

2.3. Preparation of the extracts

Extracts of air-dried and ground plant materials were prepared by using solvents of varying polarity and the extraction protocol of each is given below.

A portion (100 g) of dried plant material was extracted with hexane (HE) (3.46%, w/w), followed by dichloromethane (DCM) (2.14%, w/w) and methanol in a Soxhlet apparatus (6 h for each solvent) (Sokmen, Jones, & Erturk, 1999). In this procedure, starting material was first extracted with *n*-hexane, and after that, the same solid material with dichloromethane and methanol (subsequently). The latter extract was suspended in water and partitioned with chloroform to obtain polar (MW) (9.73%, w/w) and non-polar (MC) (4.12%, w/w) sub-fractions.

All extracts obtained were lyophilized and kept in the dark at +4 °C prior to use.

2.4. Chromatographic analysis

2.4.1. Gas chromatography (GC) analysis

The essential oil was analyzed using a Hewlett Packard 5890 II GC equipped with a FID detector and HP-5ms capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were set at 220 °C and 290 °C, respectively. Column temperature was gradually increased from 50 °C to 240 °C with a rate of 3 °C/min. Helium was the carrier gas, at a flow rate of 1 ml/min. Diluted samples (1/100 in acetone, v/v) of 1.0 μl were injected manually and in the splitless mode. Quantitative data were obtained electronically from FID area percent data without the use of correction factors.

2.4.2. Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analysis of diluted solutions of the essential oils was performed under the same conditions as GC (column, oven temperature, flow rate of the carrier gas) using a Hewlett Packard 5890 II GC equipped with a Hewlett Packard 5972 mass selective detector in the electron impact mode (70 eV). Injector and MS transfer line temperatures were set at 220 °C and 290 °C, respectively. *n*-Alkanes were used as reference points in the calculation of the Kovats indices (K.I.). Tentative identification of the compounds was

based on comparison of their relative retention times and mass spectra with those of Nist98 and Wiley275 library data and literature data (Adams, 2001).

2.5. Antioxidant activity

2.5.1. DPPH assay

The hydrogen atom-or-electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of the purple coloured methanol solution of DPPH·. This spectrophotometric assay uses the stable radical, 2,2′-diphenylpicrylhydrazyl (DPPH·), as a reagent (Burits & Bucar, 2000; Cuendet, Hostettmann, & Potterat, 1997). Fifty microliters of various concentrations of the extracts in methanol were added to 5 ml of a 0.004% methanol solution of DPPH·. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical, DPPH·, in percent (I %) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated form the graph plotted of inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.5.2. β-Carotene-linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius, Venskutonis, Van Beek, & Linssen, 1998). A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg of β-carotene was dissolved in 1 ml of chloroform (HPLC grade); 25 μl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml of distilled water, saturated with oxygen (30 min 100 ml/min), were added with vigorous shaking. Two thousand five hundred micreliters of this reaction mixture were dispensed to test tubes and 350 μl portions of the extracts prepared at 2 g/l concentrations were added and the emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with the synthetic antioxidant, butylated hydroxytoluene (BHT) as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. Anti-oxidative capacities of the extracts were compared with those of BHT and blank.

2.6. Assay for total phenolics

Total phenolic constituents of the aforesaid extracts of *N. flavida* were determined by literature methods involving Folin-Ciocalteu reagent and gallic acid as standard (Chandler & Dodds, 1983; Slinkard & Singleton, 1997). Extract solution (0.1 ml) containing 1000 μg of extract was taken in a volumetric flask; 46 ml of distilled water and 1 ml Folin-Ciocalteu reagent were added and the flask was thoroughly shaken. After 3 min, 3 ml of a solution of 2% Na_2CO_3 were added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all standard gallic acid solutions (0–1000 mg 0.1 ml^{-1}) and a standard curve was obtained with the equation given below:

$$\text{Absorbance} = 0.0012 \times \text{Gallic acid } (\mu g) + 0.0033$$

3. Results and discussion

3.1. Chemical composition of the essential oil

About 68 compounds, representing 96.4% of the essential oil, were identified. GC and GC/MS analyses revealed that 1,8-cineole (38.9%) and linalool (25.1%) were the main components, comprising 64.0% of the total oil, as listed in Table 1. β-Pinene (2.6%), β-myrcene + cineol-dehydro (2.3%) and β-caryophyllene (2.2%) were the other constituents of the essential oil studied.

There are many reports on the chemical composition of the essential oils from the members of this genus (Baser, Kirimer, Kurkcuoglu, & Demirci, 2000; Dabiri & Sefidkon, 2003; Kalpoutzakis, Aligiannis, Mentis, Mitaku, & Charvala, 2001; Rustaiyan et al., 2000; Sajjadi & Ghassemi, 1999; Sefidkon, Dabiri, & Alamshahi, 2002; Senatore & Ozcan, 2003; Thappa, Agarwal, Srivastava, & Kapahi, 2001). Based on the literature data, it appears that there are three main chemotypes for the essential oils of these plants. The first one is the nepetolactone chemotype, e.g. *N. caesarea, N. cataria, N. cadmea, N. pilinux, N. racemosa, N. betonicifolia, N. cilicia, N. fissa, N. nuda* subsp. *glandulifera, N. concolor, N. conferta* and *N. isaurica* are included in the second group (caryophyllene oxide chemotype). The last group is the 1,8-cineole and/or linalool chemotype, including those studied here and *N. italica, N. sulfuriflora, N. congesta* var. *cryptantha, N. nuda* subsp.*nuda, N. nuda* subsp. *albiflora*. This chemotype produces an essential oil of herbaceous, mildly menthole-like odour, which may be attributed to its high content of 1,8-cineole (eucalyptol).

According to a study carried out by Baser et al. (2000), major constituents of the essential oil of *N. flavida* were determined to be 1,8-cineole (22.7%) and linalool (37.7%). The results reported by these researchers are particularly in agreement with the results presented here except for the amounts of the compounds. However, it is noteworthy that the composition of any plant essential oil studied is influenced by several factors, such as local, climatic, seasonal and experimental conditions (Daferera, Ziogas, & Polissiou, 2000).

Table 1
Chemical composition of *Nepeta flavida* essential oil

No	K.I.[a]	Components	Composition (%)
1	891	α-Thujene	0.1
2	897	α-Pinene	1.1
3	918	Thuja-2,4(10)-diene	0.2
4	935	Sabinene	0.8
5	938	β-Pinene	2.6
6	953	β-Myrcene + Cineol-dehydro	2.3
7	968	α-Phelladrene	0.4
8	980	α-Terpinene	0.6
9	995	1,8-Cineole	38.9
10	1017	(*E*)-β-Ocimene	1.5
11	1027	γ-Terpinene	0.7
12	1039	*cis*-Sabinene hydrate	0.1
13	1045	*trans*-Linalool oxide	0.3
14	1061	*cis*-Linalool oxide	1.1
15	1077	Linalool	25.1
16	1081	*trans*-Sabinene hydrate	tr[b]
17	1110	α-Campholenal	0.1
18	1123	Nopinone	tr
19	1125	*cis*-Verbenol	0.5
20	1132	*trans*-Verbenol	0.4
21	1147	Sabina ketone	0.1
22	1151	Pinocarvone	0.3
23	1158	δ-Terpineol	0.6
24	1170	Terpinen-4-ol	0.5
25	1186	α-Terpineol	1.2
26	1194	Myrtenol	0.5
27	1208	Verbenone	0.1
28	1243	Pulegone	0.8
29	1266	Geraniol	0.2
30	1289	2-Phenyl ethyl acetate	0.1
31	1299	(*E*)-Anethole	0.2
32	1314	Thymol	0.2
33	1324	Carvacrol	1.1
34	1371	α-Cubebene	0.1
35	1388	Eugenol	0.1
36	1393	Piperitenone oxide	0.1
37	1399	α-Copaene	0.1
38	1409	β-Bourbonene	0.2
39	1418	β-Elemene	0.1
40	1438	β-Longipinene	tr
41	1441	α-Funebrene	tr
42	1448	β-Caryophyllene	2.2
43	1459	β-Copaene	0.1
44	1477	*cis*-Muurola-3,5-diene	0.4
45	1486	α-Caryophyllene	0.2
46	1493	*cis*-Muurola-4(14),5-diene	0.7
47	1501	β-Acoradiene	0.1
48	1512	γ-Muurolene	0.1
49	1516	Germacrene D	1.2
50	1524	γ-Amorphene	0.2
51	1531	Viridiflorene	0.9
52	1544	α-Farnesene	0.1
53	1551	γ-Cadinene	0.3
54	1560	Calamene	1.0
55	1576	α-Cadinene	0.4
56	1583	β-Calacorene	0.3
57	1602	(*E*)-Nerolidol	0.1
58	1609	Ledol	0.2
59	1617	Germacrene-D-4-ol	0.4
60	1623	Caryophyllene oxide	0.4
61	1634	Viridiflorol	0.6
62	1656	Cubenol 1,10-di-epi	0.3
63	1680	Caryophylla-4(14),8(15)-dien-5-ol	0.1
64	1683	β-Copaen-4a-ol	0.6
65	1696	α-Cadinol	0.6
66	1717	Cadalene	0.3
67	1722	Eudesma-4(15),7-dien-1-beta-ol	0.1
68	1728	Muurol-5-en-4-one, cis-14-nor	1.1
		Total	96.4

[a] K.I. = Kovats Index on HP-5ms column in reference to *n*-alkanes.
[b] tr; Trace ⩽ 0.07%.

3.2. Antioxidant activity

As shown in Fig. 1, the polar sub-fraction of the methanol extract was able to reduce the stable radical, DPPH· to the yellow-coloured diphenylpicrylhydrazine with an IC_{50} value of 63.2 ± 1.75 μg/ml, followed by the non-polar sub-fraction 102 ± 2.14 μg/ml. Of all samples studied, the essential oil had the strongest free radical-scavenging activity with an IC_{50}value of 42.8 ± 2.19 μg/ml. This essential oil concentration is far below those of some essential oils previously reported as antioxidants (Burits, Asres, & Bucar, 2001). The concentration of the positive control (BHT) required to scavenge 50% of the free radical (IC_{50}) was 18.0 ± 0.40 μg/ml. On the other hand, extracts prepared with the solvents hexane and dichloromethane exhibited moderate radical-scavenging activity when compared with those of others given above (162 ± 1.73 μg/ml and 146 ± 1.26 μg/ml, respectively).

In the case of the linoleic acid system, in general, all polar extracts seem to inhibit the oxidation of linoleic acid and that is an important issue in food processing and preservation (Fig. 2). Antioxidants minimize the oxidation of lipid components in cell membranes or inhibit the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation that are known to be carcinogenic. In general, a similar activity pattern to that seen in the first system was observed. Among the

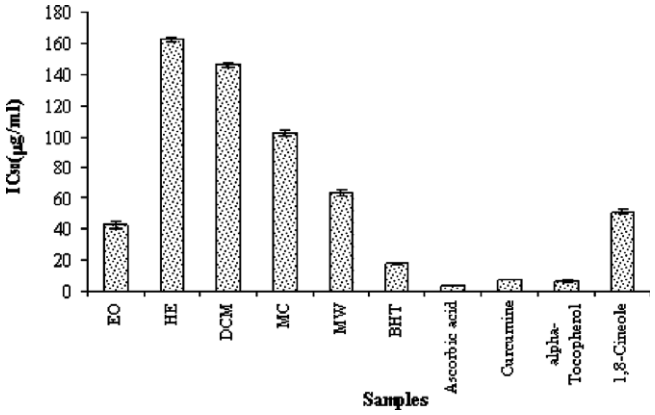

Fig. 1. Free radical-scavenging capacities of the extracts measured in DPPH assay (Results are means of three different experiments. Abbreviations: EO, essential oil; HE, hexane; DCM, dichloromethane; MC, methanol chloroform-non-polar sub-fraction; MW, methanol water-polar sub-fraction; BHT, butylated hydroxyl-toluene).

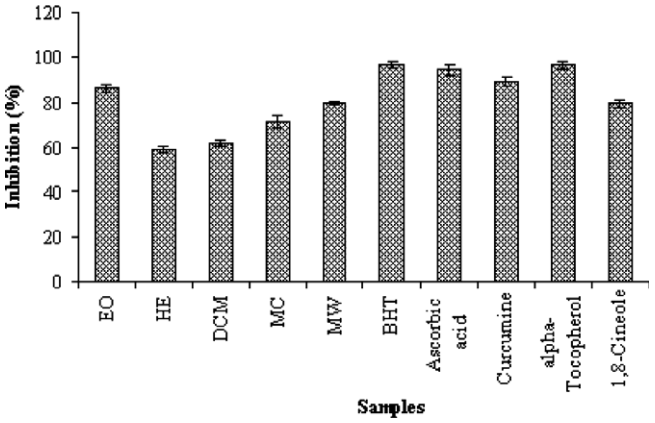

Fig. 2. Inhibition ratio of the linoleic acid oxidation by the extracts (Results are means of three different experiments. Abbreviations: EO, essential oil; HE, hexane; DCM, dichloromethane; MC, methanol chloroform-non-polar sub-fraction; MW, methanol water-polar sub-fraction; BHT, butylated hydroxyl-toluene).

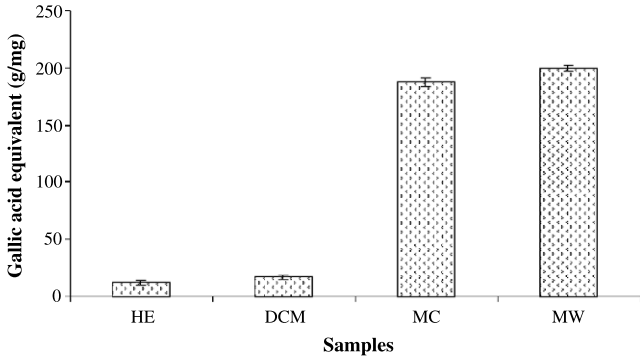

Fig. 3. Amounts of total phenolic compounds in *N. flavida* extracts (Results are means of three different experiments. Abbreviations: HE, hexane; DCM, dichloromethane; MC, methanol chloroform-non-polar sub-fraction; MW, methanol water-polar sub-fraction).

extracts prepared with various solvents, the strongest effect was supplied by the polar methanol sub-fraction (79.7 ± 0.89%). This activity was followed by the non-polar one (MC) with a percentage of 71.8 ± 2.82%. Inhibition capacity of the essential oil of *N. flavida* against linoleic acid oxidation was 86.3 ± 1.69%, which is the highest value for this system. Hexane and dichloromethane extracts showed moderate activities with percentages of 59.3 ± 1.44% and 62.1 ± 1.26%, respectively.

There are very many reports in the literature concerning the biological activities of the members of *Nepeta* genus. On the other hand, we could find only one report dealing with the antioxidant activity of *N. cataria* (Dapkevicius et al., 1998). From this point of view, antioxidant capacities of the members of this genus need a comprehensive evaluation.

As can be seen from Table 1, major compounds of the essential oil of *N. flavida* were determined to be 1,8-cineole and linalool. Therefore, we evaluated the antioxidant potentials of these compounds. The major compound, 1,8-cineole, showed remarkable activity in both systems, while linalool was almost inactive. As far as our literature survey could ascertain, some plant essential oils have proved to have various biological effects, including antioxidant activity due to the presence of 1,8-cineole (Grierson & Afolayan, 2005; Soylu, Soylu, & Kurt, 2006; Yuan, Wahlqvist, He, Yang, & Li, 1996). Moreover, antioxidant activity of this compound has been reported previously (Unlu et al., 2002).

It is well known that extracts of the plants are rich in phenolic compounds, especially terpenoids and phenolic acids. Based on the literature data, some common phytochemicals, such as isopimarane derivatives, iridoid and eugenol glycosides, abiatene diterpenes, nepetanudosides and coleons from the genus *Nepeta*, have been identified (Bhandari, Mathela, Beauchamp, Battini, & Dev, 1993; Fraga, Hernandez, Mestres, & Arteaga, 1998; Fraga, Mestres, Diaz, & Arteaga, 1994; Hussein, Rodriguez, Martinez-Alcazar, & Cano, 1999; Khalil, Gedara, Lahloub, Halim, & Voehler, 1997; Takeda et al., 1998; Takeda et al., 1996).

According to our literature survey, abiatene diterpenes and iridoid glycosides have proved to have antimicrobial (Gaspar-Marques, Rijo, Simeoes, Duarte, & Rodriguez, 2006), anti-cancer (Marques et al., 2002), antinociceptive and anti-inflammatory effects (Choi et al., 2005). No evidence was found for the biological effects of other compounds from *Nepeta* species. Additionally, antioxidative effects of iridoid glycosides have been reported previously by Tanaka, Nishikawa, and Ishimaru (2003).

3.3. Amount of total phenolics

Based on the absorbance values of the various extract solutions, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid equivalents, as described above, total phenolics are shown in Fig. 3. The amount of total phenolics was highest in the polar sub-fraction of the methanol extract (MW) (200 μg/mg), followed by the non-polar sub-fraction (MC) (188 μg/mg). The lowest value was exhibited by the hexane extract (HE) (11.9 μg/mg).

Indeed, when the results given in Figs. 1–3 are compared, it is seen that the phenolic content was high in polar extracts. It seems clear that presence of polar phenolics is fundamental in the evaluation of free radical-scavenging. Besides, the highest activity, seen for the polar sub-fraction of the methanol extract, reflects the radical-scavenging characteristics of these phenolics. The key role of phenolic compounds as scavengers of free radicals is emphasised in several reports (Madsen, Nielsen, Bertelsen, & Skibsted, 1996; Moller, Madsen, Altonen, & Skibsted, 1999). Moreover, radical-scavenging activity is one of various mechanisms contributing to overall activity, thereby creating synergistic effects. On the other hand, total antioxidant activities of the non-polar extracts (HE, DCM, MC) could also be attributed to the volatile components, since they are still present in these extracts.

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