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Phytoconstituents and biological activities of different *Silybum marianum* (L.) Gaertn. seed extracts and oils and their preventive effect on butter oxidation

Chaaba Raja *, , Ben Khaled Hayet ***◆, Nakbi Amel *,****, Amri Zahra*, Hammami Sonia *, Hammami Mohamed* and Mehri Sounira***

** University of Monastir, Faculty of Medicine, Nutrition-Functional Food and Health-Lab, LR12ES05, Tunisia*
*** Higher School of Health Sciences and Techniques, Sousse, Tunisia*
**** Department of Pharmaceutical Chemistry, Pharmacy College, Northern Border University, Rafha, Saudi Arabia*
***** Superior Institute of Applied Sciences and Technology, Mahdia, Tunisia*

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Abstract

Silybum marianum (L.) Gaertn. (milk thistle) has been widely used as a medicinal plant because of its numerous protective and curative effects in many diseases, especially in liver disorders as well as in a food remedy. The current study aimed to investigate the composition of *S. marianum* (SM) seed extracts (hexanoic, acetonetrile, methanolic and aqueous extracts) and oil. Then to determine their biological activities (antioxidant and lipase inhibition). Finally, to evaluate the antibacterial and antifungal activities of SM seed oil as well as its preventive effect on the state of butter oxidation. Our results showed that the total phenol are more concentrated in methanolic extract, followed by acetonitrile, aqueous and hexanoic extracts. Flavonoids are more frequent in acetonitrile extract, followed by methanolic, aqueous and hexanoic extracts. Regarding biological activities, acetonitrile extract exhibited the highest antiradical activity and inhibition of pancreatic lipase activity whereas methanolic extract showed the highest reducing power. Additionally, seed oils exhibited a high antibacterial activity against Gram+ bacteria strains (*Staphylococcus aureus, Staphylococcus albus*). They also showed a fungicidal activity against *Saccharomyces cerevisiae* and *Candida albicans*. Additionally, oils have an effective protective effect against butter oxidation. They decreased the degree of butter peroxide value during storage at 4°C.

1. Introduction

Silybum marianum (L.) Gaertn. (milk thistle) is considered as a medicinal plant, which is belonging to the Asteraceae family. It has been widely used in hepatoprotective remedy by its high biological activities including antioxidant and anti-inflammatory activities. This plant has been proposed as a drug by the Food and Drug Administration in Germany to treat many digestive disorders (Natural Medicines Comprehensive Database, 2012). In addition, *S. marianum* fruit extracts showed high antiviral (Polyak *et al*., 2010) and antitumor (Scambia *et al.,* 1996) activities. Numerous studies have concentrated on their constituents in the therapy of cancer for chemoprevention, treatment and amelioration of side effects related to chemotherapy. According to the study of Bahmani *et al*. (2015), *S. marianum* has been considered as a remedy for all diseases. It is a very potent antioxidant by its capacity of scavenging free radicals and preventing lipid peroxidation.

Different countries in the world employed the *S. marianum*'s leaves, stems, flowers, and seeds for a variety of purposes. Numerous substances, including silybin, silibinin A and B, silicristin, silidianin, apigenin, dehydrosilybin, deoxysilyn cristin, and deoxysilyn dianin, are found in the plant's seeds (Kroll *et al.,* 2007; Braun *et al.,* 2010). Many diseases, such as cirrhosis and viral hepatitis, can be cured with the oil extracted from the SM seeds (Elwekeel *et al*., 2013; Gurbuz *et al*., 2000). Furthermore, SM derivatives have been proposed for the enrichment of food products because of their high proportion of fiber, flavonolignans, and beneficial unsaturated fatty acid content (Andrzejewska *et al.,* 2015).

Recently, great interest has been attributed to natural antioxidants (Brewer., 2011). For example, *Moringa oleifera* Lam. (Moringaceae) leaf extract (Nadeem *et al.,* 2013**)**, Sesame seed oil (Subrahmaniyan *et al*., 2024), orange peel extract (Asha *et al*., 2015) and extract from tomato processing by-products (Abid *et al.,* 2017), were used to prevent oxidation in food. In this context, SM extracted oils could be used as an antioxidant in lipid-rich food such as butter.

The chemical composition of SM seed extracts and oils was investigated in our current study. Their biological activities were evaluated. The antibacterial and antifungal activities of two different seed oil extracts (butanol and chloroform) were assessed along with their ability to inhibit butter oxidation.

2. Materials and Methods

2.1 Seed extracts preparation

Silybum marianum (SM) is authenticated by Dr. Mohamed Ashour, the Phytochemist in Pharmacy College of Rafha, Pharmacognosy Department, University of Northern Border Region, Saudi Arabia.

Corresponding author: Dr. Ben Khaled Hayet
Assistant professor Department of Pharmaceutical Chemistry, Pharmacy College, Northern Border University, Rafha, Saudi Arabia
E-mail: hayet.khaled@nbu.edu.sa; hayet.mohamedbenkhaled@yahoo.fr
Tel.: +966-534826769

The Voucher Number of Specimen is PHC-2024-01. 20 g of SM seeds are used for extraction and are percolated with 300 ml hexane. Then, they are filtrated to obtain F1 and residue R1. R1 was percolated with 300 ml acetonitrile and filtrated yielding R2 and F2. Then, R2 is mixed with 300 ml methanol and filtrated. The last step was to concentrate the filtrates F1, F2 and F3 by evaporation till dryness to yield three different extracts (hexanoic, acetonitrile and methanolic). Regarding Aqueous extract, it was prepared by mixing seeds with water at high and low temperature.

2.1.1 Oil preparation

SM oil seeds were obtained by cold pressing extraction.

2.2 *S. marianum* seeds characterization

2.2.1 Carbohydrate content

The total sugar content of the samples is evaluated by the method of phenol-sulfuric acid. Glucose is used as a standard (Dubois *et al.,* 1956).

2.2.2 Protein content

The content of protein is determined according to the method of Bradford. BSA (bovine serum albumin) is used as a standard (Bradford**,** 1976).

2.2.3 Moisture content

The SM seeds content is performed in an oven at 105°C. The moisture content of SM seeds is calculated by measuring the mass loss of the sample in triplicate (Duraisankar and David, 2015) and expressed as the following formula:

MC (%) = $(w_0 - w_1 / w_0) \times 100$, where w_0 and w_1 represent the respective masses (g) of the sample before and after drying

2.2.4 Total ash content

A porcelain capsule containing 3 g of dried powder of SM seeds is incinerated in an oven at 550 ± 5°C for 8 h until it turns gray or whitish. The total ash content is calculated in triplicate as mg/g of the dried material. The amount is represented in a percentage using the following formula:

DM% = $(w_1 - w_2)/w_0 \times 100$

Total ash content = 100 – DM %

DM%: Dried material

w_1: weight of capsule + weight of sample after drying (g)

w_2: weight of capsule + Ash (g)

w_0: weight of sample after drying (g)

2.2.5 Lipid content

The lipid content is determined using the weight of the hexanoic extract and the initial weight, according to the following formula:

[w (extract)/w (initial)] × 100

2.2.6 Total phenol composition

Total phenol composition in SM seed extracts is determined according to the method of Folin Ciocalteu (Montedoro *et al.,* 1992). About 800 µl of distilled water and 5 ml of folin reagent (10-fold dilution with distilled water) are added to 200 µl of each extract. After one minute of dark incubation, 4 ml of sodium carbonate Na_2CO_3 (7.5%) are added. After stirring for a few seconds with the vortex, the mixture is incubated for 2 h in the dark and then the OD is read at 765 nm.

2.2.7 Flavonoids composition

The composition of flavonoids is estimated by the aluminum trichloride $AlCl_3$ method. 4 ml of distilled water and 0.3 ml $NaNO_2$ (5%) are added to 1 ml of each extract. After 5 min of incubation, 0.3 ml of $AlCl_3$ (10%) is added and the mixture is incubated for 6 min. Then, 2 ml of NaOH (1M) are added. The volume is adjusted to 10 ml by the addition of 2.4 ml of distilled water. The mixture is homogenized with a vortex and the OD is read at 500 nm (Dewanto *et al.,* 2002).

2.3 Biological activities of SM seed extracts

2.3.1 DPPH radical scavenging activity

A previously developed test with minor adjustments is used to assess SM seed extracts (DPPH) radical scavenging activity (Senol *et al.,* 2011). 100 µl of each extract is combined with a DPPH solution (1400 µl). After mixing, the mixture is incubated at room temperature for half an hour in the dark. The absorbance is measured at 517 nm. Ascorbic acid was the positive control. Results are expressed as free radical scavenging activity or free radical inhibition in percentages using the following formula:

% = [(Control Abs – Sample Abs)/Control Abs] × 100

Blank = 100 µl of the solvent of each extract + 1400 µl methanol

Control = 100 µl of each solvent + 1400 µl of DPPH

Sample = 100 µl of each extract + 1400 µl of DPPH

2.3.2 Reducing power

The method of Barbosa *et al.* (2009) is used with slight modifications, to estimate the reducing power test. 625 µl of phosphate solution (200 mM; pH 6.6) and 625 µl of 1% potassium ferricyanide ($K_3Fe(CN)_6$ are added to 250 µl of each extract. The mixture has been incubated for 20 min at 50°C. Then, 625 µl of trichloroacetic acid (TCA) (10%) are added and centrifuged for 10 min at 3000 rpm. To a volume of 625 µl of the supernatant, 625 µl of distilled water and 125 µl of ferrichloride ($FeCl_3$) (0.1%) are added. The absorbance is carried out at 700 nm, and the result is calculated as a function of the increase in OD.

2.3.3 Antilipase activity

To evaluate the antilipase activity of the seed extracts of *S. marianum*, the following steps are followed:

200 µl of the enzyme are added to 500 µl of each extract in Tris HCl (0.01 M) and incubated for 30 min at 4°C. 2 ml of substrate (the substrate is composed of 10 ml of olive oil, 10 g of gum arabic, 1.57 g Tris-HCl, 2.92 g NaCl and 0.2 g $CaCl_2$) are added and the mixture is incubated for 30 min at 37°C. To stop the reaction, 1 ml of ethanol/acetone is added and the titration is carried out with NaOH (0.02 M). The anti-lipase activity which is the degradation of olive oil from the substrate is calculated by dividing the acid concentration, is calculated following the titration by NaOH over 30 min (incubation time). The results are compared with the activity of the enzyme in the absence of the extracts.

2.3.4 Antibacterial and antifungal activities of SM seed oils

Antimicrobial activity is investigated using the Agar (Mueller Hinton) well diffusion method in accordance with the described protocol (Collins and Lyne, 1970). 20 µl of butanol and chloroform seed oil extracts are utilized (10 µl SM oil + 10 µl of butanol or chloroform). After incubation during 24 h at 37°C, the inhibition zones are expressed in millimeters (mm). The activity of the tested samples are determined against *Escherichia. Coli, Serrita* sp. *Staphylococcus aureus and Staphylococcus albus.*

Sensitivity to different oils is classified according to the diameter of the inhibition zones as follows (Hassan *et al.,* 2006):

- Not sensitive (-) for diameters less than 10 mm
- Sensitive (+) for diameters between 10 and14 mm
- Very sensitive (+ +) for diameters between 15 and19 mm
- Extremely sensitive (+ + +) for diameters greater than 20 mm

Regarding the antifungal activity, the protocol is the same but Sabouraud Agar is used as medium. This method is mostly used because of its specificity and composition; it ensures good growth of fungi and gives clear results (Horikawa *et al.,* 1999). The antifungal capacity of SM seed oil extracts are assessed against *Penicillum* sp, *Aspergillus niger, Candida albicans and Saccharomyces cerevisiea*.

2.4 Effect of SM seed oil extracts on butter oxidation

SM seed oil was added in two different ways:

First way

SM oil was added to commercial butter in different concentrations; 100 g of butter without SM oil (blank); 1 g of SM oil is added to 100 g of butter (butter preparation 1); and 10 g of SM oil are added to 100 g of butter (butter preparation 2). Then, each preparation was divided into two samples (50 g each) to be stored at 20°C and 4°C for 21 days.

Second way

To obtain butter, SM oil was added to fresh cow milk in different concentrations; without SM oil (blank); 0.1 g of SM oil is added to 1 liter of milk (milk 1) and 10 g of SM oil are added to 1 liter of milk (milk 2). After that, milk was maintained at room temperature and darkness to become a single block, and then agitated until the butter is formed at the top. So, three traditional butters are obtained. Finally, they are stored at 4°C for 21 days.

The analytical procedures described in European Union Regulations EEC 2568/91 and EEC 1429/92 (EEC, 1991) are used to measure the peroxide value (PV) and UV absorption properties at 232 nm (K232) and 270 nm (K270).

2.5 Statistical analysis

Statistical analyses were used with SPSS ver. 17.0, professional edition using ANOVA analysis at a $p = 0.05$.

3. Results

3.1 Phytoconstituents of different *S. marianum* seed extracts and oils

Medicinal plants have been practiced for many centuries and they are becoming more and more popular in the world. In our study, the composition of different SM seed extracts and oil, is evaluated. As shown in table 1, SM seeds are rich in lipid (38 g/100 g) and protein (34.2 g/100 g). This plant was considered as a very rich source of protein and lipid. Regarding the phyto-constituents of SM seed extracts, the total phenolic composition is more concentrated in methanolic extract (28.31 mg GAE/g DW), followed by acetonitrile (13.04 mg GAE/g DW), aqueous (3.31 mg GAE/g DW) and hexanoic extracts (1.78 mg GAE/g DW) (Table 2). Whereas, the flavonoids are more frequent in acetonitrile followed by methanolic, aqueous and hexanoic extracts.

Table 1: SM seed characterization

Parameters	values	Composition	values
Moisture (g/100 g DW)	3.22	Protein (g/100 g DW)	34.2 ± 5.7
Ash (g/100 g DW)	3.57	Carbohydrate (g/100 g DW)	21.45 ± 0.43
		Lipids (g/100 g DW)	38.95

DW: dried weight

Table 2: Composition of SM seed extracts and oil

	Hexanoic extract	Acetonetrile extract	Methanolic extract	Aqueous extract	oil
Total phenol	1.78 ± 0.02	13.04 ± 0.5	28.31 ± 2.7	3.31 ± 0.07	5.1 ± 0.85[f]
Total flavonoid	0.69 ± 0.31	3.11 ± 0.31	2.36 ± 0.09	1.25 ± 0.01	3.92 ± 0.03[f]

1. Values for methanole oil fraction; 2. Total phenol (mg GAE/g); 3. Total flavonoid mg equivalent catechin per g of dried weight.

3.2 Antioxidant and antilipase activities of different *S. marianum* seed extracts and oil

The antioxidant activity of medicinal plant extracts and their extracted oil is evaluated using a variety of techniques. For our study, seed extracted oil exhibited the highest antiradical scavenging activity (64%) (Table 3). Due to its high antioxidative property, SM seed oil can be used in cosmetic, pharmaceutical and food industry. Furthermore, there is a difference of antiradical ability between SM seed extracts. The acetonitrile extract is more effective against free radicals (47.54%) and pancreatic lipase activity (87.87%), while the methanolic extract showed the strongest reducing power (0.896).

3.3 Antibacterial and antifungal activities of SM seed oils

All of the extracts exhibit an antimicrobial activity on at least one of the bacterial tested strains. However, only zones of inhibition greater than 10 mm are regarded as positive. The results presented in table

4, showed that *S. albus* exhibit the highest inhibition with 18 mm for the chloroform extract and 19 mm for the butanol extract. The smallest inhibition is obtained with *S. aureus* with 17 mm and 16 mm for the chloroform and butanol extracts, respectively.

Table 3: Biological activity of SM seed extracts and oil

	Hexanoic extract	Acetonetrile extract	Methanolic extract	Aqueous extract	oil
Biological activities					
DPPH (%)	33.89	47.54	35.51	28.17	64^{f}
Reducing power	0.021	0,751	0.896	0.031	0.306^{f}
Lipase inhibition (%)	75.45	87.87	66.7	0	75.45

Table 4: Antimicrobial activity of SM seed oil extracts plant against selected microorganisms

Zones of inhibition (mm)		
Bacterial strains	**Chloroform oil extract**	**Butanol oil extract**
Gram +ve bacterial strains		
Staphylococcus albus	18 mm	19 mm
Staphylococcus aureus	17 mm	16 mm
Gram -ve bacterial strains		
Escherichia coli	< 10 mm	< 10 mm
Serrita sp.	< 10 mm	< 10 mm

Table 5: Antifungal activity of SM seed oil extracts plant against selected microorganisms

Zones of inhibition (mm)		
Fungi strains	**Chloroform oil extract**	**Butanol oil extract**
Candida albicans	17 mm	18 mm
Saccharomyces cerevisiae	15 mm	16 mm
Penicillum sp.	< 10 mm	< 10 mm
Aspergillus niger	< 10 mm	< 10 mm

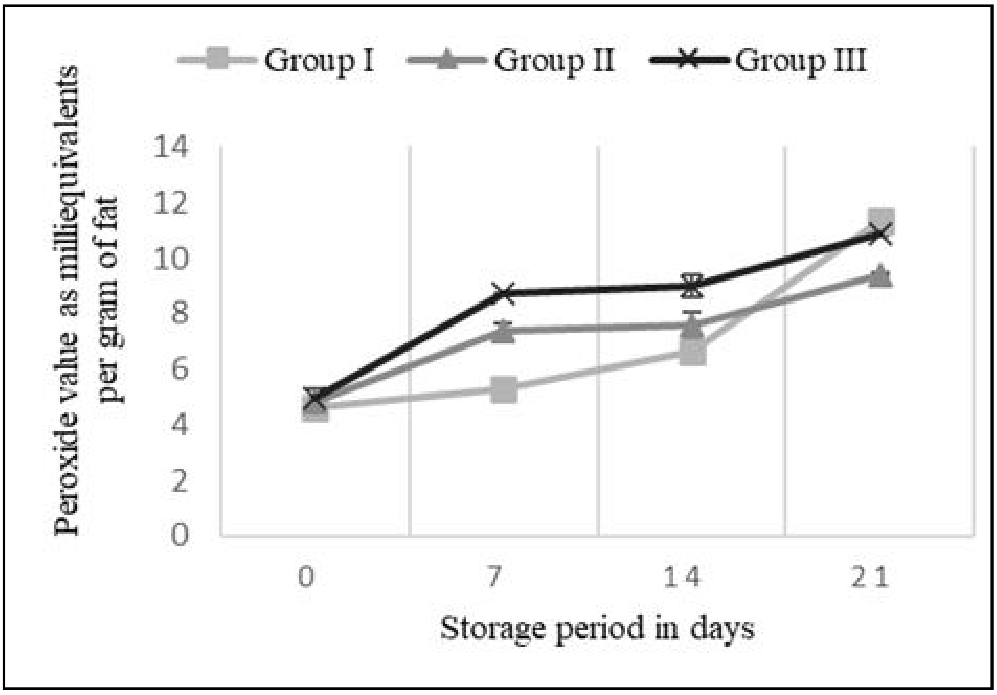

Figure 1a: Variation of peroxide value during storage: Commercial butter at 20°C.

Regarding antifungal activity of the different extracted oils, both butanol and chloroform extracts have an activity against *Candida albicans* and *Saccharomyces cerevisiae* (Table 5), but these vary according to the extract. It is noted that with inhibitory diameters of 18 and 17 mm for *C. albicans* and 16 and 15 mm for *S. cerevisiae*, respectively, it is shown that the butanol and chloroform extracts significantly affect the two microorganisms.

3.4 Effect of SM seed oils on butter peroxidation

The oxidation of milk and milk products as well as its extent, depend on many parameters such as oxygen, light, endogenous compounds like antioxidants, temperature. To evaluate the status of oxidation particularly in the early stage, peroxide value (PV) is determined. According to Figure 1, PV increases during the storage of commercial butter at 4°C and 20°C. Also, PV increases during the storage of traditional butter at 4°C. The increase of PV is more pronounced in 20°C (143 % from day 0 to day 21 for commercial butter) compared to 4°C (40 % from day 0 to day 21). As known, 4°C is ideal for butter conservation.

After adding SM oil, the degree of increase of PV is reduced in all samples. In 20°C the variation was not significant (143 % without SM oil vs 96 % with 1 g of SM oil vs 119 % with 10 g of SM oil) (Figure 1a). The same result has shown for traditional butter (Figure 1c). In 4°C, the best result has shown after adding 1 g of SM oil (percentage of variation is about 2 only: initial PV = 4.81 ± 0.02 vs final PV = 4.93 ± 0.10; $p < 0.05$). In all cases, the use of 1% oil is the best concentration. To minimize the oxidation of butter, the ideal conditions are storage at 4°C in the obscurity, adding only 1 g of SM oil to final butter and not during manufacturing process.

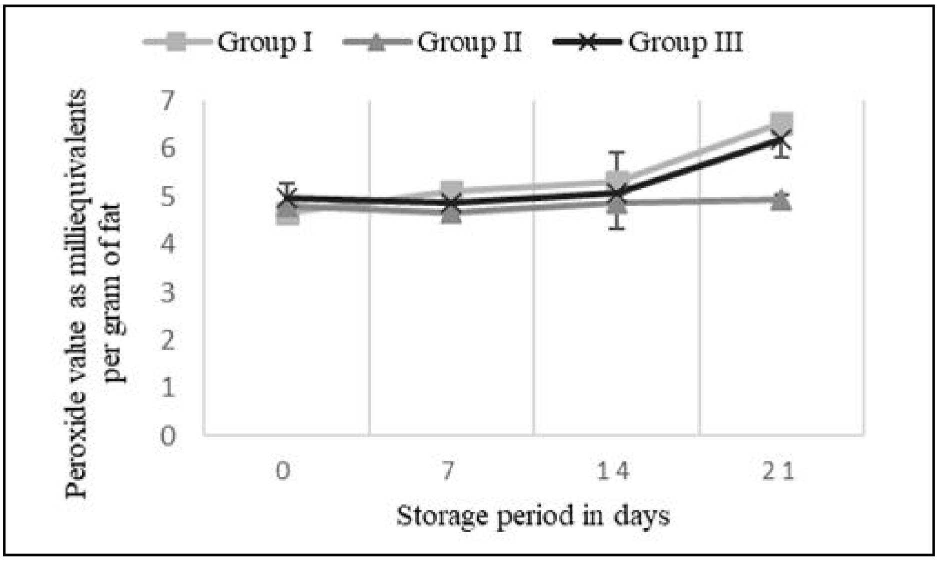

Figure 1b: Variation of peroxide value during storage: Commercial butter at 4°C.

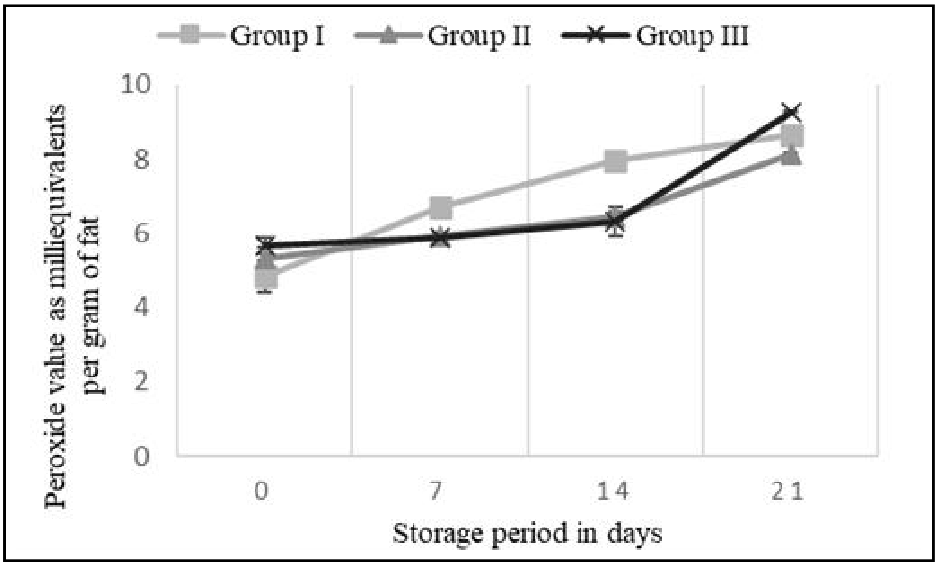

Figure 1c: Variation of peroxide value during storage: Traditional butter at 4°C.
[Group I: Butter without SM oil (control), Group II: Butter with 1 g of SM oil (1%), Group III: Butter with 10 g of SM oil (10%)]

4. Discussion

Our results are similar to those found by Wallace *et al.* (2005) and Abenavoli *et al.* (2010), 15 - 30 % lipid and 30 % protein (20.5 g/100 g protein and 10.8 g/100 g residual oil). Additionally, SM seeds are rich in carbohydrate (21.45 g/100 g) but the content is lower than those found by Denev *et al.* (2020) (42.2 g/100 g). It has been found in literature that SM seeds are a high source of hetereo-polysaccharides exhibiting different biological activities (Fan *et al.,* 2019).

According to the phytoconstituents of SM seed extracts, an optimization of extraction method from SM seeds is necessary to better explore the benefit of SM seeds. Jahan *et al.* (2016), demonstrated in their study that silymarin seed compositions are high in extracts prepared with methanol using the microwave assisted extraction technique followed by Soxhlet in both spectrophotometric and HPLC quantification.

Total polyphenol content in methanolic seed extract is comparable to those reported by Mhamdi *et al.* (2016), (29 mg GAE/g DW). While, they are higher than those found by Lucini *et al.* (2016), (3.6 mg GAE/g DW) and lower than those found by Beniwal *et al.* (2024). This difference in polyphenol content might be explained by the geographical location of cultivation and agronomic conditions of *S. marianum* (Martin *et al*., 2006).

Regarding the antioxidant activity of the different extracts, the increase in absorbance of the reaction mixture indicates an increase in the reducing capacity (Gülçin, 2006). Biological activity variations could be explained by the difference in extract composition. To accurately assess the antioxidant capacity, it is essential to consider the extraction method, the solvent utilized and the specific assay technique employed (Škrovánková *et al*., 2012).

Additionally, we notice that the butanol and chloroform oil extracts, have a significant action against Gram-positive bacteria (inhibition diameter greater than 10) and no effect against Gram-negative bacteria. This result could be explained by the accessibility of these molecules to the peptidoglycans constituting the wall of Gram-positive bacteria. The bacteria then lose their rigidity and lyse under the effect of their internal osmotic pressure, which ruptures their cytoplasmic membrane. However, in Gram-negative bacteria, the spatial configuration of the molecules prevents the transport proteins (porins) from crossing the external membrane of Gram-negative bacteria, and therefore cannot reach the peptidoglycan of the bacterial wall (Fivenson *et al.,* 2023).

The sensitivity of bacteria (*S. albus and S. aureus*) to chloroform and butanol extracts can be explained that the plant flavonoids exert growth inhibition via several mechanisms: inhibition of the biosynthesis of membrane proteins and phospholipids, inhibition of their nucleic acid. This is can be explained the sensitivity of bacteria (*S. albus and S. aureus*) to chloroform and butanol extracts (Hassan *et al.,* 2006). The flavonoid extracts of *S. marianum* probably exert their action by one of these two mechanisms.

Different extracts have a significant action on yeasts and no action on molds. However, this can be explained, by the accessibility of the yeast wall and not for the molds, which are protected by the rigid mycelial structure. In fact, some authors have mentioned that their wall is made up of three polysaccharides: β-1,3 glucan, chitin and mannan associated by chemical bonds (Aimanianda *et al.,* 2009). It is likely that the molecules contained in the extracts inhibit the synthesis of chitin and then act like antibiotics (Chaudhary *et al.*, 2013). On the other hand, the mechanism of action of antimicrobial agents on yeasts remains poorly understood (Hassan *et al.,* 2006).

Numerous investigations were carried out to assess how various products affected the oxidation of butter. According to Soulti and Roussis, butter's phenolic components, particularly gallic acid, may act as antioxidants (Soulti and Roussis, 2007). In addition, Papadopoulou and Roussis (2008), showed that N-acetyl-cysteine and glutathione exhibit a great effect on butter oxidation. More else, Ozkan *et al.* (2007) determined that essential oil of *Satureja cilicica* could be used as antioxidant for butter. The antioxidant effect of natural products, on butter would provide new approaches to maintain quality and shelf-life of butter and to minimize the side effects of use of synthetic antioxidant like butylated hydroxytoluene (BHT) (Nahm *et al.,* 2012). In the case of use of natural products having medical benefit, the medical value will be added to the final product. In our case of SM oil, the question is "in addition to the antioxidant effect, could the oil confer its medical properties to butter?". To confirm such hypothesis, additional *in vitro* and *in vivo* research should be carried out.

5. Conclusion

Based on our findings, to better explore the effect of *S. marianum seeds* in various diseases, it is necessary to choose the appropriate extract. As aqueous extract for the treatment of dyslipidemia and acetonitrile for antioxidant use (many diseases). Studying extract composition is needed. Furthermore, oils extracted from SM seeds could be exploited as an effective antibacterial and antifungal agent rather than as a preventive additive on butter oxidation.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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