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Research Article

Optimisation Of Solvent Extraction Of Antioxidants (Phenolic Compounds) From Algerian Mint (*Mentha spicata* L.)

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ABSTRACT: **Introduction:** The mints are herbs rich in phenolic compounds that appear to be capable of preventing many diseases. This study allowed for quantifation of the levels of total phenolics and flavonoids from *Mentha spicata* L. harvested in Bejaia (Algeria) using pure solvents (methanol, ethanol, acetone) and their aqueous mixtures at 50 and 75%. Furthermore, the antioxidant levels were evaluated by two methods. **Materials and methods:** The total phenolics content (TPC) was evaluated by the Folin-Ciocalteu method. Total Flavonoids content (TFC) was determined by aluminium chloride method Radical scavenging activity (RSA) and total antioxidant activity (TAA) of the extracts were determined by standard methods. **Results and conclusion:** The solvents ethanol and acetone (at 75%) gave the best extraction rates for *Mentha spicata* (20.02 and 20.20% respectively). The ethanol extract (at 50%) presented the highest TPC (39.47 ± 1.81 mg EAG/g DW). Regarding TFC, acetone 75% was the best extractor (7.68 ± 0 .02 mg EQ/g DW). The acetonic extract at 50% manifests the highest RSA, whereas, the TAA was attributed to the acetonic (0.23 ± 0.01 AU) and ethanolic extracts (0.22 ± 0.02 AU) without significant difference. The variation in the antioxidant capacity between extracts was probably due to the difference of the nature of the compounds extracted with different solvents.

KEYWORDS: spearmint, total antioxidant activity, solvent extraction, flavonoids, total phenolics, radical scavenging activity.

INTRODUCTION

Spearmint (*Mentha spicata* L.) is a long-used medicinal herb found in many African countries including Algeria. *M. spicata* (synonymous of *M. viridis Linn.*) is a herbaceous perennial with a pungent smell. It is commonly used as a herbal tea, flavoring agent, and as a medicinal plant.[1] *Its* leaves are generally given for fever and bronchitis and its decoction is used as lotion in aphthae. The herb is considered to have stimulant, carminative, antispasmodic, stomachic and diuretic properties. It is also used for gas pain, rheumatism, toothache, muscle pain and as a mouth wash.[2] It is cultivated all over Algeria for culinary purposes and to treat gastric troubles.[3]

The role of the phenolics and flavonoids as natural antioxidants and free radical scavengers has attracted considerable recent interest due to their pharmacological behavior.[4] According to Mata et al. (2007)[5], extracts of *M. spicata* showed high antioxidant activities. The phenolic compounds found naturally in Mentha were suggested to be the major contributors to the antioxidant activities of the plant. However, the extraction method of phenolic compounds differs from plant to plant and an ideal extraction method for a particular phenolic source has to be individually designed and optimized.[6]

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The aim of an extraction process should be, of course, to provide for the maximum yield of substances and of the highest quality (concentration of target compounds and antioxidant power of the extracts).[7] Extraction efficiency is influenced by various factors such as method of extraction, solvent type, solvent concentration, contact time, extraction temperature, solid to solvent ratio and particle size.[8,9] Nevertheless, solvent type has a major importance in extraction efficiency. Solvent extraction is frequently used for isolation of antioxidant and extraction yield is dependent on the solvent and method of extraction, due to the different antioxidant potentials of compounds with different polarity.[10] The most broadly applied extraction procedure is solvent extraction using, extractants such as methanol, ethanol and acetone or mixtures of these with water for the recovery of a wide range of polyphenols of diverse phenolic structures.[11] Furthermore, the use of water in combination with other organic solvents contributes to the creation of a moderately polar medium that ensures the extraction of polyphenols.[8]

An optimisation study for phenolic compound extraction from spearmint has not previously been reported. Consequently, the effects of solvent type on the extraction efficiency of phenolic compounds from spearmint needed to be investigated. It is also necessary to extract polyphenolic compounds effectively when antioxidant activities are measured. Moreover, an optimum extraction method for phenolic compounds is utmost important from the pharmaceutical and industrial point of view.

In this context, we chose nine different polar solvents to extract the bioactive compounds from spearmint for the quantification of phenolic compounds and measurement of antioxidant activity. As the polarities of antioxidant components from individual samples are likely to be different, the choice of extraction solvents is critical. Furthermore, solvent extraction is frequently used for isolation of antioxidants and the antioxidant chemical activity of extracts is strongly dependent on the solvent due to the different antioxidant potentials of compounds with different polarity.[12] The main objectives of this study were (i) to examine the efficiency of different solvents for the extraction of phenolics from spearmint using methanol, ethanol, acetone, acetone-water, methanol-water and ethanol-water system (1:1 and 3:1 v/v). (ii) to determine the total phenolic and flavonoids contents and to assess their relation with antioxidant capacity of spearmint (*Mentha spicata* L.) in these various extracts in order to choose the optimal extraction conditions.

MATERIALS AND METHODS

Plant collection

The *M. spicata* plant material was collected in August 2009 from Tichy: Lat: 36° 40' N, Long 5°10' E, Bejaia (Algeria) and was then identified by its vernacular name and later validated by Professor J. Lejoly in the Laboratory of Systematical Botany and Phytosociology, Free University of Brussels (ULB), Belgium. A voucher specimen was deposited in the Herbarium of the National Botanical Garden of Meise (Belgium) under the number BR 0000006946227.

Extraction solvent

The following solvents were trialed to test their ability to extract antioxidant phenolics from leaves of *M. spicata*: ethanol, methanol, acetone and their mixture with water. All solvents and chemicals used were of analytical grade. and obtained from either Sigma–Aldrich or Merck. The solvent extraction procedure was carried out according to the extraction procedures described by Soares et al. (2009).[13] A required amount (5g) of mint (*Mentha spicata* L.) dry powder was weighed accurately using analytical balance (Radwag, Poland) and each sample was mixed with 100 mL of methanol, ethanol, acetone, aqueous methanol (50 and 75%), aqueous ethanol (50 and 75%) and aqueous acetone (50 and 75%) to investigate the effect of solvent on the content of phenolic compounds in a conical flask (which was wrapped with parafilm and aluminium foil to prevent spilling of mixture and light exposure) respectively. The mixture was then shaken for 24 h at ambient temperature. After extraction, the mint (*M. spicata*) extract was filtered and the filtrate was evaporated to dryness under vacuum in a rotary evaporator (Buchi R 210, Switzerland) at 40°C.

Determination of total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu (F-C) colorimetric method described [14] with slight modifications. The crude extracts obtained from extraction were diluted before use. A volume of 100 µL diluted crude extract was added to 6 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent (FCR) was subsequently added. After 4 min, 1.5 mL of sodium carbonate (20%, w/v) was added and the final volume was made up to 10 mL with deionized water. The test tube was then vortexed (EV-102, tehtnica zelezniki, Germany) for 10 s, followed by a 2 hour incubation at 25°C in a dark environment. The absorbance was measured at 765 nm against the blank reagent using Uvi light spectrophotometer (SpectroScan 50, United Kingdom). The measurements were carried out in triplicate. Gallic acid was used for calibration of a

standard curve. The results were expressed as gallic acid equivalent (GAE) in mg/ g dry weight (DW) of sample.

Determination of total flavonoids content (TFC)

TFC was determined using standard procedures.[15] Briefly, the crude extract was diluted and 1.5 mL of 2% (w/v) aluminium chloride ($AlCl_3$) was added to 1.5 mL of diluted crude extract or quercetin (positive control) and then mixed using vortex mixer (EV-102, tehtnica zelezniki, Germany) for approximately 10 s. The mixture was allowed to stand for 15 min. Absorbance of the mixture was determined at 430 nm versus the prepared blank using Uvi light spectrophotometer (SpectroScan 50, United Kingdom). Total flavonoid content was expressed as mg quercetin equivalent (QE)/g dry weight of sample (mg QE/g DW). Samples were measured in triplicate.

Antioxidant assays

DPPH radical scavenging activity assay (RSA)

The DPPH assay was performed as previously described[16] with minor modifications. The DPPH solution (0.1 mM) was prepared and a 2 mL aliquot of this solution was mixed with 200 µL of the extract. The solution was shaken well and incubated in the dark for 30 min at room temperature. The absorbance was subsequently recorded at 517 nm. The scavenging activity was estimated based on the percentage of DPPH radical scavenged using the following equation:

$$\text{Scavenging effect \%} = [(\text{control absorbance} - \text{sample absorbance})/(\text{control absorbance})] \times 100.$$

The DPPH radical scavenging activity of ascorbic acid and α-tocopherol were also assayed for comparison. All tests were performed in triplicate.

Phosphomolybdate assay (TAA)

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method.[17] Briefly, a 0.1 mL of sample aliquot was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity was expressed as the absorbance of the sample. The antioxidant activity of ascorbic acid and α-tocopherol were also assayed for comparison.

Statistical analysis and Experimental design

All assays were carried out in triplicates and results are expressed as mean ± SD. One way ANOVA testing was used to analyze statistical differences amongst the various extracts for phenolic compound contents and different antioxidant assays with least significance difference (LSD) ($P < 0.05$) as a level of significance. Correlations between content of components and antioxidant attributes were determined by linear regression analysis employing Microsoft excel software.

For the optimization of extraction procedure, the influence of the process solvent (methanol (50 – 100% v/v), ethanol (50 – 100% v/v), acetone (50 – 100% v/v)) was separately investigated in experiment. The prediction profiler plot was established to determine the optimal extraction conditions for the maximum yield (%), total phenolic compounds, flavonoids contents and antioxidant activity. The results were fitted by first order polynomial equation. JMP software (version7.0, SAS) was used for the development and evaluation of the results of the experimental design.

RESULTS

Extraction yield, total phenolics and flavonoids contents

The percentage yield was obtained from this formula:

$$(W_2 - W_1/W_0) \times 100$$

Where, W_2 is the weight of the extract and the container, W_1 is the weight of the container alone and W_0 is the weight of the initial dried sample.

The percentage yields of the extracts of *M. spicata* are shown in Table 1. The extraction yield of these samples varied from 2.6% to 20.2% weight extracted/weight of original dried plant material with a descending order of acetone (75%) > ethanol (75%) > methanol (75%) > ethanol (50%) > methanol (50%) > acetone (50%) > methanol > ethanol > acetone. Thus extraction with hydro-alcoholic solvents resulted in the highest amount of total extractable compounds, whereas the extraction yield with methanol, ethanol and acetone extracted less material in comparison with the other solvents.

Phenolics or polyphenols are plant secondary metabolites and are very important by virtue of their antioxidant activity by chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversions into reactive oxyradicals.[18] Figure 1 summarizes the total phenolic compounds in the extracts (expressed as gallic acid equivalents (GAE)). These varied between 5.91 ± 0.12 mg and 39.47 ± 1.81 mg/g dry weight of extract. The ethanolic extract at 50% exhibited the highest TPC

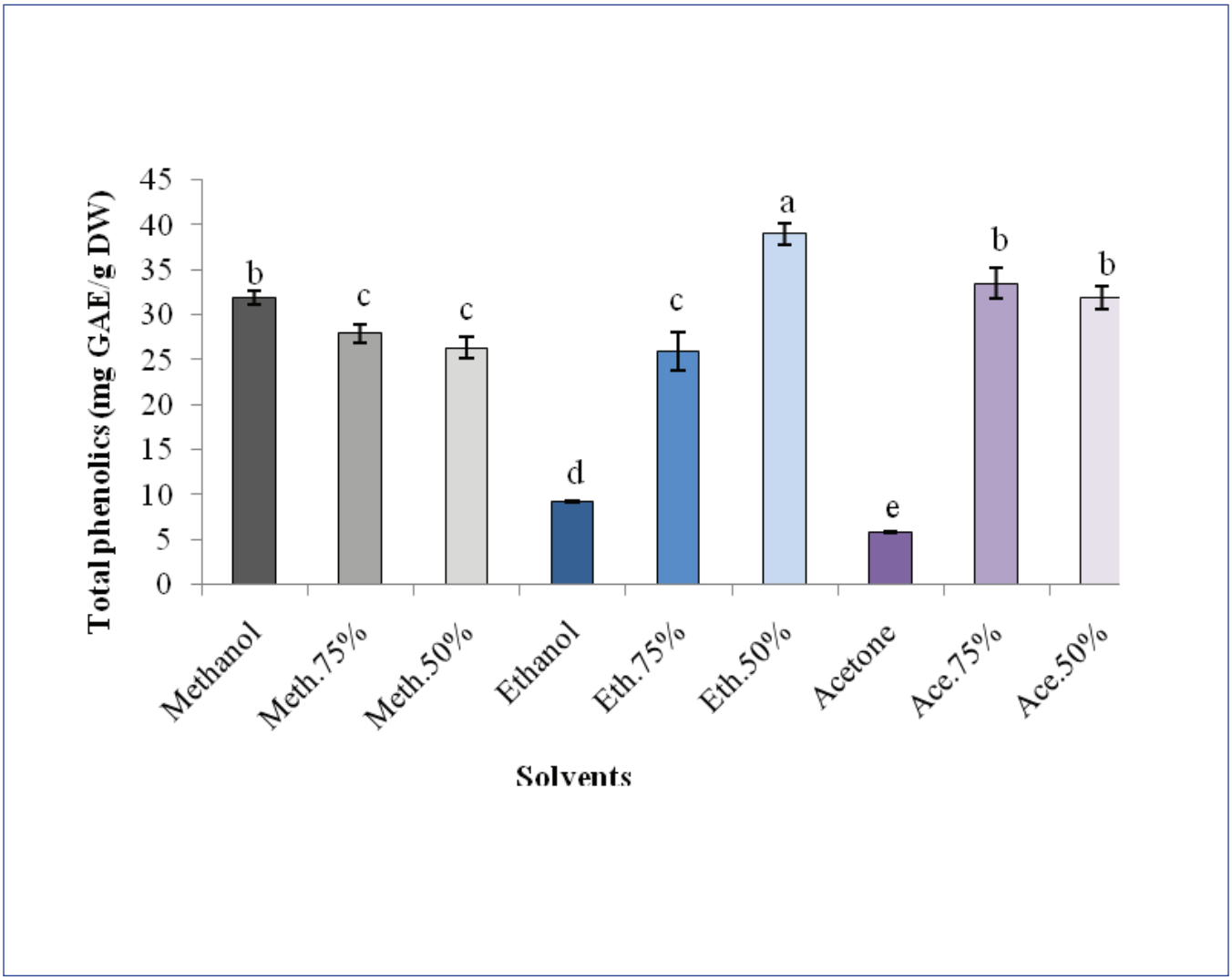

Figure 1: Influence of solvent on extraction efficiency of phenolics from *M. spicata* leaves. Values are expressed as the mean ± SD of three determinations. Values marked by the same letter are not significantly different ($p < 0.05$) from each other. DW = dry weigh; GAE = gallic acid equivalent.

Table 1: Solvent effects on the yield of *Mentha spicata* L.extraction

Extraction solvent	Snyder's solvent polarity index[a]	Extract yield (% g/g sample)
Organic		
Methanol	6.6	11.6
Ethanol	5.2	4.6
Acetone	5.4	2.6
Aqueous		
75% methanol (75:25 v/v methanol–water)	7.2	19.42
75% ethanol (75:25 v/v ethanol–water)	6.15	20.02
75% acetone (75:25 v/v acetone–water)	6.3	20.2
50% methanol (50:50 v/v methanol–water)	7.8	13
50% ethanol (50:50 v/v ethanol–water)	7.1	14.1
50% acetone (50:50 v/v acetone–water)	7.2	12.5
Water		9.0

[a] Snyder's solvent polarity index cited from Markom et al. (2007).[22]The aqueous solvent mixture indexes were calculated from equation $(I_A/100\times P_A) + (I_B/100\times P_B)$ where I_A and I_B are polarity index of solvents A and B, respectively, and *P*A and *P*B are percentage of solvents A and B, respectively, in the solvent mixture.

(39.47 ± 1.81 mg GAE /g DW), whereas the content obtained with acetone was much smaller ($P < 0.05$) (5.91 ± 0.12 mg GAE /g DW).

The TFC expressed as quercetin equivalents, varied from 1.98 ± 0.22 to 7.68 ± 0.02 mg quercetin equivalent/g dry weight. The acetonic (75%) extract showed the highest TFC (7.68 ± 0.02) and the methanolic at 50% the lowest (1.98±0.22) with significant difference at $p < 0.05$ (Figure 2).

Antioxidant activity

DPPH radical scavenging activity

Figure 3 shows that the DPPH radical scavenging ability of samples can be ranked as ascorbic acid > α-tocopherol > acetone (50%) > ethanol (50%) > methanol > ethanol > acetone (75%) > acetone > methanol (50%) > ethanol (75%) > methanol (75%).

Ascorbic acid showed the highest percentage DPPH radical scavenging activity with a significant difference at $p < 0.05$ (82.98 ± 3.17%). The percentage of scavenging DPPH radical for the α-tocopherol, 50% acetone, 50% ethanol and methanol extracts were 34.54± 0.78, 30.41 ± 2.92, 26.69 ± 2.05 and 26. 42 ± 0.62 % , respectively. The ethanol, acetone (75%), acetone, methanol (50%), ethanol (75%) and methanol (75%) extracts had percentages of 21.88 ± 1.81, 20.56 ± 0.05, 19.92 ± 0.78, 19.20 ± 2.33, 16. 91± 1.75 and 15.31 ± 0.80 % respectively.

Phosphomolybdate assay (TAA)

The total antioxidant capacity of standards and extracts of *M. spicata* can be ranked in the order ascorbic acid > α-tocopherol > acetone > ethanol > methanol > aqueous ethanol (50%) > aqueous acetone (50%) > aqueous methanol (50%) > aqueous methanol (75%) > aqueous acetone (75%) > aqueous ethanol (75%) extracts (Figure 4).The absorbance values of the antioxidant capacity for acetone, ethanol and methanol extracts were 0.23 ± 0.01, 0.22 ± 0.02 and 0.18 ± 0.01 AU, respectively, while for the aqueous ethanol, acetone and methanol they were significantly lower. However, the total antioxidant activity of all the extracts were found to be much low ($P < 0.05$) when compared to ascorbic acid (0.68 ± 0.07 AU).

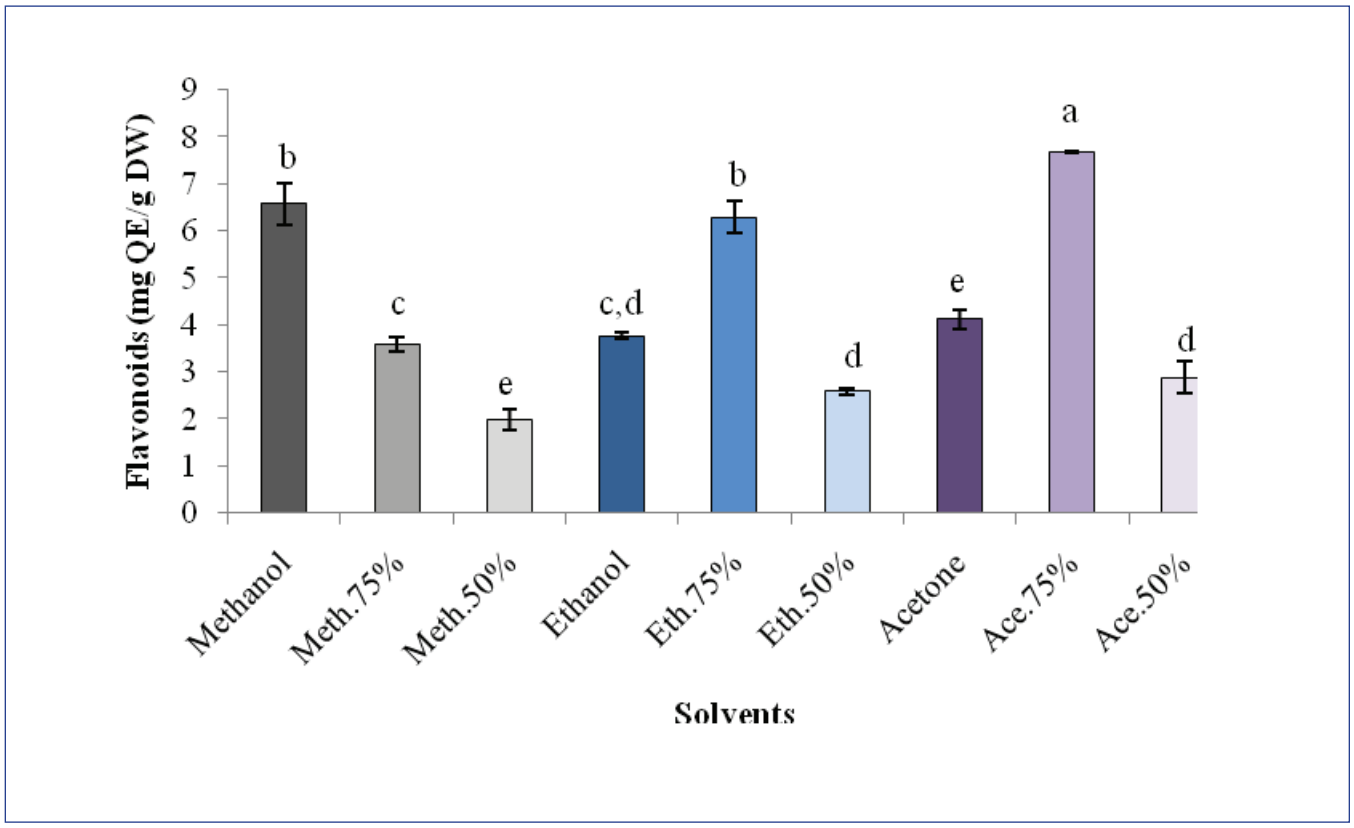

Figure 2: Influence of solvent on extraction efficiency of flavonoids from *M. spicata* leaves. Values are expressed as the mean ± SD of three determinations Values marked by the same letter, are not significantly different ($p < 0.05$) from each other. DW = dry weight; QE = quercetin equivalent.

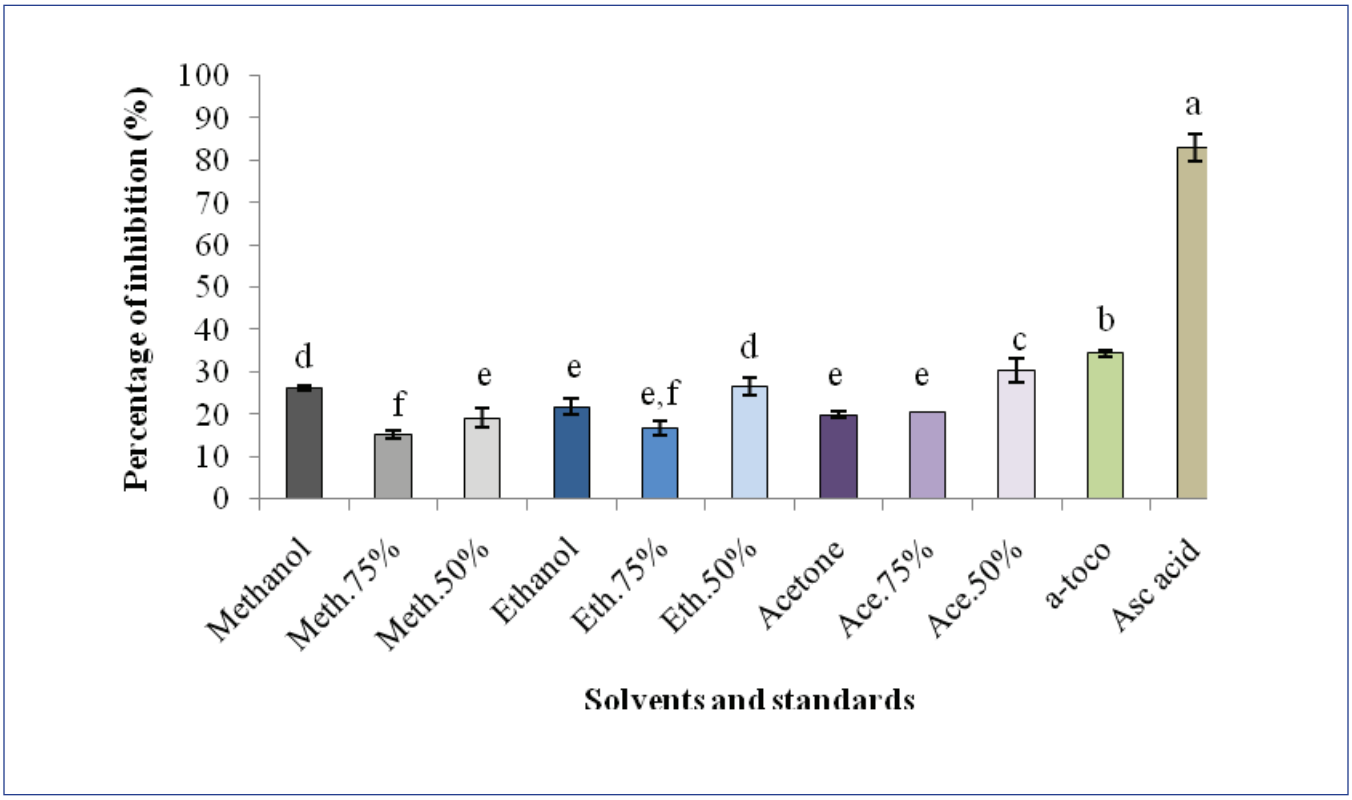

Figure 3: Radical scavenging activity (RSA) of different mint extracts and standards. Values are expressed as the mean ± SD of three determinations Values marked by the same letter, are not significantly different ($p < 0.05$) from each other. α-toco = α-tocopherol; Asc acid = ascorbic acid.

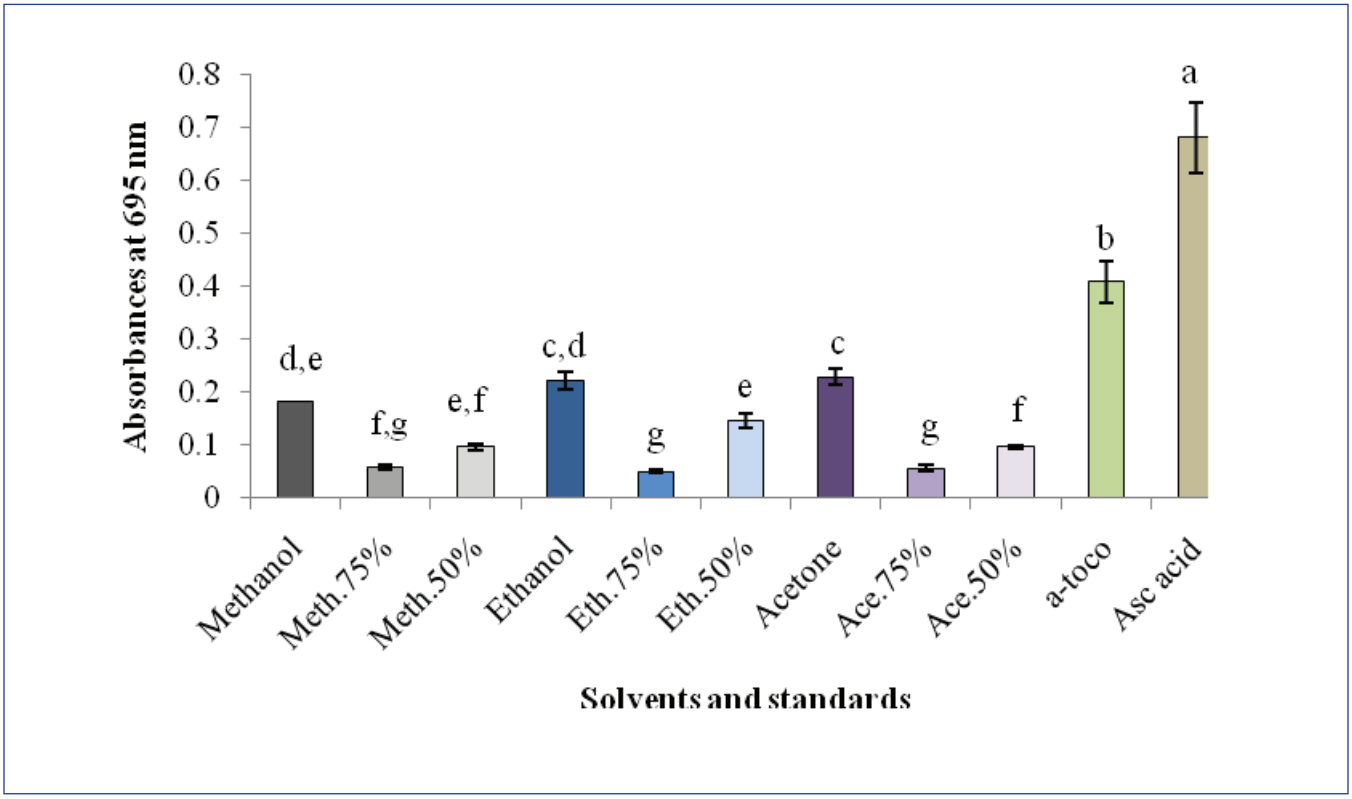

Figure 4: Total antioxidant activity (TAA) of different mint extracts and standards. Values are expressed as the mean ± SD of three determinations, Values marked by the same letter, are not significantly different ($p < 0.05$) from each other . α-toco = α-tocopherol; Asc acid = ascorbic acid.

Correlation between antioxidant activities and phytochemical contents for different solvents extractions

Correlation analysis for phytochemical contents with antioxidant activity of various extracts of *M. spicata* showed that the contents of phenolics and flavonoids exhibited good correlation with DPPH and TAA only for some extracts: pure methanol (TPC with TAA and TFC with TAA), pure ethanol (TPC with RSA and TFC with TAA), pure acetone (TFC with RSA), aqueous methanol (75%) (TFC with RSA), and aqueous ethanol (50%) (TFC with TAA (Figure 5).

Optimization of extraction solvents

According to Figure 6, methanol showed the highest predicted values for yield: 13.97%, TPC: 31.51 mg GAE/g DW, TFC: 6.35 mg QE/g DW , RSA: 23.92%. Besides, total phenolics content, flavonoid content and the capacity to scavenge DPPH increases by increasing the methanol concentration in the mixture (methanol-water). For the ethanol and acetone, results were respectively: yields: 8.16, 6.82%, TPC: 9.88, 10.78 mg GAE/g DW, TFC: 4.82, 5.52 mg QE/g DW, RSA: 19.42, 18.39% and TAA: 0.18, 0.19 AU.

DISCUSSION

Extraction yields

Extraction is the first step in the isolation of phenolic compounds from plant materials. Extraction is influenced by the chemical nature of the compounds (simple and complex phenolics), the extraction method employed, the storage time and conditions, and the presence of interfering substances. Solvent extraction is a process designed to separate soluble phenolic compounds by diffusion from a solid matrix (plant tissue) using a liquid matrix (solvent). This process is widely employed for phenolic extraction from various vegetable materials.[8] The effects of different extracting solvents have been tested for the extraction of polyphenols from plant material. It is well known that the yield of chemical extraction depends on the type of solvents with varying polarities, pH, extraction time and temperature, as well as on the chemical compositions of the sample. Under the same conditions of time and temperature, the solvent and the chemical properties of the sample are two most important factors.[19] So, processing efficiency is quantitatively related to extraction yield.[20]

Variation in the yields of various extracts is attributed to polarities of different compounds present in the plant and such differences have been reported in literature.[21] In our study, different solvents with different polarities were used to determine which gave the greatest recovery of phenolic compounds. Nine solvents were tested: (1) pure methanol, (2) methanol (75%), (3) methanol (50%), (4) pure ethanol, (5) ethanol (75%), (6) ethanol (50%), (7) pure acetone, (8) pure acetone (75%) and (9) acetone (50%). The extraction yield is dependent on the nature of the solvent used. Based on our results reported here, the highest extraction yield was found with hydro-alcoholic solvents. This indicates that most of the components in *M. spicata* are hydrophilic or water-soluble. The extract yield increases with the solvent polarity. The addition of water into acetone and ethanol tremendously increases the extract yield. It was also found that the highest yield can be achieved at 75% solvent. These yields were higher than those seen using pure solvents, and slightly higher than by using solvent at 50% since both the polar and less polar compounds were co-extracted together.[22]

Alcoholic solvents have been commonly employed to extract phenolics from natural sources where they gave quite a high yield of total extract even though they are not highly selective for phenols. Mixtures of alcohols and water have revealed to be more efficient in extracting phenolic constituents than compared to mono-component solvent system.[7] Addition of small quantity of water to organic solvent usually creates a more polar medium which facilitates the extraction of polyphenols as suggested by Spigno et al.[7] By increasing the proportion of water, the polarity of the solvent also increases. When this is achieved, the solvent system is able to extract phenolic substances from both ends of the polarity range (highest polarity substances and low polarity substances), as well as those of moderate polarity.[23]

Similar results were reported previously.[24] Previous reports studied extracts from defatted wheat germ and noted that the yield of 50% ethanol extract did not show significant difference with the 30% and 70% ethanol extracts; but the yield of the aqueous extract was significantly ($P < 0.05$) different from the 100 % ethanol extract. Moreover, these results agree with further studies which reported that aqueous ethanol (50%) showed the highest yield in extraction of phenolic compounds from *Phyllanthus niruri*.[22] This is followed by methanol, ethanol and acetone respectively. Bimakr et al. [25] have used different methods for the extraction of major bioactive flavonoid compounds from spearmint (*Mentha spicata* L.) leaves. They found that the highest extraction yield (267.3 mg/g) was found with methanolic extract, which was followed by ethanolic (70%) one (257.6 mg/g).

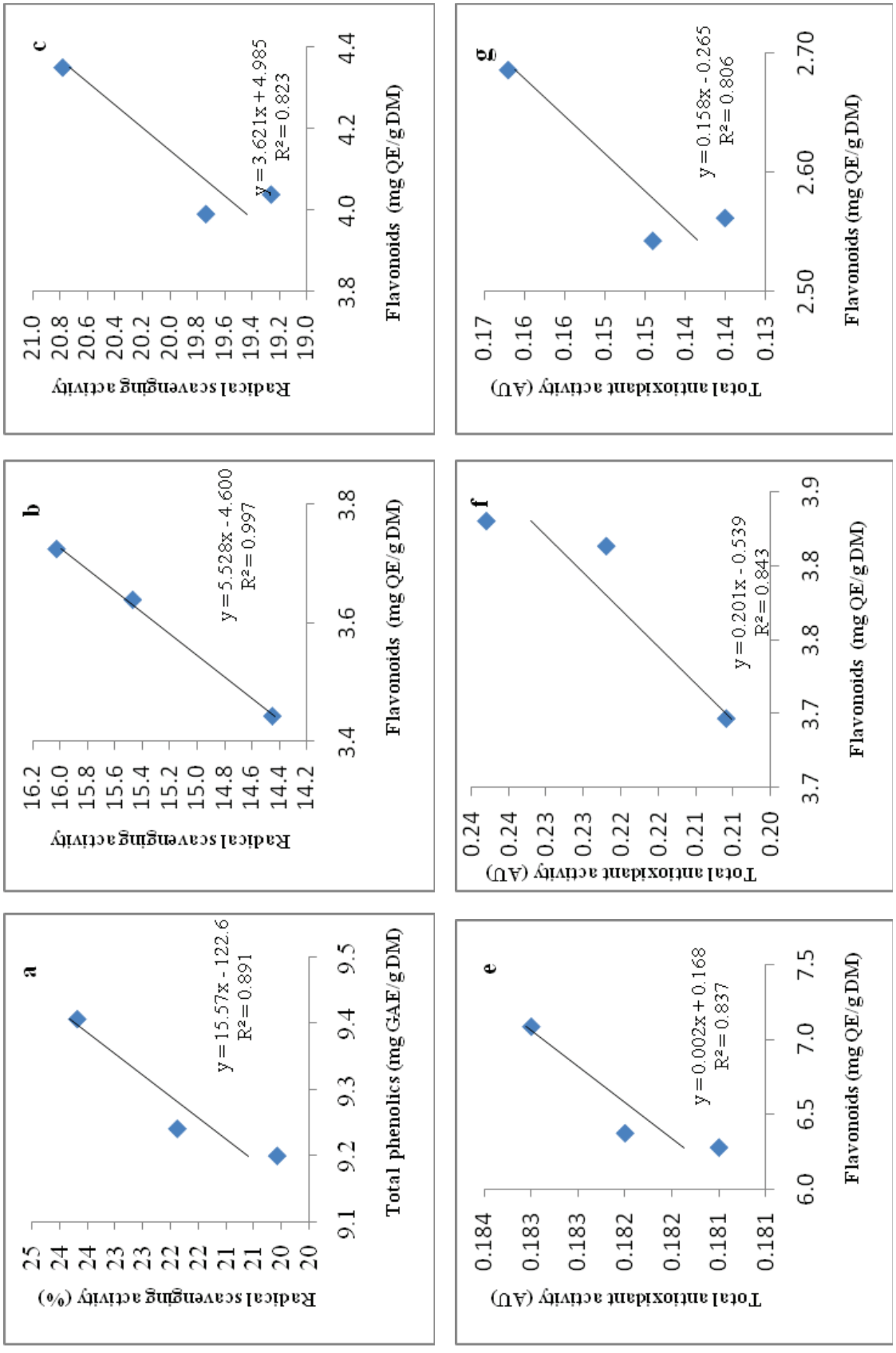

Figure 5: Correlation analysis between mint total phenolics and flavonoids and their antioxidant activity (RSA and TAA): (a) Ethanol extract; (b) Methanol (75 %) extract; (c) Acetone extract; (d) Methanol extract; (e) Methanol; (f) Ethanol and (g) Ethanol (50%) extract.

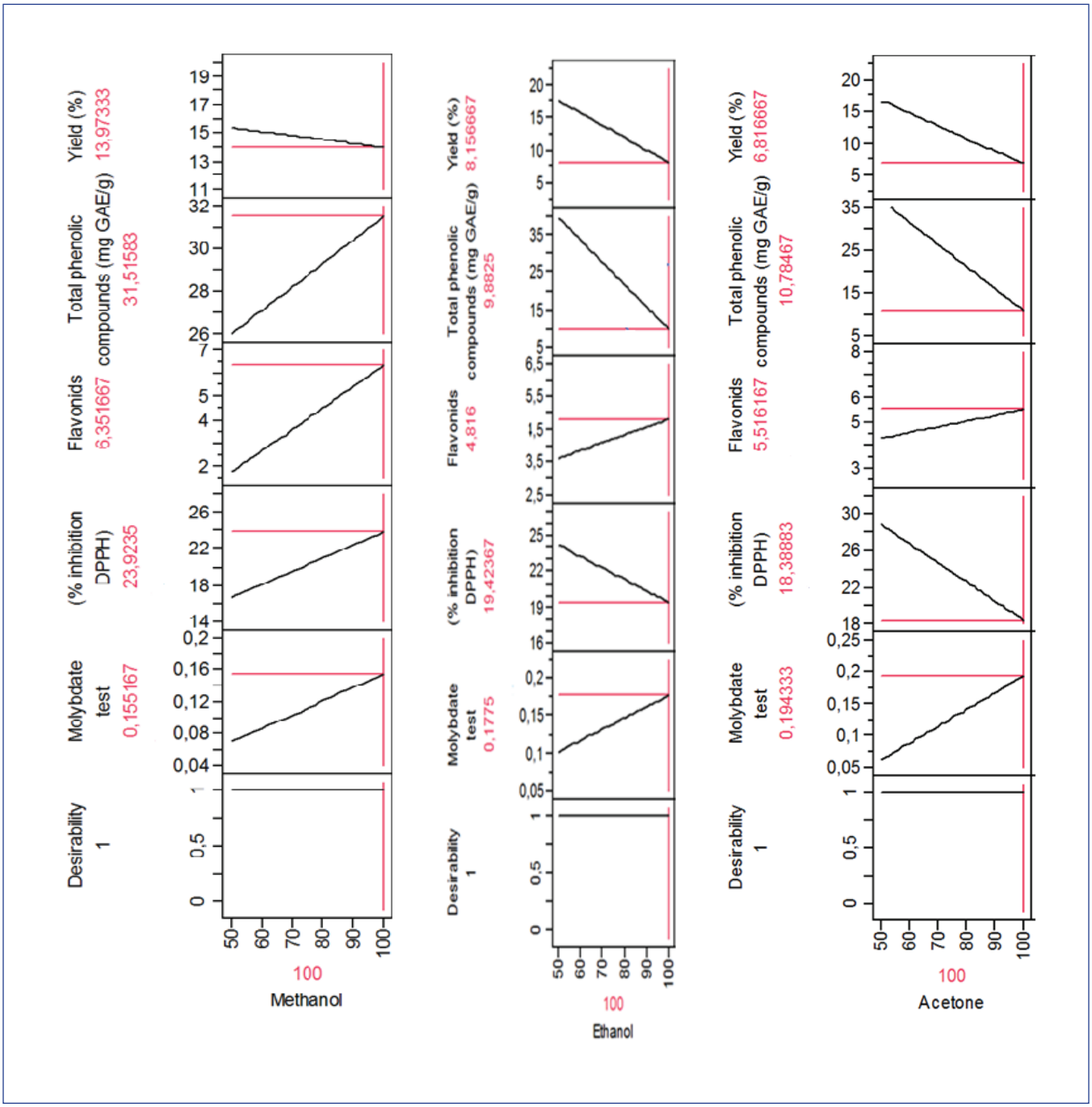

Figure 6: Prediction profiler plot showing the effect of methanol, ethanol and acetone concentrations on the yield, TPC, TFC, RSA and TAA from *Mentha spicata* L.

The lowest extraction yield was obtained by using petroleum ether (30.4 mg/g), suggesting that polar compounds in the plant matrix would be easier to extract with a more polar solvent while lower polarity solvents enable to obtain the extracts with higher concentration of bioactive compounds.[25]

Thus solvent polarity plays an important role in this and other extraction studies. The net molecular polarities of solvents are measured by their dipole moments. The polarities of solvents used are listed in Table 1. Nevertheless, the separation of components by solvents depends on the polarity of both the solvent and the component. According to Markom et al.,[22] a single solvent might or might not be selective for the separation of two components as shown in Figure 7. For example, if the desired compound *x* is non-polar, it can be selectively extracted using solvent with polarity *P'1*. On the other

hand, if the more polar compound *y* is desired, *P'x* solvent can be used to remove compound *x* first, followed by solvent *P'2* to extract compound *y*. However, for plant materials that consist of multi-components with complex interactions, 100% recovery of individual component is probably not achieved since a single solvent may not be selective for a single compound.[22]

When we compared between absolute solvents, methanol was the more efficient. The phenolic compounds in the extract are more often associated with other biomolecules (proteins, polysaccharides, terpenes, chlorophyll, lipids and inorganic compounds) and a solvent must be found that is suitable for extracting them. The extraction of other biomolecules probably could explain the very high percentage yield obtained in the methanol extract of this study which was higher than ethanol, acetone. The high percentage yield of the methanol extract in this study could be due to the ability of these solvents to dissolve endogenouse compounds together with the phenols.[26] Moreover, methanol has been reported to be the most suitable solvent in the extraction of polyphenolic compounds from plant tissue due to its ability to inhibit the action of polyphenol oxidase that causes the oxidation of polyphenols.[26]

Effect of extraction solvent on total phenolic and flavonoids extractants

Methanol, ethanol and acetone separately or mixed with water are commonly used to extract phenolic compounds from sample.[23] The ability of different solvents in extracting phenolic compounds was compared by performing Folin-Ciocalteu assay method. The results were expressed as gallic acid equivalents (mg GAE/g dry weight of spearmint leaves). Data show that extract of phenolic compounds is significantly affected by the extractant used. The concentration of polyphenols was determined by means of the Folin-Ciocalteou reagent, made from a mixture of phosphotungstenic and phosphomolybdenic acids. The addition of the Folin-Ciocalteou reagent to the polyphenol solution leads to the formation of chromophore compounds which have a maximum absorbance at wavelengths of 700 nm.[14]

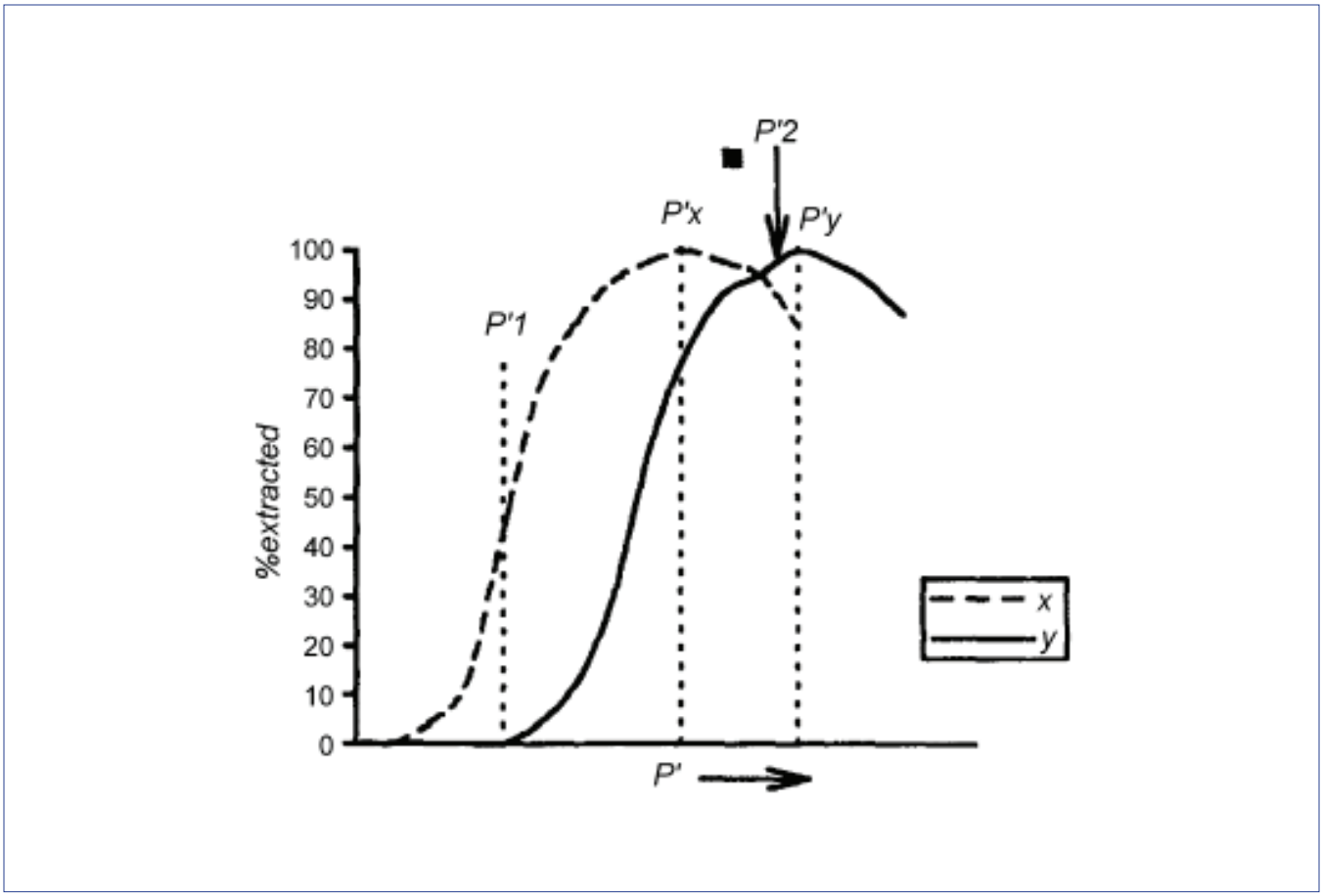

Figure 7: Separation of compound x and y as a function of solvent polarity. P'x = polarity of compound x, P'y = polarity of compound y.[22]

Several studies have shown that polyphenol content differs with different solvent polarities.[27] Hayouni et al.[21] reported that water and organic solvents used individually or in mixture significantly affected total polyphenols contents of *Quercus coccifera* L. and *Juniperus phoenicea* L. fruit extracts. In our study, ethanol-water (50 %) extracted markedly greater amounts of phenolic compounds (39.47 ± 1,81 mg GAE/g DW) compared with pure ethanol and pure acetone which presented the lowest contents (9.28 ± 0.11, 5.90 ± 0.12 mg GAE/g DW respectively). Similar studies have that found that ethanol (50%) was examined the extraction of phenolics from citrus peels.[28] This study reported that the recovery of total phenolics increased with increasing ethanol concentration, until the concentration reached 85%; after which, the recovery reduced with the increase of ethanol concentration.

When we compared between pure solvents, it should be noted that the ability of methanol for extraction of phenolics (31.97 ± 0.76 mg GAE/g DW) was better than ethanol (9.28 ± 0.11 mg GAE/g DW and acetone (5.90 ± 0.12 mg GAE/g DW). Our data are in accordance with those reported previously which has shown that methanol had better recoveries and is specifically effective in extracting polyphenols.[29] A recent report studying the influence of neat solvents on extractability of total phenolics from the black cohosh matrix reported similar results.[30] According to Anokwuru et al.[26] the result of the total phenolic content of *Hibiscus sabdariffa* calyx in different solvents demonstrated that the methanol extract yield was higher than the yields both of ethanol and acetone. Similarly, it has been reported that the methanol extract of *Bridelia Retusa Spreng* Bark contained relatively higher levels of total phenolics than the other extracts.[31] Conversely, the aqueous mixtures of the ethanol and acetone tested in our study showed higher polyphenolic concentrations than the pure solvents. The findings were in accordance with other studies which reported that ethanol and acetone at 50% were the most efficient solvents compared to 100% ethanol and acetone for extracting phenolic compounds from black and black mate tea.[32] In addition, similar results have been reported in studying the phenolic content of selected tropical fruits from Malaysia.[33] Still in this context, Mukhopadhyay et al.[30] in studying the extraction efficiency of black cohosh have examined the aqueous effect by employing ethanol: water mixture (50:50, v/v) and obtained a high total phenolic content value compared with pure ethanol. Furthermore, the results with acetone: water (50:50, % v/v) was found to be similar to that with ethanol: water (50:50, v/v).

Aqueous methanol, due to its polarity, is more effective at extracting polyphenols linked to polar fibrous matrices.[34] In contrast, acetone/water mixtures are more useful for extracting polyphenols from protein matrices since they appear to degrade the polyphenol–protein complexes.[34] In general, ethanol or methanol solutions containing some water, particularly those consisting of 40–80% ethanol or methanol, had greater efficiency in the extraction of polyphenolic compounds compared to water or pure ethanol or methanol.[35] Addition of water is known to cause the plant material to swell thereby allowing the solvent to penetrate more easily in the solid matrix and increase extractability.[30] A literature search was unable to find other studies that compared all the nine solvents used in this study.

Flavonoids are large compounds occurring ubiquitously in foods plants. Many flavonoids are found to be strong antioxidants capable of effectively scavenging reactive oxygen species because of their phenolic hydroxyl groups.[26] In the present investigation, the content of flavonoids in extracts of *M. spicata* obtained from solvents values was experimentally determined. The highest concentration of flavonoids was obtained from aqueous acetone (75%) as solvent (7.68 ± 0.02 mg QE/g DW) and the lowest concentration of 1.98 ± 0.22 mg QE/g DW was observed in the aqueous methanol (50%). Our data are in accordance with those reported in the literature which revealed that acetone and aqueous acetone were the best solvents for the extraction of flavonoids.[26, 33] However, the methanolic extract also allowed the extraction of a considerable amount of flavonoids (6.58 ± 0.44 mg QE/g DW) in our study, which was almost twice as much as the ethanolic solvent. This is in agreement with results reported by in other studies[36] from *Vitis vinifera* wastes extracts. Moreover, our results showed that except of acetone and ethanol at 75% , pure solvents extracts had the highest amount of flavonoids. According to Abaza et al.[29], solvent polarity plays a key role in increasing phenolic solubility. The least polar solvents are considered to be suitable for the extraction of lipophilic phenols. This can also be explicated by the nature of flavonoids present in the plant studied. It might have least polar aglycones like the high mythoxylated isoflavones, flavanones and flavonols that have tendency to be soluble in pure solvents.

Antioxidant activity

Extraction is an important step for obtaining extracts with acceptable yields and strong antioxidant activity.[24] Since the methods used to measure antioxidant activity are extremely dependent on the reaction conditions and

the substrates or products, not all methods yield the same values for the activity.[29] In order to obtain the most relevant data about the antioxidant capacity of *M. spicata* extracts, two different methods were used in this study. Radical scavenging potential of the *M. spicata* extracts was tested by the DPPH method. The proton radical-scavenging action is known to be one of various mechanisms for antioxidation. DPPH is one of the compounds that possess a proton free radical and shows a characteristic absorption at 517 nm (purple). When DPPH encounters proton radical scavengers, its purple color would fade rapidly.[37] The percentage of inhibition of DPPH° within the assay time will reflect the antioxidant capacity of the extract assessed. The assay time would vary from 10–20 min up to about 6 h.[33]

As shown in Figure 3, *M. spicata* extracts are a source of radical scavenging activity (RSA), this activity changed with the solvent used to prepare the extract. Our results showed that the 50% acetone extract exhibited significantly higher RSA than the other solvent systems trialled. Acetone–water mixtures have previously been shown to be good solvent systems for the extraction of polar antioxidants.[33] This is followed by 50% ethanol and pure methanol extracts respectively, without significant differences between them. Similar results have been reported by Alothman et[33] for Thai seedless guava extract. Moreover, the methanolic extract presented higher RSA than the acetonic and ethanolic ones in our study. These results were comparable with those described in the literature for Rio Red grapefruits and Sour orange fruit extracts.[36] Furthermore, methanol extracts have previously been reported to have high RSA, followed by acetone and ethanol extracts of fruit powder of citron and blood orange RSA.[17] It was also shown that methanol extract exhibited maximum radical scavenging activity (92.5%) at 100 ppm concentration, followed by acetone of green coffee extracts.[38]

Phenolic acids and flavonoids have been reported to be the main phytochemicals responsible for the antioxidant capacity of fruits and vegetables. Plant derived polyphenols display characteristic inhibitory patterns toward the oxidative reaction *in vitro* and *in vivo*.[18] In our study, the observed differential scavenging activities of the extracts against the DPPH system may be due to the presence of different compounds in the extracts. The RSA activity of mint extracts studied would depend on the chemical structure of phenolic compounds and the availability of phenolic hydroxyl groups which have the capacity to donate their electron or hydrogen thereby forming stable end product.[35]

The phosphomolybdate method or total antioxidant activity (TAA) has been routinely used to evaluate the antioxidant capacity of extracts. In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green coloured phosphomolybdenum V complex, which shows a maximum absorbance at 695nm.[17, 39] The high absorbance values indicated that the sample possessed significant antioxidant activity. In our study, maximum antioxidant capacities were observed in acetonic and ethanolic extracts without significant difference. This trend was similar to that observed in other studies examining the antioxidant capacity of Rio Red extracts.[35]

Extracts with absolute solvents used exhibited considerably higher total antioxidant activity than those with concentrations of 50% and 75% ones. Variations in antioxidant capacity of different extracts may be attributed to differences in their chemical composition such as phenolic acids, and flavonoids. Polyphenolic and antioxidant index is a combined measure of the quality and quantity of antioxidants in vegetables.[35]

The differential responses between the methods used to evaluate the antioxidant activity in this study may partially be attributed to qualitative variations of the phenolic compounds as the structural differences may modify the antioxidant potential of the phenolic. Furthermore, some of the extracts have hydrophilic and hydrophobic compounds and those samples may not work efficiently in some *in vitro* model systems.[35] Hence, the trend of antioxidant activity of *M. spicata* extracts cannot be compared from one method to another method due to their different mechanisms involved in the assay.

Correlation for different solvents used between antioxidant activities and phytochemical contents

Correlations were tested to link the antioxidant activities measured by the DPPH and phosphomolybdate methods with the phenol and flavonoid contents of different extracts of *M. spicata*. The highest correlation coefficients were found between the RSA and TFC for the methanolic (75%) extract and TAA and TFC for pure methanolic one which were 0.997 and 0.944 respectively (Figure 5). There were also a positive correlation between RSA and TPC, TAA and TFC for the pure ethanolic extract, TAA and TFC for the aqueous ethanolic extract (50%). For the acetone extract, we have only observed a correlation between RSA and TFC. All of these data indicated that phenolic compounds in these *M. spicata* extracts were the major contributors of RSA and TAA. Nickvar et al.[1] showed that there was a positive correlation between anti-DPPH° potency and TPC of the ethanolic extracts

of *Mentha* species. Several studies found also even higher correlations between the RSA and TPC values.[17,35,40] Phenolic compounds have been reported to be responsible for the antioxidant activities of grain, vegetables, and other botanical materials.[41]

In contrast, other extracts have not presented a similar correlation, or presented a correlation (data not shown), where the antioxidant capacity could be related to other antioxidant compounds contained in these extracts. According to Zhu et al. [24] the use of different extraction solvents resulted in differences in compositions, and consequently the antioxidant activities of the extracts. It was suggested that phenolic compounds extracted by these solvents might be the weak scavengers of DPPH and reducers of Mo (VI) to Mo (V). Some of the studies have reported no correlation between the total phenolic content and the radical scavenging activity.[42] This may be explained by the differences between samples and matrices and additional factors that can modify reactions among individual compounds. The relationship between antioxidant compounds and antioxidant capacities are often complicated. Antioxidant capacity does not only rely only on the amounts of antioxidants, but also on the structure and interactions among each other.[41] In general, the phenolic compounds at low concentration show antioxidant behavior, while presenting pro-oxidant behavior at higher concentrations. Upon further increasing of their concentration, they may again show antioxidant behavior. This always depends on the type (position and number) of hydroxyl groups in the molecule and the concentration of the phenolic compound.[35]

Moreover, the poor correlations between antioxidant activity and TPC can be explained by the fact that the Folin-Ciocalteu assay is specific not only to just phenolics, but also to any other substances that could be oxidized by the Folin-Ciocalteu phenol reagent.[42] In addition, phenolic compounds, depending on the number of phenolic groups they have, respond differently to the Folin-Ciocalteu reagent.[26] Therefore, the total phenolic content of the extracts in this study may not directly correlate with the exact content of the phenols present. The no/ moderate correlations found between TFC and antioxidant activity can also be explained by the choice of method used to estimate flavonoid content. The aluminium chloride method used in this study is specific only for flavones and flavonols, while flavanones and flavanonols react better with 2,4-dinitrophenylhrdrazine method.[40] Moreover, we suggest that the flavonoid content of these extracts may be glycosylated, making the flavonoids not freely available for antioxidant activity.[26]

Optimization of extraction solvents

All collected data were submitted to the modelisation with response surface methodology by using experimental design which enables us to identify the suitable solvent extraction for each component. The prediction profiler plot provides a graphic representation on different parameters tested and solvents relationships, it provides a way to reduce the complexity of the data. Methanol provided the best results in almost all cases. However, there was not a great differences between ethanol and acetone. Furthermore, the trend for the total phenolic content was similar with the trend obtained in DPPH radical scavenging capacity assay for these extracts (ie they decreased by increasing the solvent concentration). This finding suggested the polar character of the total phenolics extracted with these solvents and that these compounds are potentially responsible for RSA.

Adding water to the solvent tremendously increased the phenolic compound concentration for the ethanolic and acetonic extracts, but decreased the amount obtained for the methanolic extract. A solids solubility may be affected by changes in the activity coefficient, which varies with the composition of the solution. Interactions of compounds with the solvent may have modified the activity coefficient, and thus the solubility of the compounds to the solvent.[40] This suggests that the compounds extracted were soluble in pure methanol which was the most polar. However, to be able to extract them with pure ethanol and acetone its preferable to add water to them in order to increase their polarity. Consequently, the experimental design used was efficient in determining optimal extraction conditions and response surface methodology (RSM) can be employed to optimize other factors including extraction time and extraction temperature from *M. spicata*.

CONCLUSIONS

In this study, the extraction solvent significantly affected the levels of total phenolics, flavonoids and antioxidant contents extracted from mint (*M. spicata* L.) leaves harvested in Algeria. Acetone and ethanol (75 %) were the most efficient solvents for phenolic extraction. Further, antioxidant potential evaluation by both the methods showed that both acetone and ethanol extracts were promising. Nevertheless, according to the correlation study, the activity of the aqueous ethanolic extract is attributed to the phenolic and flavonoid contents but this is not the case for the aqueous acetonic extract. Moreover, acetone is regarded as a low toxicity solvent. There are safety concerns associated with the use of aqueous ethanol

for extraction of phenolic compounds from mint. In cases where the extract is used for medicinal or ingestion purposes, pure ethanol or a mixture of ethanol and water has typically been used. Ethanol is also more acceptable for use in food industry.

From the results obtained in the extraction of the antioxidant compounds from spearmint with different solvents at laboratory level, a mixture of ethanol with water at a ratio of 50:50 (v/v) was the best choice among other solvent compositions evaluated in this study. So, the present results provide evidence for potent antioxidative effect of ethanolic at 50 % extract under *in vitro* conditions, although this antioxidant activity was lower than that of ascorbic acid and α-tocopherol. Consequently, our results suggested that the extract can be utilized as an effective and safe antioxidant source.

The phenolic compounds obtained for this procedure could be used as additives in food products as natural antioxidants to extend their shelf-life. The present research renews interest in the increased use of naturally occurring antioxidants. Furthermore, it can be concluded that leaves of mint consumed as a foodstuff in different areas of Algeria can be used as an accessible source of natural antioxidants with consequent health benefits. However, the components responsible for the antioxidative activity of aqueous ethanolic extract are currently unclear. Therefore, it is suggested that further work could be performed on the isolation and identification of the antioxidative components in *M. spicata.*

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