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Physicochemical Properties, Antioxidant and Antimicrobial Activities of Milk Thistle (*Silybum marianum* L.) Seed and Leaf Oil Extracts

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Key words: Antioxidant activity, antimicrobial potential, DPPH, inhibition zone, minimum inhibitory concentration, test pathogens

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Abstract: Milk thistle (*Silybum marianum* L.) fruit extract (silymarin) is a flavonolignan called silybin that has been indicated to conserve animal livers against the harming impacts. The present study was aimed to examine physicochemical properties antioxidant and antimicrobial activities of oil extracted from *S. marianum*, seeds and leaves. The oil extraction was done in Soxhelt apparatus using petroleum ether as a solvent. Then, physicochemical properties of the oil extracts were conducted based on determination of oil content, specific gravity, acid value, percent free fatty acid and peroxide values. The antioxidant activity was investigated based on determination of ascorbic acid content, DPPH and hydrogen peroxide free radical scavenging activities. The antimicrobial activity test were conducted using disc diffusion method and broth dilution method. The result of physicochemical properties of *S. marianum* seed and leaf oils indicated significance differences between seed and leaf oils were obtained for all measured parameters. It was found that oil yield (44.5%), specific gravity (0.89), acid value (1.54) and free fatty acids (0.78% were significantly higher for seed oil extract. The antioxidant activities of *S. marianum* seed and leaf oil extracts presented significantly higher antioxidant activities with respect to ascorbic acid content (55.05±1.47) and DPPH value (18.00±2.55) for seed oil than for leaf oil. Stronger antibacterial activity with maximum zone of inhibition (15.50mm), bactericidal activity with MIC (0.06 µL/mL) and MBC (0.12 µL/mL) were recorded for leaf oil extract against *S. aureus.* The stronger antifungal activity with maximum zone of inhibition (13.83 mm), MIC (0.25 µL/mL, the least value) and MFC (0.50 µL/mL) were recorded for seed oil against *A. versicolor.* Thus, milk thistle has low peroxide and acid values which show its low oxidation rancidity and high biological activities make it suitable for nutraceutical and drug development.

INTRODUCTION

Silybum marianum (L.) Gaertn, a member of the Asteraceae/ Compositae family, is an herb whose fruits have been used medicinally for thousands of years[1, 2]. Its common name is milk thistle. It is native to the North Africa and Mediterranean area and found as annuals or biennials[3]. Milk thistle can also be cultivated for several purposes[2]. Because of its attractive, beautiful spiny and creamy foliage it is cultivated ornamentally[4]. Its stems, roasted seeds and inflorescence are consumed either raw as well as cooked[5].

The seeds yield 1.5-3% of a polyphenolic healthy component with highly medicinal properties called silymarin. The silymarin is used for liver detoxification to remove exotic chemicals[6]. "Silybin" is the main component of silymarin containing 30% calcium and is extracted from fruit of milk thistle[7]. It is hepatoprotective and used for the treatment of liver disorders[8]. Silymarin has antioxidant, anticancer, Anti-metastasis, anti-inflammatory potentials[9] and also enhances the immunity against hepatitis C[10]. Silymarin prevents infiltration of neutrophils and control spread of inflammatory agents evaluation[11].

The increasing demand of better quality oils, milk thistle seeds oil is cheap, healthy and beneficial for human consumption, having no side effects[2]. Compounds derived from *Silybum marianum* seed extract are found to have a promising effect in nutrition as well as in therapeutics. Previous studies on *S. marianum* focus on proximate analysis, physicochmical parameters and its medicinal uses[12]. Therefore, the present study was aimed to examine physicochemical properties antioxidant and antimicrobial activities of oil extracted from milk thistle (*Silybum marianum* L.), seeds and leaves.

MATERIALS AND METHODS

The experiment was conducted in Biotechnology Laboratory, Haramaya University. The milk thistle (*Silybum marianum* L.) plant sample was collected from Kemise district, South Wollo, Ethiopia. The seeds and leaf samples were manually washed with distilled water and residual moisture was evaporated at room temperature. Then after, ground to a fine powder in a grinder for 2 min, the process was stopped for 15 sec avoid heating of sample. Determination of moisture (on dry basis) was carried out[13].

The oil extraction was done in Soxhelt apparatus using petroleum ether as a solvent. Then, physicochemical properties of the oil extracts were conducted based on determination of oil content, specific gravity, acid value, percent free fatty acid and peroxide values. The antioxidant activity was investigated based on determination of ascorbic acid content, DPPH and hydrogen peroxide free radical scavenging activities.

Antimicrobial activity of the oil extracts: The antimicrobial experiment was arranged as 2×1×4 (2 source extracts: seeds and leaves of from milk thistle at three concentration levels, 1 solvent system i.e. petroleum ether, 4 test organisms (2 bacteria: *Escherichia coli* (gram negative), *Staphylococcus aureus* (gram positive), two fungal spp: *Aspergillus niger and A. versicolor*) completely randomized factorial design in three replications.

Test pathogens including two bacteria: *Escherichia coli* (gram negative), *Staphylococcus aureus* (gram positive), two fungi *(Aspergillus niger* and *A. versicolor)* were obtained from Ethiopian Institute of Food and Health, Addis Ababa, Ethiopia. The fungal and bacterial pathogens were subcultured and maintained on Potato Dextrose Agar (PDA) and Nutrient Agar, respectively. Then, the fungal and bacterial cultures were incubated for 72 h at 27ºC and for 18-24 h at 37ºC, respectively.

Media preparation and standardization of inoculum: Nutrient Agar (NA), Potato Dextrose Agar (PDA) and Muller Hinton agar (MHA) was used for sub-culturing of bacterial test organism, fungal test organism and determination of antimicrobial activities, respectively. These media were prepared and sterilized using an autoclave according to the manufacturer's instructions. The bacterial colonies and spores of the test fungi were harvested by washing the surface of the fungal colony using 5 mL of sterile saline solution. This procedure repeated until the turbidity of each bacterial and fungal spore suspension matched the turbidity of 0.5 McFarland Standards as described by the Clinical Laboratory Standards Institute[14]. The resulting suspension will be used as inoculums for the test pathogen in the antimicrobial susceptibility test.

DISC diffusion method: the discs of 6 mm diameter was prepared from sterile filter paper cut into small, circular pieces of equal size by a perforator and then impregnated each of them was impregnated with 0.01 mL of the prepared test extract solution. The extract impregnated discs were placed onto MHA plates evenly inoculated with test pathogens[15]. Following this step, the impregnated discs were dispensed onto the surface of the inoculated agar plates using sterile forceps[14]. Discs of commercial gentamycin (1 µL/disc) and fluconazole (1 µL/disc) were used as positive controls for bacterial and fungal pathogens, respectively and distilled water

impregnated discs were used as negative controls. Then the MHA plates were sealed with parafilm and incubated at 37°C for 24 h and 27°C for 72 h for bacterial and fungal pathogens, respectively. The diameters of the zone of inhibition around each disc were measured to the nearest millimeter along two axes (i.e., 90° to each other) using a transparent ruler and the means of the two readings were be recorded.

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC): The MICs of the oil extracts were determined by broth dilution method. In the broth dilution method, the oil extract solution for example at 1 µL/mL(v/v) was serially diluted in a two-fold dilution in downward direction as 1%(v/v), 0.50 µL/mL and 0.25 µL/mL, 0.125 µL/mL, 0.0625 µL/mL concentrations. Two milliliter of nutrient broth and potato dextrose broth for bacteria and fungi, respectively were added into all test tubes and 0.1 mL of the prepared concentration of each oil extract were mixed with the nutrient broth and potato dextrose. Thereafter, standardized inoculums of 0.1 mL of the respective test pathogens were dispensed into the test tubes containing the suspensions of the broth and the oil extract. Then, all test tubes were properly corked and incubated at 37°C for 24 h for bacteria and 27°C for 72 h for fungi. After that, they were observed for absence or presence of visible growth. The lowest concentration at which no visible growth of organisms were regarded as the MIC.

For the determination of the MBC and MFC, fresh nutrient agar and potato dextrose agar plates were inoculated with one loop full of culture taken from each of the broth cultures that showed no growth in the MIC tubes. That is MBC/MFC values were determined by subculturing from respective MIC values if for example MIC = 0.50 µL/mL (v/v) subculturing was performed as 0.50 µL/mL, 1.00 µL/mL, 1.50 µL/mL, 2.00 µL/mL up to four acceptable concentration levels. Since antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC. While MBC assay plates were incubated for 48 h, MFC assay plates were incubated for 3 days. After the incubation periods, the lowest concentration of the extract that did not allow any bacterial or fungal growth on solid medium was regarded as MBC and MFC for the extract[14]. This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation for bacteria or spore germination after 3 days of incubation for fungi.

The experimental data were analyzed using SAS version 9.2.[16] to investigate statistical significance between the different oil quality parameters. Differences between means were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Physicochemical properties of milk thistle (*Silybum marianum* L.) seed and leaf oil extracts: The result of physicochemical properties of *Silybum marianum* seed and leaf oils were determined based on oil yield, specific gravity, acid value, free fatty acids and peroxide values as in shown in Table 1. Significance differences between seed and leaf oils were obtained for all measured parameters. It was found that oil yield (44.5%), specific gravity (0.89), acid value (1.54) and free fatty acids (0.78% were significantly higher for seed oil extract. The peroxide value (1.90) was found to be significantly higher for leaf oil extract. Low peroxide value of seed oil shows its low oxidation, rancidity and high antioxidant activity. The acid value is the weight of potassium hydroxide in mg required to neutralize the organic acids present in 1g of the substance. The acid value is often a good measure of hydrolytic rancidity which has an adverse effect on the quality of many lipids. In general, it gives a Specific gravity is a parameter used to identify, measure concentration and confirm purity of substances. In the present study, the value of specific gravity obtained 0.73 for leaf oil and 0.89 for seed oil is <1 indicating that the oil is less dense than water suggesting that the oil composed of light molecular weight components and therefore volatile. Acid value is used as an indication of edibility of oil and suitability to be used in the paint industry and that are within range of (1.26-2.95), falls within the recommended codex of 0.6 and 10 for virgin and non-virgin edible oils and fats[12]. The peroxide value of *S. marianum* seed oil is low (1.10 meqKOH/g) compared to the maximum acceptable value of 10 meqKOH/g set by the Codex Alimentarius Commission for groundnut seed oils[17].

Antioxidant activities of milk thistle (*Silybum marianum* L.) seed and leaf oil extracts: The antioxidant activities of *S. marianum* seed and leaf oil extracts presented significantly higher antioxidant activities with respect to ascorbic acid content (55.05±1.47) and DPPH value (18.00±2.55) for seed oil than for leaf oil. However, insignificantly higher hydrogen peroxide free radical scavenging activities (15.90±0.14) was obtained for seed oil extract. The higher DPPH antioxidant activities in seed oil extract indicates the presence of higher essential omega-3 fatty acids in *S. marianum* seed oil extract (Table 2).

Antimicrobial activities of milk thistle (*Silybum marianum* L.) seed and leaf oils: The diameter of inhibition zone for *S. marianum* seed and leaf oils presents significance differences were observed for both seed and leaf oil extracts at different concentration levels

Table 1: Physicochemical properties of milk thistle (*Silybum marianum* L.) seed and leaf oil extracts

Oil extract	Oil yield	Spgr	ACV	FFA	PV
Seed	44.50±0.71a	0.89±0.01a	1.54±0.20a	0.78±0.10a	1.10±0.14b
Leaf	36.25±1.77b	0.73±0.03b	0.70±0.19b	0.35±0.09b	1.90±0.14a

Means followed by same letter within a column were not significantly different at 0.05 probability level based on LSD (Least Significance difference) test. Small letters: significance within column; Spgr: specific gravity; ACV: acid value; FFA: free fatty acids; PV: peroxide value

Table 2: Antioxidant activities of milk thistle seed and leaf oils

Oil extract	DPPH	HPSA	AA
Seed	18.00±2.55a	15.90±0.14a	55.05±1.47a
Leaf	10.95±3.18b	14.60±0.56a	45.18±1.96b

Means followed by same letter within a column were not significantly different at 0.05 probability level based on LSD (Least Significance difference) test. Small letters: significance within column; DPPH: 2, 2- diphenyl-1-picrylhydrazyl; HPSA: hydrogen peroxide scavenging activity; AA: ascorbic acid

Table 3: Antimicrobial Activities oil extracts from milk thistle seed and leaf oil extract as mean diameter of zone of inhibition against test pathogenic microbial spp.

		Concentrations of the oil extract (v/v)			
Test pathogens	Oil extract	1 µL/mL	2 µL/mL	3 µL/mL	Gentamycin (1 µL/mL)
E. coli	Seed	0.00±0.0cD	7.50±0.45dC	12.00±0.50cB	18.33±0.10aA
E. coli	Leaf	10.17±0.55bC	13.10±0.50bB	14.50±0.36bB	18.17±0.28aA
S. aureus	Seed	9.50±0.76bD	11.50±0.36cC	13.90±0.50bB	18.16±0.30aA
S. aureus	Leaf	11.83±0.80aC	14.50±0.50aB	15.50±0.25aB	18.33±0.12aA
Fluconazole (1 µL/mL)					
Aspergillus niger	Seed	0.00±0.0cD	9.17±0.29cC	12.43±0.40bB	18.67±0.76aA
A. niger	Leaf	0.00±0.0cD	8.50±0.45cC	11.50±0.50bB	18.60±0.58aA
A. versicolor	Seed	10.00±0.50aC	12.00±0.45aB	13.83±0.76aB	18.50±0.87aA
A. versicolor	leaf	8.83±0.76bD	10.50±0.50bC	12.50±0.40bB	18.83±0.28aA

Means followed by same letter within a column were not significantly different at 0.05 probability level based on LSD (Least Significance difference) test. Small letters: significance within column; capital letters: significance across row. *E. coli*: *Escherichia coli; S.aureus*: *Staphylococcus aureus*

(Table 3). The mean zone of inhibition at highest concentration (3 µL/mL) against bacterial test pathogens ranged from 12.00±0.50-15.50±0.25 mm while 11.50±0.50-13.83±0.76 mm against fungal test pathogens. Stronger antibacterial activity with maximum zone of inhibition (15.50 mm) at highest concentration (3 µL/mL)of the oil was recorded for leaf oil extract against *S. aureus* while the weaker antibacterial activity (12.00 mm) was observed for *S. marianum* seed oil against *E. coli* indicating that *S. aureus* was more susceptible than *E. coli*. Hence, leaf oil has exhibited more antibacterial potential than seed oil in milk thistle (*S. marianum* L.). On the other hand, the stronger antifungal activity with maximum zone of inhibition (13.83 mm) was recorded for seed oil against *A. versicolor* as the weaker antifungal activity with minimum zone of inhibition (11.50 mm) was observed for leaf oil against *A. niger* suggesting seed oil extract might be more effective antifungal potential than leaf oil extract in *S. marianum*.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), Minimum Fungicidal Concentration (MFC) of milk thistle (*Silybum marianum* L.) seed and leaf oil extracts: The effectiveness of *S. marianum* seed and leaf oil extracts against pathogenic microbes was evaluated by MIC, MBC and MFC (Table 4). The leaf oil extract has exhibited strongest bactericidal activity with MIC (0.06 µL/mL) and MBC (0.12 µL/mL) against *S. aureus* while the weakest bactericidal activity with MIC (0.75 µL/mL, the largest value) and MBC (1.25 µL/mL) was recorded for seed oil against *E. coli* indicating that *S. aureus* is more susceptible to the oil extract than *E. coli* and also indicating leaf oil extract possesses stronger antibacterial potential than seed oil in milk thistle.

Table 4: MIC, MBC and MFC of milk thistle seed and leaf oil extracts

Test pathogens	Oil extract	MIC (µL/mL)	MBC/MFC (µL/mL)
E. coli	Seed	0.75	1.25
	Leaf	0.12	0.25
S. aureus	Seed	0.50	1.00
	Leaf	0.06	0.12
A. niger	Seed	0.50	1.50
	Leaf	1.00	2.00
A. versicolor	Seed	0.25	0.50
	Leaf	0.50	1.00

By contrast, *S. marianum* seed oil extract has demonstrated stronger antifungal activity with MIC (0.25µL/mL, the least value) and MFC (0.50µl/ml) against *A. versicolor* whereas the weakest antifungal activity with MIC (1.00 µL/mL) and MFC (2.00 µL/mL) was observed for the leaf oil extract against *A. niger* showing that *A. versicolor* was more susceptible to the oil extract than *A. niger* and the seed oil was more effective antifungal potential than the leaf oil in milk thistle.

CONCLUSION

Milk thistle has low peroxide and acid values which show its low oxidation rancidity and high antioxidant activity and its suitability for the use in soap industry. Reducing power of *S. marianum* increases with the increase in concentration. The *S. marianum* seed and leaf oil extract was evaluated for physicochemical properties, antimicrobial and antioxidant activities. *S. marianum* oil extracts were active against tested bacteria and fungi, suggesting its broad-spectrum biological activity. The oil also showed dose-dependent antioxidant and antimicrobial activities. The results reveal that extracted oils of *S. marianum* could be valuable sources of bioactive compounds with substantial biological activities. Milk thistle seed can be used as a promising multipurpose medicinal source whereas further clinical trial is required to prove its efficacy.

Authors' contribution: Zekeria Yusuf: initiation and design of the study, Lab experiment, data analysis; Gebremeskel Mebrate: Lab experiment, data collection and write up of the document; Sewnet Mengistu and Mulugeta Desta: Analysis and interpretation of data. All authors contributed to drafting the article and revising it critically for important intellectual content.

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Conflict of interest: The authors declare no conflict of interest.

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