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Research Article

Effects of Different Extraction Methods and Solvents on the Phenolic Composition and Antioxidant Activity of *Silybum Marianum* Leaves Extracts

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Abstract:

Objective: The objectives of this study were essentially to evaluate the best technique for extracting phenolic compounds from *Silybum marianum* leaves.

Subjects and Methods: Two extraction methods (maceration and decoction), using four extraction solvents. The efficiency of the extraction methods was estimated by quantifying the total polyphenol contents by the Folin -Ciocalteu method and by determining the antioxidant power of the various extracts by the ability to trap the free radical DPPH °, and the influence of these parameters on the antioxidant activity.

Results: The results showed that the extracts of the leaves by maceration have contained the highest percentage yield (24.68%); the highest content in the aqueous -methanol polyphenols extract (18.75 ± 0.55 mg GAE / g DW) against the decoction is more effective for the extraction of flavonoids and condensed tannins. The highest content of flavonoids had been recorded for the aqueous- ethanolic extract (5.08 ± 0.57 CE mg / g DW). The water had the highest content of condensed tannin (03.86 ± 0.22 TAE / g DW). On the other hand, the aqueous -ethanolic and aqueous -methanol extract of the leaves obtained by maceration have the best antioxidant capacity (73.84 ± 5.90 EAG / g DW and 73.64 ± 5.93 mg EAG / g DW respectively) and the aqueous extract of the leaves by decoction had a better antiradical power (48.64 µg / ml).

Conclusion: The extraction of phenolic compounds from Silybum marianum leaves was strongly influenced by the type of solvent and the extraction method. In addition, it is very difficult to choose an appropriate extraction solvent for all plant samples.

Key words: *Silybum marianum* - phenolic compounds - extraction solvent - maceration-decoction - antioxidant activity.

INTRODUCTION

The use of medicinal plants is as old as human civilization. Continuous efforts are being made to improve medicinal plants or produce their products in high amounts through various technologies. [1] Thus, plants can be considered as reservoirs of bioactive molecules still little explored. [2] Indeed, the scientific community has carried out several scientific works to study medicinal plants for the research and the valorization of new molecules, which represent the principal groups endowed with a powerful activity antioxidant or scavengers of free radicals or others of activity in order to provide solutions to oxidative stress [3]. Phenolic compounds are present in all higher plants and they are very heterogeneous compounds both by their compositions and by their structures [4]. They correspond to a very wide range of chemical structures and are characterized by a very unequal qualitative and quantitative distribution according to the considered species but also the organs, the tissues and the physiological stages, [2, 4] of very different physicochemical properties influencing the extraction of polyphenols. [5] The extraction technique is a very important step in the isolation and recovery of the active ingredients of these existing metabolites in the plant material, as well as in their identification. [6]Conventionally, the phenolic compounds were extracted by maceration or extraction at reflux with a hydro-alcoholic mixture. [7] Numerous studies have been devoted to the study of the effect of some experimental parameters on the quantitative variation of the phenolic compounds of plant extracts on the one hand, and on its antioxidant power on the other hand, such as the mode of extraction and type of solvent. The extraction of phenolic compounds from the different plant samples is strongly influenced on the one hand by the nature and the concentration of extraction and penetration solvents in the plant material, and on the other hand the capacity of the plant sample to solubilize in solvents. [8, 9] For this purpose, it is very difficult to select a suitable solvent for the extraction of polyphenols from all plant samples.

Silybum marianum is an annual biennial plant belonging to the family of Asteraceae or composed, used for more than two thousand years in traditional European medicine. [10] Its geographical distribution is wide; it is found in a majority of European countries as well as North Africa, North and South America, South Russia and Asia Minor. In Algeria, according to Belouahem (2009), [11] the milk thistle is particularly widespread in the highlands, the steppe, the south of the Saharan Atlas, the sandy pastures and places a little wet. This plant is of medicinal and pharmaceutical interest thanks to its richness in bioactive substances; its properties are due to the presence of silymarine with a concentration of about 70 - 80%, responsible for most of the therapeutic effects of the plant. [12] Milk thistle has a significant biological and medicinal value, but remains little studied in Algeria.

In this context, we were interested in evaluating the best solvent and extraction technique of phenolic compounds in *Silybum marianum* leaves, and evaluate the influence of these parameters on the antioxidant activity.

Material and methods

Plant material

The leaves of *Silybum marianum* were harvested during the month of June in the TESSALA Mountain of the region of SIDI BEL ABBÉS- Algeria. Mr. MAHDADI Zouheir, Professor at the Department of the Environment, Djillali Liabès University, - SIDI BEL- ABBÈS (Algeria), provided us with a botanical identification of the species. The plant material was freshly collected, dried in the shade for 2 to 3 weeks, then pulverized with the MF 10 BASIC IKAWERKE grinder to obtain a fine vegetable powder.

Extraction methods

Two methods of extracting polyphenols were used, namely maceration and decoction, using four extraction solvents (methanol / water (70/30), ethanol / water (70/30: v / v); acetone / water (70/30: v / v) and distilled water.

The extraction by maceration is carried out according to the protocol described by Romani et al., 1995 and Mahmoudi et al., 2013[08, 13]: 10 g of powder of the plant material is extracted in 100 ml of the solvent (renewal twice every 2.5 hours) at room temperature with magnetic stirring, and protected from light.

The extraction by decoction: was carried out according to the protocol described by Chavane et al. (2001) [14]: 1 g of plant material is contacted with 20 ml of different solvents. The preparation is refluxed and heated (100 ° C.) for 30 minutes, repeated twice the various extracts were combined, recovered and filtered with some filter paper. The maceras and decocts thus obtained are concentrated in a rotavapor at a temperature of 50 ° C and then stored at 4 °C.

Calculation of extraction yields

Yield percentage of the various extracts obtained was defined as being the ratio between the mass of the extract and that of the dry vegetable matter subjected to the extraction [15]. It is calculated by the following formula:

Percent yield = $[W_1-W_2 / W_3] \times 100$

$\mathbf{W_1}$: Weight of the flask after evaporation, $\mathbf{W_2}$**:** Weight of the empty balloon, $\mathbf{W_3}$: Weight of the starting dry vegetable matter.

Quantitative analysis of phenolic compounds

Determination of Total Polyphenol Contents

The total polyphenol content of the various extracts was achieved by the use of the Folin-Ciocalteu reagent, according to the method described by Vermerris et al., (2006) [16]: A volume of 100 µl at different concentrations of extracts was added to 2 ml of Na_2CO_3 (2%). After stirring and a rest of 5 min, 100 µl of the Folin-Ciocalteu reagent were added to the reaction medium. The absorbance was measured at 750 nm after 30 min of incubation at room temperature in the dark. A calibration curve is established using gallic acid at various concentrations (0 to 400 µg / ml) as standard. Thus, the results were expressed in milligram gallic acid equivalent per gram of dry weight (mg GAE/ g DW).

Determination of total flavonoids

The determination of total flavonoids was carried out according to the method described by Dewanto et al., (2002). [17] An aliquot of 250 µl of each diluted extract was added to 75 µl of a $NaNO_2$ solution (7%). After 6 minutes, 150 µl of an $AlCl_3$ solution (10%) was added and after standing for 5 minutes, 500 µl of NaOH were added to the mixture. The final volume of reaction medium is subsequently adjusted with distilled water at 2500 µl. The absorbance of the mixture was read at 510 nm. A calibration curve was performed with catechin at concentrations of 0 to 400 µg / ml. The flavonoid contents are expressed in milligram catechin equivalent per gram of dry weight (mg CE/g DW).

Determination of Condensed Tannins

The condensed tannins were determined by the colorimetric method of Folin - Denis, described by Joslyn, 1970. [18] One milliliters of the extract was mixed with 75 ml of distilled water, 5 ml of Folin - Denis reagent and 10 ml of the saturated solution of CO_3Na_2. The mixture was stirred by a vortex and was incubated at room temperature for 30 minutes. The absorbance of the mixture was read at 760 nm. Condensed tannins contents were expressed in milligram (mg) equivalent of tannic acid (TA) per gram of dry weight (mg TAE / g DW).
Determination of the antioxidant activity:

Total antioxidant capacity (TAC)

It is evaluated by a spectroscopic method leading to the formation of a phosphomolybdenum complex. [19] A volume of 1 ml of reagent solution (sulfuric acid (0.6 N), sodium phosphate (28 mM) and ammonium molybdate (4 mM)) was combined with 0.1 ml of each diluted extract. After 90 minutes of incubation at 95°C and a rest of 6 minutes at room temperature, the absorbance was measured at 695 nm against a

white containing methanol instead of the extract. The total antioxidant activity was expressed in mg of ascorbic acid equivalent per gram of dry weight (mg EAG / g DW).

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay for antioxidant activity

The experimental protocol followed is that according to Sanchez-Moreno et al (1998) in [20]. An aliquot of 50 µl of different concentrations prepared in methanol of each extract were added to 1950 µl of the methanol solution of DPPH freshly prepared. A negative control is also prepared, in parallel. Absorbance was read at 515 nm after 30 min incubation. Ascorbic acid was used as a positive control. The results were expressed as inhibition percentage, calculated according to the equation below:

$$IP\ \% = ((A_{blank} - A_{sample})/A_{blank}] \times 100.$$

A_{blank}: Absorbance of the control reaction
A sample: Absorbance in the presence of plant extract.

The study of the variation of the percentage of inhibition as a function of the concentration of the extracts allows the determination of the concentrations corresponding to 50% inhibition (IC 50).

Statistical Analyses

The results of the analyzes carried out in triplicate were expressed in mean ± standard deviation. The ANOVA statistical analyzes were carried out by Excel 2003 and was applied to determine the significant difference at p <0.05.

Results

Extraction yield

The best averages of extraction yields by different solvents were for the aqueous extracts (24.68%) followed by the aqueous -methanol extract (23.24%) which were close for the – aqueous-acetonic and the aqueous -ethanolic extracts (17.31 and 16.02% respectively). In the case of maceration, water is the best extraction solvent with values of about 24.71%, followed by methanol 22.03%, ethanol (19.92%) and finally the acetone 15.43%. With regard to the decoction, the results show that water and methanol gave better extraction yields with very close values: 24.65% and 24.45% respectively. However, ethanol, showed the lowest yield (12.13%) (Figure 1). The averages of extraction yields, of the two methods used are very close and they are practically similar for maceration and decoction (20.52% and 20.10% on average respectively).

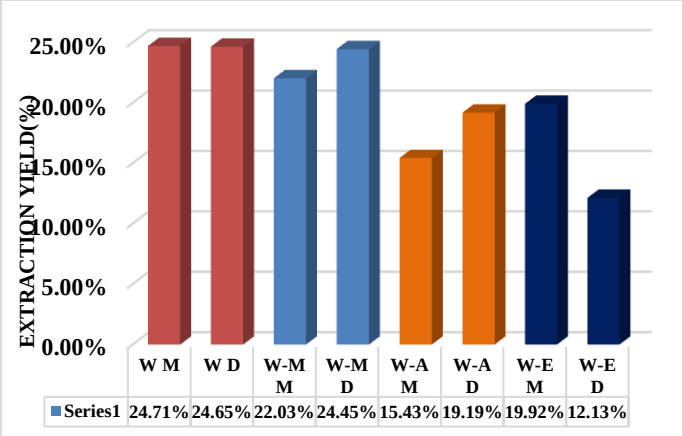

Figure 1: Extraction yields of Silybum marianum leaves by maceration and decoction

W: Water (100%), W-M: Water-Methanol (30-70; v-v), W-E: Water-Ethanol (30-70; v-v), W-A: Water-Acetone (30-70; v-v), L: Leaves, M: *Maceration, D: Decoction.*

Quantitative phytochemical analysis

Total polyphenol assays

the results of the determination of total polyphenols reveal that the highest levels of polyphenols were recorded in the extracts by maceration method: in which, the aqueous-methanol and aqueous acetonic extracts have amounts of 18.75 ± 0.55 and 16.97 ± 5.46 mg GAE / g DW respectively. Significant levels were also observed in the decoction extracts in which the aqueous- ethanol and aqueous -methanol extracts have the amounts of 16.12 ± 2.50 and 15.31 ± 0.78 mg EAG / g MS respectively. However, the aqueous extract by maceration had the lowest phenolic compounds content with a rate of 07.52 ± 0.24 mg GAE / g DW. Among the four solvent systems used, the methanol extracts offered the highest content of polyphenols. (Table 1).

Statistical analysis showed that there is no significant difference in phenolic content (P> 0.05) for the leaves extracts obtained by maceration and decoction.

Table 1: The contents of total polyphenols, flavonoids and condensed tannins in the leaves of Silybum marianum extracted by maceration and decoction.

Part of the plant	Extraction solvents	Total polyphenols (mg GAE/ g DW)		Flavonoids (mg CE/g DW)		*Condensed tannins* (mg TAE / g DW).	
		By maceration	By maceration	By Decoction	By decoction	By maceration	By decoction
Leaves: (L)	Water	07.52 ± 0.24	04.23 ± 0.37	02.63± 0.39	10.93± 2.78	02.30 ± 0.22	03.86± 0.22
	Water-Methanol	18.75± 0.55	02.70 ± 0.32	04.09 ± 0.60	15.31 ± 0.78	00.75 ± 0.05	00.73 ± 0.18
	Water-Acetone	16.97 ± 5.46	0.95 ± 0.06	05.06 ± 0.44	12.18 ± 3.89	01.66 ± 0.23	01.15 ± 0.20
	Water-Ethanol	11.95 ± 1.76	03.04 ± 0.45	05.08 ± 0.57	16.12 ± 2.50	00.54 ± 0.06	01.04 ± 0.26

Flavonoids content

The highest content of flavonoids was recorded for aqueous ethanol and aqueous acetonic, extract by decoction, with similar values of the order of 5.08 ± 0.57 and 05.06 ± 0.44mg CE mg / g DW respectively. The aqueous acetonic extract by maceration presented the lowest content (0.95 ± 0.06 EC / g MS mg / g). With regard to the extraction solvent, ethanol is the best flavonoid extractor. The results shown in table 1 indicate that the extracts of the leaves by maceration had significantly different flavonoid contents (P <0.05), closely related to the nature of the solvent and the extraction method used. While the results were insignificant (P> 0.05) for the extracts obtained by decoction.

Content of condensed tannins

The results illustrated in the table below, show that the decoction is more effective for extraction of condensed tannins than maceration. We noticed that the water had the highest contents of condensed tannin, whatever the extraction mode used, with a value of the order of 03.86 ± 0.22 TAE / g DW by decoction and 02.30 ± 0.22 mg TAE / g DW by maceration. The lowest tannin content was that of ethanol by maceration (00.54 ± 0.06 mg TAE / g DW (Table 1). Statistical analysis had shown that there was a significant effect of the polarity of the extraction solvent (P <0.05) on condensed tannin concentration of leaves extracts by maceration and by decoction.

Determination of antioxidant activity

Total antioxidant capacity

The results of this activity (Figure 2) indicate that aqueous - methanol and aqueous ethanol extracts leaves by maceration have the best antioxidant capacity compared to other extracts and had almost the same value (73.84 ± 5.90 EAG / g DW and 73.64 ± 5.93 mg EAG / g DW respectively). The other extracts showed considerable activities, except for the aqueous acetonic and aqueous extracts by decoction, which were less active with a very low total antioxidant capacity (Fe Aq D: 0.69 ± 0.041; Fe Ac D: 0.75 ± 0.04 mg EAG / g MS). The statistical study revealed that the total antioxidant capacity of leaves extracts by decoction were significantly (P <0.05) influenced by the type of solvent used, whereas the effect of the solvent was not significant for the maceration extracts.

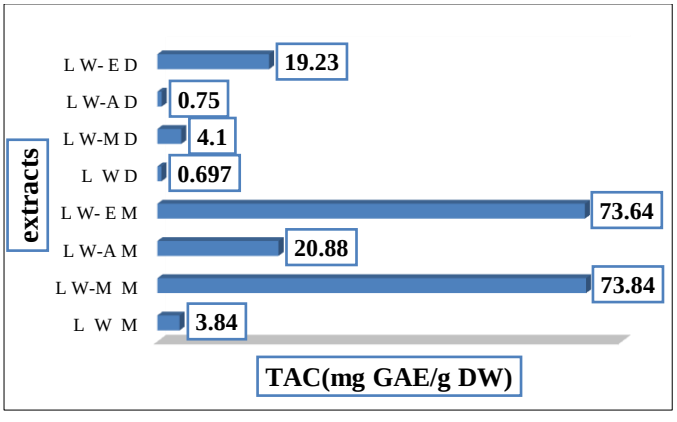

Figure 2: Comparison of the total antioxidant capacity of the extracts of Silybum marianum leaves.

DPPH radical scavenging activity

Leaves extracts by decoction of *Silybum marianum* showed significant DPPH inhibition percentage values: the leaves aqueous extract obtained by decoction had the highest value (Fe Ac D: 71.57%), followed by aqueous ethanol extract with a DPPH inhibition percentage equal to 68.88% at the concentration of 200 µg / ml. In contrast, the aqueous acetonic extract by decoction had a very low antioxidant activity showed by a percentage inhibition of 43.65% at the same concentration. The DPPH inhibition rates recorded for the standard substance (ascorbic acid) were higher than those of the various extracts of the leaves with a value of 92.06%. From the figure 3, it is noted that the percentages of free radical inhibition increase significantly (p≤0.05) with the increase of the concentration of extracts by maceration and by decoction.

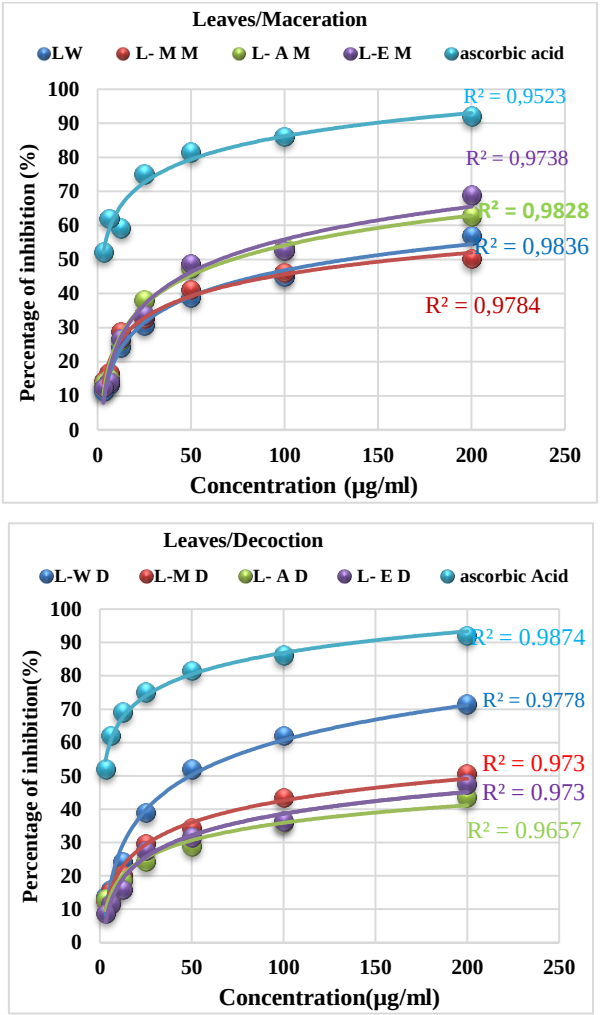

Figure 3: Percentage inhibition of DPPH radical by ascorbic acid and extracts of Silybum marianum leaves obtained by maceration and decoction (each value represents the average of three trials).

In order to compare the antioxidant efficiency of the extracts, the IC50 of the different extracts were compared. Indeed a low value of IC50 indicates an important antiradical activity. The results shown in figure 4 were classified into two groups:

The first group: extracts with IC50 values between 48.64 to 72.65 µg / ml, have significant antioxidant activities, this is the case of: L W D (48.64 µg / ml) > L W- E M (65.15 µg / ml) > L W- A M (72.65 µg / ml) extracts. The second group of extracts showed a very low ability to scavenge the DPPH radical, with IC50 values higher than 133.75 µg / ml. This is the case for all extracts, except the aqueous extract by decoction, the aqueous ethanol and aqueous acetonic extracts by maceration. All extracts tested were less active compared to the IC50 of ascorbic acid (2.17 µg / ml). The results revealed that the antiradical activity of leaves extracts obtained by maceration and decoction was significantly influenced by the nature of the extraction solvent.

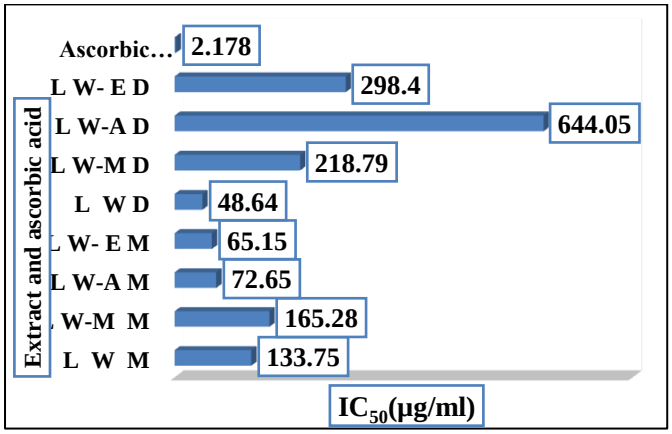

Figure 4: Minimum inhibitory concentrations 50 (IC50) of the extracts tested and the standard (ascorbic acid) by the free radical scavenging method (DPPH °).

DISCUSSION

In recent years, interest in natural antioxidants, in relation to their therapeutic properties, has increased considerably. Scientific researches in various specialties has been developed for the extraction, identification and quantification of these compounds from several natural substances namely; medicinal plants and agri-food products .[21]The results showed that the extraction yield differs according to the method and the extraction solvent. The average extraction yields of the two methods used were very similar and close for maceration and decoction. Maceration extraction is one of the simplest and most widely used extraction method for determining the content of medicinal plants as secondary metabolites, [22] and preserves metabolites and their structure, in contrary the extraction by the decoction where the heat is likely to degrade certain thermosensitive chemical compounds. [28]However, this does not mean that the decoction does not remain effective for the extraction of phenolic compounds, because it is an operation, which can be reserved for the extraction of non-thermolabile active principles. It is very fast, requiring only contact times of ten minutes. [32] In our case, the best averages of extraction yields by different solvents were for aqueous extracts. This can be explained by the fact that water is a highly polar solvent known to extract a wide range of molecules of which a significant amount of non-phenolic compounds such as carbohydrates and proteins which are one of the main causes of the increase in yield corresponding to the extraction method with hot water or by decoction. [23] .The results of the total polyphenol assay showed that the highest levels were recorded in the maceration extracts. The decoction seems to be the best method of extracting flavonoids and tannins. Our results are in agreement with the work of Mahmoudi and his collaborators (2013) ,[8] who found that maceration seems to be the best method of extraction of total polyphenols .Also, Bourgou et al., (2016) [24] revealed that the decoction of the aerial part of Euphorbia helioscopia showed high levels of flavonoids. Among the four solvent systems used, methanol offered the highest content of polyphenols (18.75 ± 0.55 mg GAE/ g DW). Our results are in agreement with (Gamil E et al., 2013 and Salem et al., 2016) that showed that the maximum amount of total phenolic compounds was obtained from artichoke leaves using methanol as the solvent for extraction .[25, 2 6] However, the contents of phenolic compounds were much higher than ours. This may be due to the variation in the metabolite content of each species as well as the geographical origin, the conditions and the duration of the drying during the harvest period. [27, 23] Regarding the extraction solvent, whatever the mode of extraction, ethanol is the best flavonoid extractor. For *Silybum marianum* leaves, the flavonoid content in the hydro-ethanol extract by maceration is lower than that of the same species previously studied by Sun et al (2016) ,[28]in the range of 03.04 ± 0.45mg ECE / g DW. Flavonoids have a wide distribution in the plant world, they are present in all parts of higher plants [29] and the great distinction between the parties appears in the level of wealth of some and the poverty of others. [08] Several previous studies have shown that the extraction yield increases significantly with the use of aqueous ethanol or aqueous methanol compared to pure organic solvent extractions. [30] More specifically, the addition of water to the polar solvents improves the extraction of phenolic compounds, in particular with ethanol, methanol or acetone. [31] The aqueous extract of leaves by decoction and maceration, had the highest contents of condensed tannins (03.86 ± 0.22, 02.30 ± 0.22 mg TAE / g DW) respectively followed by the aqueous acetonic extract (01.66 ± 0.23 mg TAE / g DW). This result is in agreement with numerous results of researchers who indicated that the aqueous extract had the highest contents of condensed tannins followed by the aqueous acetonic extract. The disadvantage is that water extracts undesirable substances such as proteins and non-phenolic pigments that cause interference when tannin is being measured. [14] The antioxidant power of our extracts was evaluated through two methods (TAC and DPPH radical scavenging). A comparison of the antioxidant activities of the extracts obtained by the two techniques described above was carried out. The extraction by maceration gave extracts, which have a good antioxidant capacity compared to the decoctions. This may be due mainly to the richness of its extracts in biomolecules particularly: polyphenols, flavonoids and tannins endowed with important antioxidant activities and depending on the chemical structures of the bioactive molecules. The results of this activity indicate that the two extracts: aqueous-

ethanol extract and aqueous- methanol extract of the leaves by maceration had the best antioxidant capacity compared to other extracts and had almost the same value (73.84 ± 5.90 and 73.64 ± 5.93 mg GAE / g DW respectively). This result can be explained by the fact that leaves extracts by polar organic solvents such as ethanol (polarity of 5.2) and methanol (polarity of 6.6) contain molecules such as polyphenols endowed with important antioxidant activities in comparison with less polar solvents. In addition, the nature of the solvents used, affects significantly the total antioxidant activity of the extracts. For the DPPH free radical scavenging test, the work done by Salem et al. (2016) stated that the percentage of free radical inhibition (DPPH) increases with increasing concentration. [26] This work shows that aqueous methanol extracts and aqueous acetonic extracts of *Silybum marianum* leaves from Tunisia have a great ability to reduce free radicals and can be used as remarkable antioxidants. Nisar et al (2013b) [32] report that the percentage of inhibition (PI%) of the stable radical (DPPH °) recorded by the aqueous ethanol extracts for the leaves of *Silybum marianum* was 52% and the work of Jing Sun, (2016) and his collaborator,[28] showed that the aqueous ethanol extracts of the leaves recorded an inhibition rate of DPPH of 56.82%. These results were a little lower compared to our extracts with a value of about 68.88%. [29] We have noticed that the aqueous leaves extract by decoction had the most important antioxidant power compared to the other extracts. This can probably be related to the quantity and quality of the polyphenols, flavonoids and condensed tannins. The availability of these can act as radical scavengers. [34] The aqueous ethanol and aqueous acetonic extracts by maceration showed an important antioxidant potential. These results are in agreement with previous work that concluded that extracts obtained using polar solvents have a more efficient activity than less polar solvents. [32] In the same optics, Bourgou et al. (2016) and Bettaieb et al. (2016) studied the influence of different solvents on the antiradical activity where ethanol was the most active, because of the good polarity and solubility of the antioxidants in this solvent. [3, 24]These authors proved that the addition of water at low ratio to organic solvents improves the extraction of powerful antioxidants.

Conclusion

From our results, we find that the leaves of *Silybum marianum* contain variable contents in phenolic compounds and consequently have different antioxidant activities. The average extraction yields of the two methods used are very similar. The results showed also, that the aqueous extracts represent the highest yield, and that the total polyphenol contents were important in methanol extract, obtained by maceration. Ethanol is the best extractor of flavonoids by decoction while the aqueous extracts (by decoction and maceration) had the highest contents of condensed tannins. On the other hand, the aqueous methanol and aqueous ethanol extracts of leaves obtained by maceration have a good antioxidant capacity compared to the decoction. Concerning the DPPH technique, the aqueous extract obtained by decoction was the most active in scavenging the free radical. The extraction by decoction seems to be a better method than maceration. Therefore, differences in the antioxidant activities of the extracts may reflect differences in their composition of phenolic compounds. In addition, the extraction of these compounds and the estimation of the antioxidant activity of the crude extracts obtained from *Silybum marianum* leaves is strongly influenced by the nature of the solvent and the extraction method used. Our results are very promising and encouraging reflecting the richness of the leaves in phenolic compounds, which can play a powerful antioxidant role, considering the nutritional and therapeutic virtues of these compounds. For a better valorization of *Silybum marianum* leaves, and in order to reduce certain diseases related to oxidative stress, further works are needed to isolate and determine the active compounds responsible for this antioxidant activity.

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