Total Phenols, Flavonoids Contents and Antioxidant Activity of *M. suaveolens* (Ehrh.) Extracts

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Abstract

In this study, the flavonoids and phenolic contents and the antioxidant activities of M. suaveolens extracts from Morocco were evaluated. These extracts were obtained by mixing areal parts in powder with methanol/water solution. They subjected thereafter to the liquid-liquid extraction with solvents of progressive polarity (chloroform, ethyl acetate and n-butanol) by maceration and Soxhlet methods. The total phenol and flavonoids contents from crude extracts and its fractions were determined by using Folin-Ciocalteu and $AlCl_3$ assays respectively. The antioxidant activity of extracts was evaluated by DPPH• (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging test. The crude extracts obtained by maceration and Soxhlet showed the highest total phenol yields and both total phenols and flavonoids contents followed by ethyl acetate or n-butanolic fractions. Moreover, the highest contents were recorded by extracts from Soxhlet method.

Similarly, the crude extracts followed by ethyl acetate ones exhibited the highest antioxidant capacity; the degree of this activity depended on the solvent and extraction method. These results suggest that M. suaveolens has promising antioxidant activity and could serve as potential source of natural antioxidants.

Keywords: *M. suaveolens* Ehrh., total phenol, flavonoids content, antioxidant activity.

Introduction

Due to the toxic effects resulting from the use of synthetic antioxidants, many researchers have focused on seeking for natural compounds with antioxidant properties such as phenolic compounds including flavonoids reported to be more potent antioxidants than vitamins C, E and β-carotene which are widely used routinely. The consumption of flavonoids and its potential importance as antagonists of oxidative stress has been the subject of several studies. Indeed, some flavonoids in tea infusions may have protective effects against coronary heart disease, cancer, or allergy[19].

One of the best approaches to discover new antioxidants is the screening of plant extracts. The antioxidant power of these extracts is developed as a substitute in food preservation. It is mainly polyphenols that are responsible of this power[36]. In this context, this study was conducted to investigate the antioxidant potency of crude extracts and its fractions obtained from leaves and flowers of *M. suaveolens*(Ehrh.) growing spontaneously in the Azrou region (Middle-Atlas) by the stable free radical DPPH• scavenging method.

Material and Methods

Plant material: The areal parts (leaves and flowers) of *M. suaveolens* (Ehrh.) were collected on August from Azrou region in Moroccan Middle-Atlas (Latitude: 33° 25′ 59″; Longitude: 5° 13′ 01″; Altitude: 1278 m). The climate is semi-humid with strong continental influence with an annual average temperature of 20°C. The dried leaves and flowers were pulverized and then used for preparation of various extracts.

Preparation of extracts from leaves and flowers of *M. suaveolens* (Ehrh.) by maceration and Soxhlet: For solid liquid extraction of total phenols and flavonoids in the solvents, 30 g of ground material from a dry pulverized sample was macerated in aqueous methanol solution (80%) at room temperature every 48 hours (3 replicates). After filtration and vacuum concentration, the aqueous phase was subjected to successive extractions (splitting) of liquid-liquid using organic solvents with increasing polarity (chloroform, ethyl acetate and *n*-butanol).

By Soxhlet, the mixture of methanol/water (80/20) (v/v) was added to the plant material (30 g) already dried and ground and then refluxed for three hours in a Soxhlet apparatus; the hydromethanolic extract (the crude extract) is filtered and then evaporated by a rotary evaporator. Thereafter, the same protocol of maceration was followed for the polyphenols fractionation.

The determination of total phenols was performed by a method adapted by Singleton and Rossi using the Folin-Ciocalteu reagent[34] while the content of total flavonoids in the samples is evaluated by the aluminum trichloride ($AlCl_3$) method adapted by Djeridane et al[10].

Determination of PPC: The amount of phenolic total in the extracts of *M, suaveolens* (Ehrh.) leaves and fruits was determined by the method described by Dehpour et al[9] slightly modified. They used the Folin-Ciocalteau method to determine the polyphenols content of a plant extract.

Different concentrations: 0,08, 0,04, 0,16, 0,32, 0,48, 0,6, 0,96 and 1,28 µg/ml, were prepared in volumetric flasks, from 50 mg/l of gallic acid by adding to each solution a volume of 1,5 ml of Folin-Ciocalteu (10%). The mixture was stirred and allowed to stand for 6 minutes before the addition of 1,5 ml of Na_2CO_3 solution (7,5%). The solutions were adjusted with distilled water to a final volume of 100 ml, shaken immediately and kept in the dark for 2h at room temperature.

The absorbance of each solution was determined at 765 nm with a spectrophotometer Shimadzu UV-MINI 1240. The quantitative analysis of total phenols in our extracts was carried out by adapting the same procedure used for the preparation of the curve calibration, replacing gallic acid with a volume of extract to an appropriate concentration.

The total polyphenols concentrations of each extract was calculated from the regression equation of the calibration range established with gallic acid (y = 0,095x + 0,003).

The results are expressed in milligrams of gallic acid equivalent/ gram of dry matter (EGA mg/g plant). These results were used to provide estimates on total polyphenols contained in the leaves and flowers of *M. suaveolens* (Ehrh.). The total phenol content is calculated according to the following formula:

$$T = \frac{\mathrm{C} \times \mathrm{V}}{\mathrm{m}_{\mathrm{dry\ material}}} \times \mathrm{D}$$

where C = Concentration evaluated according to the calibration curve, V = Volume of overall extract and D = Dilution Factor.

Determination of flavonoids content: The quantification of flavonoids was carried out by a colorimetric method adapted by Djeridane et al[10]. From the methanolic solution (0,1 g/l) of quercetin, different concentrations: 5, 10, 15, 20, 25 and 30 µg/ml were prepared in volumetric flasks (50 ml) by adding to each solution 20 ml of distilled water. After 5 min, 100 µl of aluminum trichloride ($AlCl_3$) at 10% (w/v) is added.

The solutions were adjusted to 50 ml with methanol, shaken immediately and then kept in the dark for 30 minutes at room temperature. The absorbance of each concentration was determined by a spectrophotometer at 333 nm as mentioned previously for the determination of total phenolic content.

Quantitative analysis of flavonoids in our extracts was carried out by adapting the same procedure used for the preparation of the calibration curve replacing the quercetin by a volume of the extract until an appropriate concentration.

The flavonoids concentrations of each extract were calculated from the regression equation of the calibration range established with quercetin (y = 0,073x – 0,081).

Antioxidant activity: The experiment was performed by the spectrophotometer at 515 nm. The solution of DPPH at 6 10^{-5} M is obtained by dissolving 2,4 mg of the powder in 100 ml of ethanol while the samples were prepared by dissolving in ethanol at 1,6 mg /ml[27].

The test is carried out by mixing 2,8 ml of the prepared solution DPPH with 200µl of the crude, ethyl acetate and *n*-butanolic extracts or standard antioxidant (ascorbic acid) at different concentrations (0 to 200 µg/ml). After 30 minutes of incubation in the dark at room temperature, the absorbance is read at 515 nm against a control containing only ethanol. The control consists of DPPH free extract and the values obtained are then converted into percentages of inhibition using the following formula:

$$AA\% = \frac{\mathrm{A}_{\mathrm{control}} - \mathrm{A}_{\mathrm{sample}}}{\mathrm{A}_{\mathrm{control}}} \times 100$$

where AA% = Percentage of antioxidant activity, $A_{control}$ = Absorbance of the solution containing only radical DPPH solution and A_{sample} = Absorbance of the sample solution to be tested in the presence of DPPH

The graph of the absorbance variation according to the concentration of extract allowed determining the IC_{50} (concentration corresponding to the loss of 50% of free radicals activity). The values of IC_{50} were obtained from the 3rd degree polynomial trend curves.

Results and Discussion

Yield of extraction: The yields of total polyphenols (TPP) in the different extracts from *M. suaveolens* are summarized in the figure 1. According to the obtained values, Soxhlet method provided the better yields of TPP than the maceration; the best extraction yield, obtained by Soxhlet, was observed for the crude extract 39,13% followed by the aqueous one. Similarly, the crude extract by maceration showed the best yield (27,17%) followed by the aqueous extract (6,1%). However, the lowest yields were recorded both by Soxhlet and maceration by the *n*-butanolic and chloroformic extracts.

The extraction yields therefore varied according to the extraction technique. The best extraction yields of both used methods were recorded by Soxhlet.

The solvent extractions are the most commonly used procedures for preparing plant extracts because of their ease of use, effectiveness and broad applicability. Many studies have reported that the extraction yield depended on the type of solvent with different polarities, the extraction time, the temperature, the sample/solvent ratio, the chemical composition and physical characteristics of the samples[8].

Barchan et al[2] found that the best yields are derived from water and methanol extracts as opposed to hexane and dichloromethane. Inversely, Stankovic et al[37] concluded that

the best solvent was methanol followed by water and ethyl acetate.

For the extraction of phenolic substances from Lamiaceae, the most widely used solvent is methanol. Çakir et al[7] in their study on phenolic compounds of *L. Teucrium* (Lamiaceae) reported that methanol gave a higher extraction yield than acetone, chloroform and petroleum ether. The use of methanol, water, petroleum ether and chloroform by Sharififar et al[35] for the extraction of polyphenols from *T. polium* showed that methanol gave the highest extraction yield. Similarly, Tay et al[39] found that the tested concentrations of the solvent (ethanol) used for the extraction of polyphenols and the sample/solvent ratio had a significant effect on yields. Likewise, Mata et al[23] also found that the aqueous extracts of some *Mentha* species from Portugal are richer in polyphenols than ethanolic extracts.

According to Franco et al[14], the highest yields are generally obtained with methanol and ethanol and its mixtures with water, although other solvents have been widely used in the extraction of plant polyphenols such as ethyl acetate or acetone. Water and ethanol are most widely used because of their low toxicity and high extraction efficiency with the advantage of modulating the polarity of the solvent using ethanol / water mixtures at different ratios. The main disadvantage of aqueous extraction is the low solubility of non-polar or non-polar lipid-soluble antioxidants such as carotenoids which give too low yields. The solubility of polyphenols depends mainly on the hydroxyl groups, the molecular size and the length of the carbon chain.

Polyphenols and flavonoids contents (PPC/FC)

Polyphenols Content (PPC): The results of the colorimetric analysis of *M. suaveolens* total phenolic compounds are shown in figure 2. They show that the crude extracts generally present the highest levels of polyphenols by both maceration and Soxhlet followed by the *n*-butanolic extract obtained by Soxhlet (24,89 mg AGE/ g DM) and ethyl acetate one (15,56 mg AGE/ g DM) obtained by maceration.

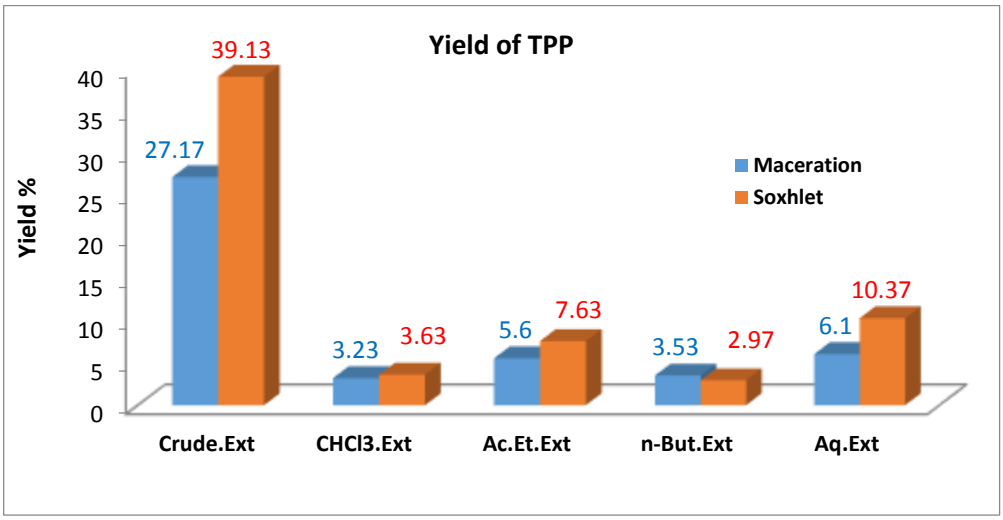

Figure 1: Yields of TPP in different extracts of *M. suaveolens* (Ehrh.)

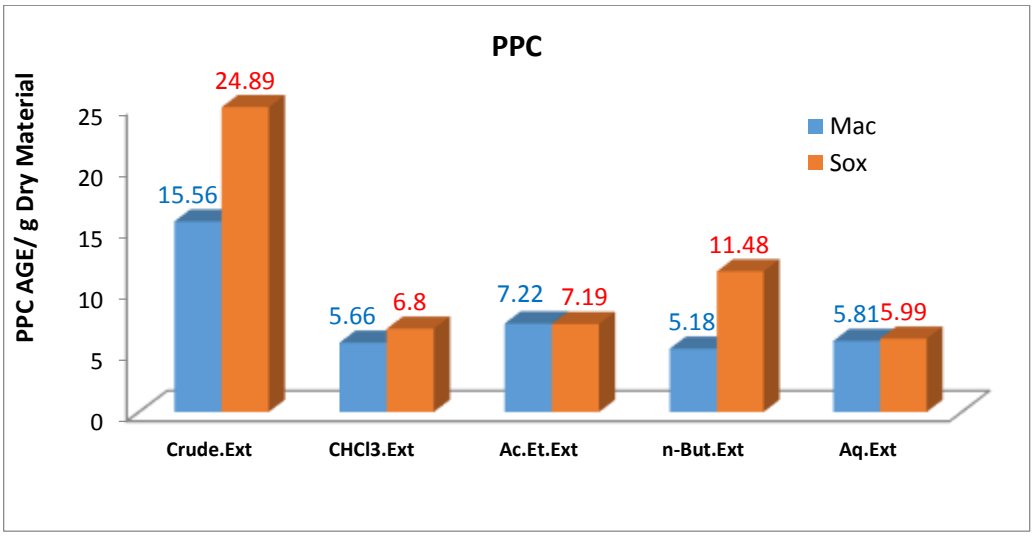

Figure 2: Contents of polyphenols in different extracts of *M. suaveolens* (Ehrh.)

The fractions obtained by the Soxhlet method showed the highest levels compared to those resulting from maceration. On the other hand, the crude extracts and the most polar fractions exhibited the large content of polyphenols. Senevirathne et al[33] investigated the antioxidant potential of the different fractions of the methanolic extract of the *Ecklonia cava* species and reported that among the organic fractions, the ethyl acetate fraction had the highest level of total phenols; the chloroform fraction and the methanolic extract also showed a high content of phenolic compounds. However, our results showed that the chloroformic extracts contain generally the lowest levels of TPP.

The variability of PPC is probably due to the phenolic composition of the extracts[16], the biotic (species, organ and physiological stage) and abiotic conditions, the soil nature and the bioclimate type and also the bioclimatic stages where this plant grows[1].

The study of PPCs in plants has been the subject of many researches; the PPCs of the various extracts of *M. suaveolens* studied by Bichra et al[4] ranged from 0,25 to 2,65 mg GAE/ g DM. While the extracts prepared from the stems and leaves of Algerian *M. suaveolens* using methanol and water revealed a significant PPC for the aqueous extract from the leaves (20,75 mg GAE/ g DM)[32]. Dorman et al[13] reported that PPCs were ranged between 128 - 230 mg GAE/ g extract from different *Mentha* species.

In fact, the PPCs obtained indicated that they therefore depended on the solvent used and the extraction method. In addition to these factors, there is the water/solvent ratio, the sample/solvent ratio, the number of extraction and the extraction conditions[31,40,44].

It seems that the different solvents make it possible to extract the different types of phenolic compounds. Thus, the more polar fractions should contain a larger amount of hydrophilic phenols while the chloroformic extracts, which exhibit low polyphenols contents, may include low molecular weight hydrophobic phenolic compounds. On the other hand, the crude extracts should have been rich in phenolic compounds of both types[18].

Flavonoids content (FC): According to the absorbance values of the various extracts solutions, compared to the quercetin equivalent standard (QE) solution, the results of the colorimetric analysis are given in table 2. The flavonoids contents (FCs) varied between 0,87 and 26,56 mg QE/ g DM by Soxhlet and between 3,99 and 11,22 mg QE/ g DM by maceration (Figure 3).

The highest levels of flavonoids were observed generally in crude extracts followed by ethyl acetate then n-butanolic extracts both by maceration and by Soxhlet and particularly by Soxhlet. However, the chloroformic extract by Soxhlet has recorded the lowest content while by maceration the content was more important than that of ethyl acetate extract. Indeed, Meziti[24] and Hossain et al[17] obtained comparable results whose chloroformic extract contains more flavonoids than the other polar fractions.

The flavonoids contents in the extracts like those of the polyphenols also depend on the extraction method and the solvent used. This is consistent with results found in many studies[12,14,22]. Several studies have reported the extraction of plants by different solvents and in the determination of polyphenols and flavonoids contents by various techniques but those concerning mints are few. In this regard, the solvents used for the extraction of polyphenols and flavonoids of the various mint species, which have been the subject of previous researches, were often methanol, water or ethanol.

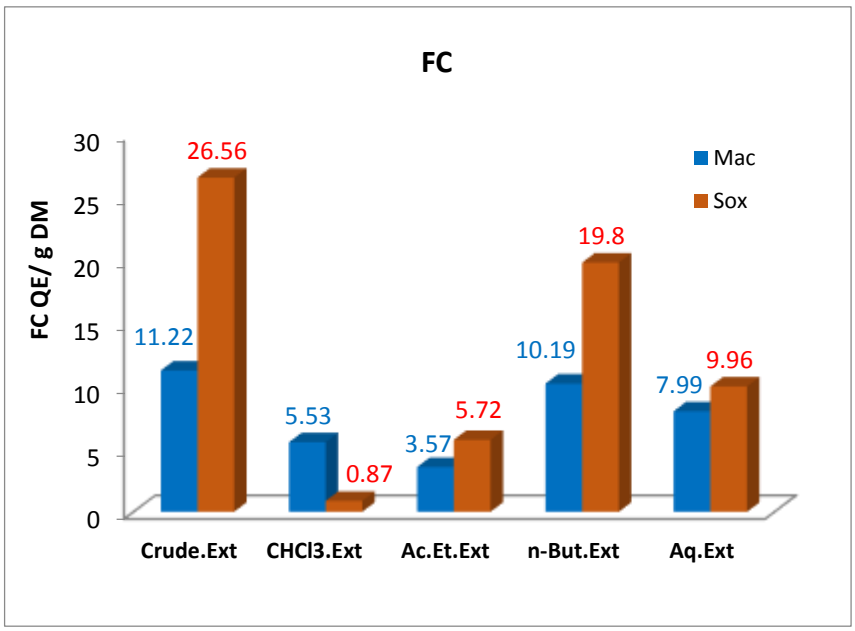

Figure 3: Flavonoids Contents in different extracts of *M. suaveolens* (Ehrh.)

The methanolic extract prepared from the leaves of *M. suaveolens* from Algeria revealed a content of 2,19 mg equivalent of Catechin (EC) / g DM compared to the aqueous extract from the same plant part[32].

The flavonoids content was higher in some extracts than that of PPC. This could be explained by the fact that all phenolic compounds could not be estimated by unique extraction or by a unique method due to the complexity of the compounds. The majority of flavonoids are phenolic compounds that mean they contain at least one phenolic group.

Moreover, the TPC measured by the Folin-Ciocalteu procedure may not be able to give a complete image of the quality or quantity of the phenolic constituents in the extracts[42]. Despite its high sensitivity, this method may present interference problems. Indeed, the Folin-Ciocalteu reagent can react with amino-acids and reducing sugars such as glucose and fructose[6].

Talbi et al[38] found that the FC is greater than that of PPC in the methanolic and aqueous extracts of *Nigella sativa* and thus for the aqueous, hydro-ethanolic and ethanolic extracts of *Cucumeropsis edulis* and *Garcinia kola*[28].

Similarly, Settaraksa et al, in their study of the effect of solvent on FC showed that the curry paste produced the highest content 81,62 ± 0,03 mg EC/ 100 g sample while that of PPC was around 34,02 ± 0,03.

Thus, the hydro-methanolic and chloroformic extracts of *Leonorus cardiaca* (L.) presented interesting FCs: 50,21 ± 0,65 and 27,25 ± 0,670 mg hyperoside equivalent/ g, while polyphenols have recorded contents about 42,95 ± 3,55 and 4,90 ± 0,98 mg GAE/ g respectively[15]. In addition, aqueous and ethanolic extracts from 44 Australian species showed higher flavonoids levels than polyphenols[29]

Antioxidant activity of *M. suaveolens* extracts

The antioxidant activity of the various extracts of the plant toward the DPPH* radical has been evaluated spectrophotometrically following the reduction of this radical at 515 nm. The reduction is determined by a decrease in the absorbance induced by antiradicals. The antioxidant power was characterized by the parameter IC_{50}. The lower is the IC_{50} value, the higher is the antioxidant activity (Figure 4). This test aims to measure the ability of the extracts to trap the stable DPPH radical formed in solution by donation of a hydrogen atom or an electron[41].

According to calculated IC_{50} values, the extracts of *M. suaveolens* (Ehrh.) were able to reduce the free radical DPPH* (Table 1). They showed a good antioxidant capacity compared to that of the standard (IC_{50} of Ascorbic Acid = 0,051 mg/ ml).

The percentages of radical inhibition, according to the concentrations of each extract, are recorded in the figures 5 to 8. The crude extracts by maceration and Soxhlet (IC_{50} = 0,335-0,484 mg/ ml) respectively were displayed to be the most effective by comparison to the ethyl acetate and *n*-butanolic fractions.

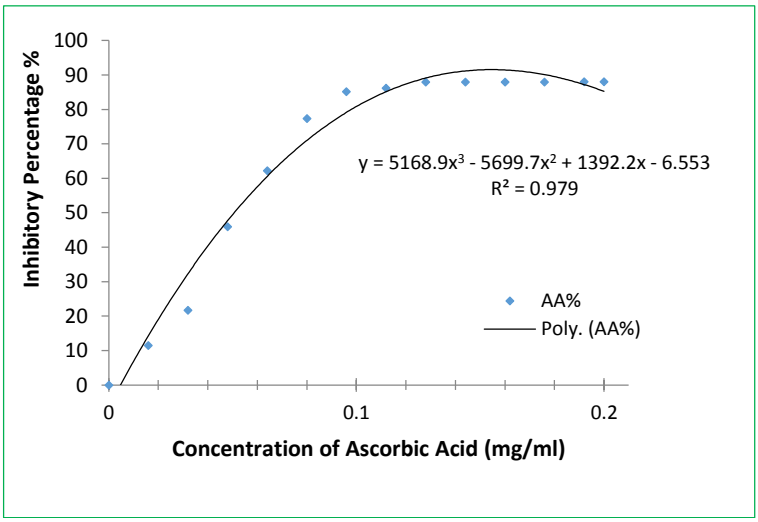

Figure 4: Inhibitory percentage of DPPH according to Ascorbic Acid concentrations

Table 1
Values of IC_{50} according to the solvent nature and extraction method

Extract	IC_{50} mg/ml	
	Maceration	Soxhlet
Crude Extract	0,484	0,335
Ethyl acetate Extract	0,677	0,371
n-Butanolic Extract	0,907	0,789

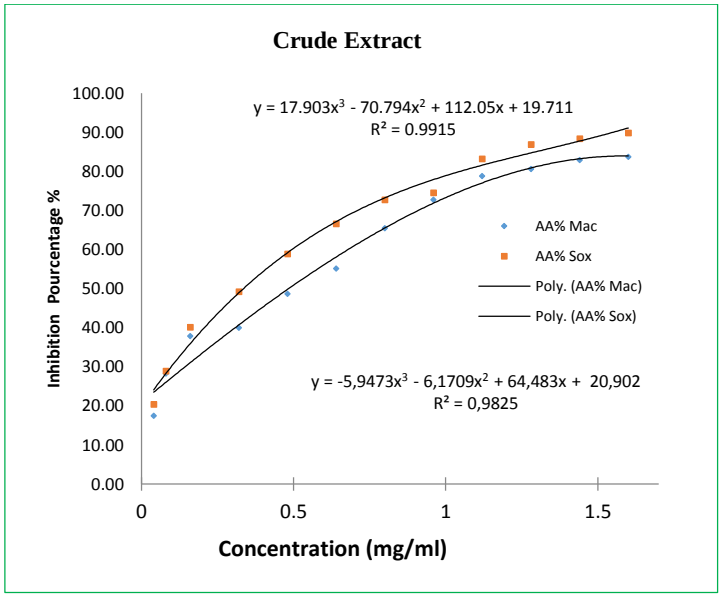

Figure 5: Percentage of inhibition of DPPH by *M. suaveolens* crude extract

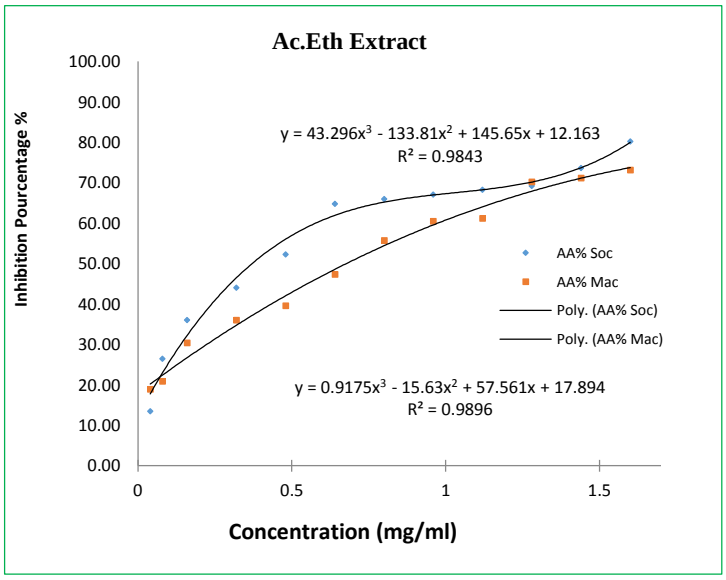

Figure 6: Percentage of inhibition of DPPH by *M. suaveolens* Ethyl acetate extract

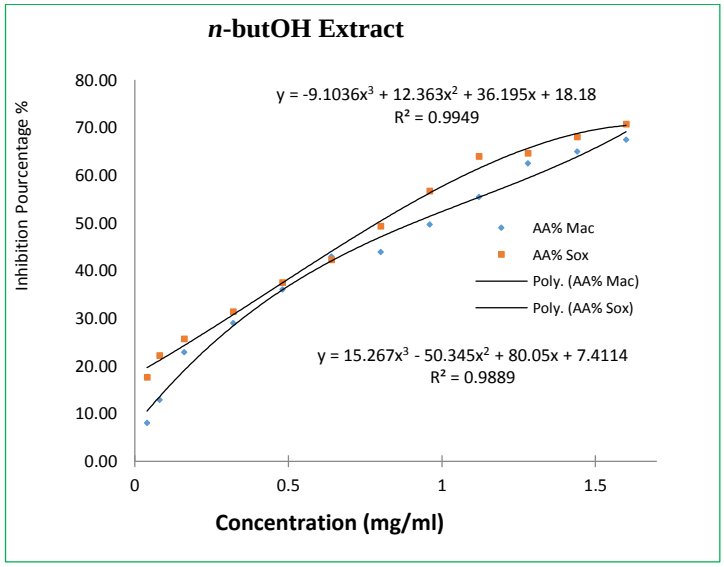

Figure 7: Percentage of inhibition of DPPH by *M. suaveolens n*-butanolic extract

Some studies have been carried out in Morocco on the antioxidant activity of the *M. suaveolens* extracts. Bichra et al[3,4] showed that *M. suaveolens* harvested from Marrakech is endowed with a high antioxidant potential and that the phenolic extract exhibited a stronger antioxidant activity ($1/EC_{50}$= 0,4) than the aqueous extract and equivalent to that of BHT ($1/EC_{50}$= 0,3) and ascorbic acid ($1/EC_{50}$= 0,35). Moreover, the methanolic extract of *M. suaveolens* (Ehrh.) from Pakistan has also revealed a significant anti-radical activity with an inhibition rate reaching 82%. Similar antiradical activity has also been observed for the ethanolic extract from Iran (IC_{50}= 21,71µg/ ml)[26] and that from Romania with an IC_{50}= 104,74 ± 1,76 µg/ ml[25].

Our results indicated that each extract reacted differently towards the free radical DPPH and according to the solvent of extraction. The crude extracts recorded the strongest antiradical activity. Moreover, according to the yields of the extracts and the contents of phenolic compounds obtained, it appears that the Soxhlet technique was the most effective compared to the maceration. Same results were obtained by Bimakr et al[5], the yields and the flavonoids levels were higher by Soxhlet than those by the supercritical carbon dioxide method.

Positive correlation was found between the PPC and the degree of antioxidant activity (Table 2). The crude extracts are exhibiting an important antioxidant activity recorded the highest PPCs and FCs.

Many researchers have also reported a positive correlation between free radical scavenging activity and PPC[11,20,21,43]. Romero-Jimenez et al[30] reported that the level of antioxidant activity was strongly associated with the PPC in the extracts. In addition, Barchan et al[2] also found that the antioxidant activity correlated positively with the PPC. Similarly, Mata et al[23] concluded that the antioxidant potential of tested mints was highly dependent in the presence of phenolic compounds.

Conclusion

The purpose of this study was to determine the polyphenols and flavonoids contents in *M. suaveolens* (Ehrh.) extracts, obtained by solvents with progressive polarity and by different extraction methods and to evaluate their antioxidant activity according to these solvents and methods. The results of this investigation have demonstrated that all extracts were rich in polyphenols and flavonoids and that the richer ones showed a significant antioxidant potential comparable with that of the reference standard (Ascorbic acid).

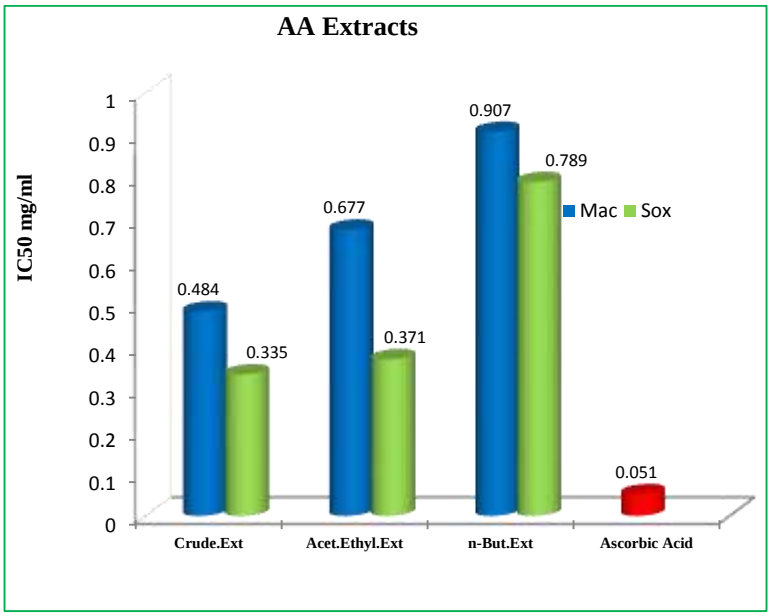

Figure 8: Values of IC_{50} by different extracts

Table 2
Correlation between polyphenols and flavonoids contents in the extracts and the IC_{50} values

Extract	Maceration			Soxhlet		
	IC_{50} (mg/ ml)	PPC	FC	IC_{50} (mg/ ml)	PPC	FC
Crude Extract	0,484	15,52	11,22	0,335	24,89	26,56
Ethyl acetate Extract	0,677	7,22	3,57	0,371	7,19	5,72
n-Butanolic Extract	0,907	5,18	10,19	0,789	11,48	19,8

Moreover, total phenolic contents have shown a positive correlation with antioxidant capacity. Consequently, *M. suaveolens* may be potent sources of natural antioxidants that could be used in food and pharmaceutical fields.

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