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ORIGINAL ARTICLE

Isolation and Biological Evaluation of Some Secondary Metabolites from Seeds of *Silybum marianum*

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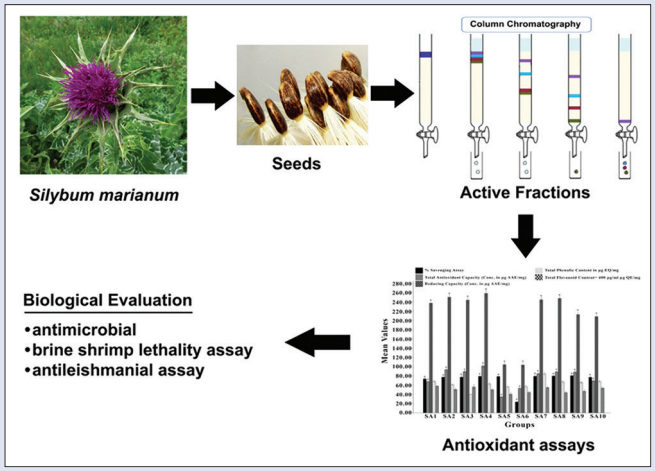
ABSTRACT

Background: Despite the widespread ethnopharmacological use and significance of *Silybum marianum* (SM) seeds, most of its phytochemical and biological properties are still yet to be confirmed using validated scientific methods. **Objectives:** The current study was designed to investigate the phytochemical and biological properties of SM seed extract. **Materials and Methods:** Methanolic extract of SM (MESM) dried seeds was fractionated by column chromatography, and fractions (SA1–SA10) were evaluated for antioxidant, antimicrobial, and cytotoxic activities (brine shrimp lethality assay and antileishmanial assay). **Results:** All fractions showed considerable level of antioxidant potential. Free radical scavenging activity of fraction (SA9) was maximum at 80.7%. Fraction SA4 exhibited substantial total antioxidant capacity (101.81 µg/mg). In ferric-based reducing antioxidant power assay, fraction SA4 showed the highest antioxidant power (258.93 µg/mg). Phytochemical screening of the fractions (SA1–SA10) inferred that total phenolic contents were maximum in fraction SA7 (85.13 µg/mg) and total flavonoid contents were found to be highest in fraction SA1 (58.24 µgQE/mg). However, mild antibacterial and antifungal activities were shown by different fractions. To evaluate cytotoxic potential, brine shrimp lethality bioassay was performed. Among all the fractions, the fraction SA9 revealed the lowest LD_{50} of 49.99 µg/mL, whereas all the other fractions tested demonstrated significant cytotoxic property. The results of antileishmanial assay showed that the fraction SA6 possesses the highest mortality percent (84%) compared to the other fractions. **Conclusion:** These findings revealed that MESM can be an important source of natural antileishmanial herb that can be used as a therapeutic alternative for leishmaniasis.

Key words: Antileishmanial assay, antimicrobial assay, antioxidant assay, cytotoxic assay, milk thistle, phytochemical analysis

SUMMARY

- With the background of the popular ethnopharmacological use of *Silybum marianum* (SM) seeds as traditional medicine against several human ailments, an attempt is made to investigate the phytochemical and biological properties of SM seed extract. Methanolic extract of SM dried seeds was subjected to column chromatography and based on thin-layer chromatography analysis, fractions (SA1–SA10) were pooled up. The collected fractions were tested for antioxidant, antimicrobial, and cytotoxic activities (brine shrimp lethality assay and antileishmanial assay). All the tested fractions demonstrated significant antioxidant activities. Fraction SA9 displayed pronounced free radical scavenging activity of 80.7%. Fraction SA4 exhibited significant total antioxidant capacity (101.81 µg/mg). In ferric-based reducing antioxidant power assay, fraction SA4 showed the highest antioxidant power (258.93 µg/mg). Phytochemical screening of the fractions (SA1–SA10) revealed that the total phenolic contents were abundant in fraction SA7 (85.13 µg/mg) and total flavonoid contents were found to be highest in fraction SA1 (58.24 µgQE/mg). On the other hand, mild antibacterial and antifungal activities were shown by the fractions. Brine shrimp lethality bioassay was performed to test the cytotoxic potentials of the fractions. Among all the fractions, fraction SA9 revealed the lowest LD_{50} of 49.99 µg/mL, whereas all the other fractions tested demonstrated significant cytotoxic property. The results of antileishmanial assay showed that the fraction (SA6) possessed the highest mortality percent (84%) compared to the other fractions. It can be concluded that based on the current study, SM seed extract has shown potential antioxidant and antileishmanic activities.



Abbreviations used: MESM: Methanolic extract of *S. marianum*; SA = Subfraction; LD_{50}: Median lethal dose; SM: *Silybum marianum*; MeOH: Methanol; $CHCl_3$: Chloroform; TLC: Thin-layer chromatography; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; DMSO: Dimethyl sulfoxide; TDP: Total phenolic content; FCR: Folin–Ciocalteu reagent; TFC: Total flavonoid content.

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INTRODUCTION

Silybum marianum (SM), previously known as *Cardus marianus,* is a member of *Asteraceae* family frequently recognized as Daisy family. It is a biennial or an annual plant indigenous to North Africa, North America, Mediterranean region, Europe, Middle East, and Australia.[1] It is also common in India at a height of 1800–2400 m.[2] Milk thistle is the common name of the plant, which is given due to the presence of "milky white" veins on the surface of leaves. The seeds of SM have been used as a therapeutic source from thousands of years, and Theophrastus was the first who reported this plant as a source of remedy and cure. The medicinal value of SM is well known for over 2000 years, and frequent use of its seeds has been reported in the West (European countries) as a therapeutic agent in the treatment of several diseases such as hepatic ailments (to remove gall stones), for pregnant women (as a bitter tonic), anxiolytic issues, stomach acidity, varicose veins, splenic congestions, uterine hemorrhage, amenorrhea, and menstrual disorders.[3-5] It has shown promising results in ameliorating pesticide-induced hepatotoxicity.[6] Similarly, its extract has exhibited tremendous cardioprotective potential by improving healing after a myocardial infarction.[7] When used in combination, SM extract has potentiated the antidiabetic and antibacterial activities of zinc oxide nanoparticles.[8]

SM has a variety of natural products with promising biological potential. Among them, antioxidants are those compounds which can stop or slow down the oxidation of biomolecules such as lipid oxidation by hindering the chain reactions of oxidation and have the ability to give protection or reconstruct cellular damage that can occur in the body because of oxygen.[9] The present study was designed to investigate the phytochemical and cytotoxic potential of SM seed extract with special focus on its antibacterial, antifungal, and antileishmanial effects.

MATERIALS AND METHODS

Collection and extraction of plant seeds

Plant material of SM was collected from Haripur district of Khyber Pakhtunkhwa (610 m above the see level), Pakistan, followed by identification by a taxonomist at Quaid-i-Azam University, Islamabad, Pakistan (herbarium voucher no. QAU/Bot-Herb-14328). The seeds were dried, crushed, pulverized into powdered form by using a heavy-duty blander, and weighed (~2.5 kg). The powdered material was macerated in methanol for 9 days with occasional shaking to achieve maximized extraction of the seed constituents and kept in a dry shaded place at room temperature. The filtrate was evaporated under reduced pressure by using a rotary evaporator at 30°C to complete dryness to yield crude seed extract. The dried seed extract was preserved till further experimentation.

Fractionation via column chromatography

A slurry of crude methanolic extract (20 g) was prepared by dissolving the extract in minimum volume of 10% (MeOH in $CHCl_3$) and adsorbed on silica gel by keeping the sample to adsorbent (1:1.5) proportion. The sample was loaded on the top of a chromatographic column packed with silica gel as stationary phase and eluted with 100% ($CHCl_3$) to 10:1 ($CHCl_3$: MeOH) followed by 50% ($CHCl_3$: MeOH) in gradient manner. Forty fractions were collected and further subjected to normal-phase thin-layer chromatography (TLC) analysis. Based on TLC investigation, the fractions with similar R_f values were pooled up to obtain a total of ten fractions from SA1 to SA10, having % yield of 12.5, 18, 11.25, 5, 7.5, 10, 12.5, 12.75, 5, and 4, respectively. The fractions were used for further phytochemical and biological evaluation.

Antioxidant assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of fractions (SA1–SA10) was determined by a previously reported method with slight modification.[10-12] Briefly, 3.2% of DPPH reagent, 4% of ascorbic acid, and 1% of each sample fraction was prepared as stock solution. A volume of 20 µL of each fraction to be tested was taken in 96-well plate followed by the addition of 180 µL of DPPH reagent in each well to make the final volume of 200 µL and incubated for 1 h at 37°C. Ascorbic acid and ethanol were used as positive and negative controls, respectively. The DPPH reduction activity was measured by reading the absorbance at 517 nm. The experiment was performed in triplicate, and the percentage of final scavenging was calculated by the following formula:

DPPH scavenging activity (%) = (A0 – A1/A1) ×100.

The antioxidant capacity of all fractions (SA1–SA10) was determined using reported methodology with slight modifications.[13] Premeasured 1.63 mL H_2SO_4 (conc.), NaH_2SO_4 (1.679 g), and ammonium-molybdate (0.247 g) were dissolved in a 100-mL volumetric flask and finally, the volume was made up to 100 mL. In 1 mL of dimethyl sulfoxide (DMSO), 4 mg of ascorbic acid was dissolved to prepare a stock solution of ascorbic acid. First, 1 mL of the reagent was taken in an Eppendorf tube, in which 0.1-mL sample was added and mixed properly. The mixture was then placed in an incubator at about 95°C for 1.5 h. The mixture was cooled to 28°C, and the absorbance of the mixture was measured at 695 nm with the help of a microplate reader. For calibration curve, ascorbic acid was tested at various concentrations (125, 100, 75, 50, and 25 µg/mL).

The reducing power of the plant extracts was determined by using the method reported previously with slight modifications.[10] Phosphate buffers (0.2 M), potassium ferric cyanide (1%), trichloroacetic acid (10%), and ferric chloride (0.1%) were used as stock solutions. Each fraction (200 µL) was taken in the Eppendorf tube and was added with buffer (500 µL). Then, potassium ferric cyanide (500 µL) was added and incubated for 20 min at 50°C. After incubation, trichloroacetic acid (500 µL) was added, and the mixture was then subjected to centrifugation for 10 min at 3000 rpm. A volume of 100 µL of the upper layer was removed and carefully poured into the assigned well. Ferric chloride (1%) was further added to each well followed by the addition of distilled water (20 µL) in each well. The absorbance was measured by a microplate reader at 630 nm wavelength.

Phytochemical analysis

The total phenolic content (TPC) was determined by using Folin–Ciocalteu assay.[14] Folin–Ciocalteu reagent (FCR) and distilled water (in 1:10 v/v ratio), 6% sodium carbonate, and 4% gallic acid in methanol were used as stock solutions. In 96-well plate, the sample (20 µL) was taken followed by the addition of FCR (90 µL) and incubated at 40°C for 5 min. After an interval of 5 min, 6% sodium carbonate solution (90 µL) was added and the reaction mixture was incubated at 40°C for 60 min. Gallic acid and DMSO were used as positive control and negative control, respectively. The absorbance was measured at 630 nm on a microplate reader.

The total flavonoid content (TFC) was measured by aluminum chloride colorimetric assay protocols as described earlier.[14] The stock solutions of aluminum chloride (10%), potassium acetate (1.0 M), and quercetin (4 mg/mL) in DMSO were prepared. To a sample (20 µL) placed in 96-well plate, aluminum chloride (10 µL), potassium acetate (10 µL), and distilled water (160 µL) were added to make a final volume to 200 µL and incubated for 30 min at room temperature. Quercetin and DMSO were used as positive control and negative control, respectively. The absorbance was measured at 405 nm using a microplate reader.

For preliminary phytochemical screening and identification of bioactive components in MESM, several phytochemical investigations were carried out by using the standard procedures described previously with slight modifications.[15-17]

For alkaloids, the following tests were performed:

a. Mayer test: Methanolic extract (5 mL) was added with 2–3 drops of Mayer's reagent (potassium mercuric iodide solution). The appearance of cream yellow or white brown color indicated the presence of alkaloids
b. Wagner's test: Methanolic extract (5 mL) was added with 2–3 drops of Wagner's reagent (iodine potassium iodide solution). Formation of reddish brown precipitates also indicated the presence of alkaloids
c. Dragendroff's reagent test: Methanolic extract (5 mL) was added with dilute HCl (2 mL) followed by the addition of Dragendroff's reagent (1 mL) in the mixture. Instantaneous formation of orange or red precipitates showed the presence of alkaloids.

Test for saponins

Methanolic extract (5 mL) was stirred vigorously with distilled water (15 mL) in a test tube and warmed gently with continuous stirring for 10 min. The formation of a stable layer of foam was taken as an indication of the presence of saponins.

Test for terpenoids

Methanolic extract (~10 mg) was taken and dissolved in chloroform (1 mL). Acetic anhydride (1 mL) was added slowly followed by the addition of concentrated H_2SO_4 (2 mL). Appearance of reddish violet color showed the presence of terpenoids.

Test for tannins

Methanolic extract (5 mL) was added with 2–3 drops of 1% lead acetate. Formation of yellow or pale yellow precipitates indicated the existence of tannins.

Antimicrobial assays

The antibacterial effect of the extracts was studied against four bacterial strains, i.e. *Staphylococcus aureus* (American Type Culture Collection [ATCC] 6538), *Escherichia coli* (ATCC 15224), *Bacillus subtilis* (ATCC 6633), and *Enterobacter aerogenes* (ATCC 13048), by following the standard disc diffusion method as advised earlier.[18] The stock solution of methanolic extract of SM (MESM) (20 mg/mL) was prepared in DMSO. Initially, nutrient agar was prepared and pH was adjusted (pH = 7) and autoclaved for 20 min at 121°C. About 25 mL was poured in each glass Petri plate separately. The freshly made inoculum for each bacterium was swabbed on a solid media. Then, the sample solution was poured on filter paper discs and carefully placed on their assigned positions in Petri plates. One disc was used as a positive control. The plates were placed in the incubator for 24 h at 37°C. The zones of inhibition caused by the test samples in the bacterial cultures were measured. The test was performed in triplicate for each sample. Cefotaxime solution in DMSO (4 mg/mL; 20 µg/disc) was used as the positive control, whereas DMSO was taken as the negative control.

The antifungal effect of the extracts was evaluated against four fungal strains, i.e., *Fusarium solani* (FCBP 0064), *Mucor* species (FCBP 0300), *Aspergillus flavus* (FCBP 066), and *Aspergillus niger* (FCBP 0198) by disc diffusion method.[19] In brief, the sample (20 mg/mL) in DMSO was prepared as a stock solution. Dextrose agar media was prepared and pH was adjusted (pH = 6.5) and autoclaved. The media was poured in each Petri plate and once solidified, the fungal strains were swabbed cautiously on the media surface of each plate. Then, 5 µL of each sample solution was poured on the surface of filter paper disc and the discs were placed carefully on its assigned place. The Petri plates were placed in an incubator for 24 h at 28°C. Then, the zone of inhibition was measured using a Vernier caliper. Terbinafine (4 mg/mL; 20 µg/disc) was used as the positive control, whereas the solvent (DMSO) was taken as the negative control. The test was performed in triplicate for each sample.

Brine shrimp lethality assay

The cytotoxic potential of methanolic extract was evaluated by brine shrimp lethality bioassay using *Artemia salina* eggs (Ocean Star International Inc., Snowville, UT, USA) as described previously with slight modifications.[20,21] From the stock solution, three dilutions of 1000, 100, and 10 µg/mL were prepared to obtain the concentrations of 10, 1, and 0.1 µg/mL, respectively. Doxorubicin was used as reference, and DMSO was used as a negative control. The eggs were hatched in continuous oxygen supply for 24 h. Ten shrimps were transferred to each well of 96-well plate by using a Pasteur pipette under a ×3 magnifying glass. The samples were applied in triplicate using 0.5, 1.5, and 3.0 µL. The 96-well plate was placed in an incubator for 24 h. Then, the shrimps were removed from the wells by using the Pasteur pipette, and survivors of each well were counted. Terbinafine (10 µg/mL, 1 µg/mL, and 0.1 µg/mL in DMSO) was used as the standard drug.

In-vitro antileishmanial assay

The *in-vitro* antileishmanial assay was performed by using protocols reported by Pulivarthi *et al.* with slight modifications.[22] *Leishmania tropica* (KWH23) strain was incubated at 24°C for 7 days in 199 medium consisting of about 10% bovine serum. A stock solution of 4000 ppm was used. In the 96-well plate, the stock solution was serially diluted. Amphotericin B was used as a positive control, whereas DMSO was used as a negative control. The 96-well plate was incubated at 24°C for 72 h. After 3 days, the test culture (~15 µL) was then transferred to improve Nubauer counting chamber, and live promastigotes were counted under light microscope. Percentage mortality was calculated, and the experiment was performed in triplicate.

RESULTS AND DISCUSSION

Antioxidant assays

The crude methanolic extract showed 63% DPPH scavenging effect, however, the fractions demonstrated interestingly differential percentage of antioxidant effects. In DPPH free radical assay, all fractions were examined for scavenging activity. It was observed that contents in methanolic extract of the selected plants were observed to be potent DPPH reducing agents. The crude methanolic extract showed 63% DPPH scavenging effect, however, the maximum DPPH radical scavenging activity was exhibited by fraction SA9 (80.7%). The lowest DPPH radical scavenging activity was shown by fraction SA6 (23.7%). All the other fractions showed DPPH reducing activity in the range of 78.9%–80.0% when compared with ascorbic acid. Our findings are in line with those of a previous study carried out for the antioxidant activity of fractions of methanolic extract of *Silybum* species having significant DPPH value.[23] The obtained fractions were having different proportions of phenolic contents, which might vary in the number and the position of hydroxyl groups. This variation might affect the DPPH % scavenging activity.[24] The unit for showing antioxidant potential was % scavenging/mg. The results of the analyzed samples are depicted in Figure 1.

In total antioxidant capacity assay, the crude methanolic extract presented statistically significant antioxidant capacity ($P < 0.05$) (73.47 µg/mg), whereas the fraction SA4 demonstrated maximum antioxidant capacity which was 101.81 µg/mg followed by other fractions SA2 (93.85 µg/mg), SA3 (89.76 µg/mg), and SA8 (89.12 µg/mg). SA5 exhibited the least total antioxidant capacity (34.91 µg/mg). These results are in line with those of a previous study where *Silybum* species showed significant antioxidant activity.[25] To the best of our knowledge, it is the first study to evaluate the different activities on various fractions of SM extract eluted with solutions of different polarities. The total antioxidant capacity was expressed in ascorbic acid equivalent/mg (AAE/mg). The results of the tested fractions are depicted in Figure 1.

In reducing power assay, the crude extract showed statistically considerable activity (138.33 µg/mg), while the fraction SA4 (258.93 µg/mg) showed the maximum reducing power followed by fractions SA2 (250.90 µg/mg), SA5 (248.26 µg/mg), and SA7 (245.10 µg/mg). The lowest reducing power was shown by fractions SA5 (104.49 µg/mg) and SA6 (103.77 µg/mg). Our findings are relevant with those of a previous study, in which only a single extract with 4.77% of phenolic content was analyzed.[23] However in our study, we examined the reducing power assay of ten different fractions with different phenolic concentrations. The unit AAE/mg was used to express the reducing power. The tested sample results are depicted in Figure 1.

Phytochemical analysis

Total phenolic contents

In the following results, the TPC was found maximum in fraction SA7 (85.13 µg/mg) followed by fractions SA10 (68.37 µg/mg) and SA1 (68.14 µg/mg). Fraction SA3 (40.03 µg/mg) showed the least phenolic content. The findings of the current study are in support with those of a previous report showing varied phenolic contents in ethyl acetate, methanol, and ethanolic extracts in Iraqi species of SM.[26] TPCs were expressed in gallic acid equivalents (GAE). The results of the tested samples in species obtained from Haripur, Pakistan, are depicted in Figure 1.

Total flavonoid contents

In this assay, TFCs were found maximum for fraction SA1 (58.24 µg/mg) followed by fractions SA3 (56.48 µg/mg), SA7 (55.16 µg/mg), and SA10 (53.92 µg/mg). The least TFC was found in fraction SA5 (40.61 µg/mg). Our results are in strong agreement with the findings obtained by Sun *et al.*, who studied that higher phenolic content was present in seeds (17.10 mg/g dry weight) as compared to roots (15.134 mg/g dry weight).[27] The elevated flavonoid contents in our sample might be due to the species that was obtained from Haripur, Pakistan, and it is the first study to examine the properties of this species gathered from this locality. TFCs were expressed as QE/mg. The results of the analyzed samples are depicted in Figure 1.

Phytochemical screening tests

In case of qualitative screening of phytochemicals, all methanolic extract fractions (SA1–SA10) showed significant result and indicated the presence of alkaloids, saponins, terpenoids, and tannins. Our findings are in correlation with a previous study that showed the presence of phytochemicals in MESM.[28] The results are summarized in Table 1.

Antibacterial assay

In antibacterial assay, disc diffusion method was applied. The result of crude extract demonstrated significant antibacterial activity with 6 mm, 9 mm, 5 mm, and 11 mm zone of inhibitions for *S. aureus, E. coli, E. aerogens,* and *B. subtilus*, respectively. Most of the fractions showed poor activity against bacterial strains which were used in this assay except the fractions SA9 (10 mm in case of *S. aureus*), SA7 (11 mm in case of *E. coli*), and SA7 (9 mm in case of *E. aerogenes*) which showed mild zone of inhibition. In case of *Bacillus* species, all fractions showed no activity against *B. subtilis* as shown in Figure 2. The results of our study are in partial agreement with a previous study that showed mild or non-significant activity against Gram-negative and Gram-positive bacteria.[28] The results of the tested samples on the four different bacteria are depicted in Figure 2.

Antifungal assay

In antifungal activity assay, the extract displayed significantly poor antifungal effect on the tested fungal. Whereas, most of the fractions (SA1–SA10) showed no activity against selected fungal strains, but maximum activity was found against *Mucor* species (8 mm) and *A. niger* (8 mm). Our findings are in correlation with a previous study that showed non-significant zone of inhibition against fungal strains.[28] All results from sample fractions are depicted in Figure 3.

Cytotoxicity assay (brine shrimp lethality assay)

Results of the cytotoxic assay showed that the crude extract was found to be poorly toxic on brine shrimp with LD_{50} 311 µg/mL. Whereas, the

Table 1: Phytochemical screening of methanolic fractions from seeds of *Silybum marianum*

Tests	Observations	Results
Mayer test	Cream yellow color	+++
Wagner test	Reddish brown precipitate	+++
Dragendorff"s test	Orange or red precipitate	+++
Saponins	Foam layer	+++
Terpenoids	Reddish violet color	+++
Tannins	Yellow precipitate	+++

+++ is presence

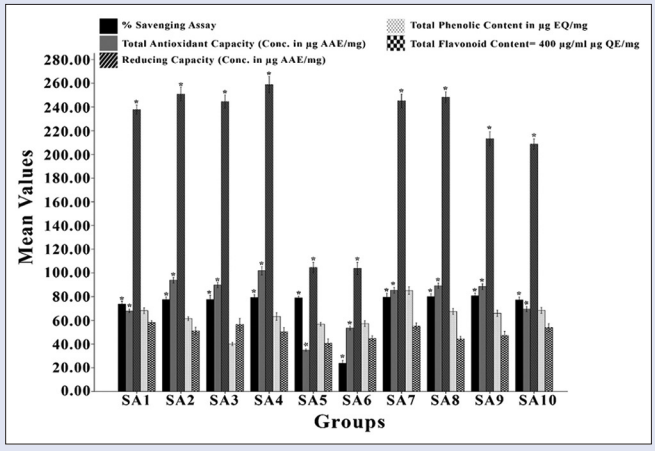

Figure 1: Antioxidant, total phenolic, and flavonoid contents of the fractions of *Silybum marianum* seed extract

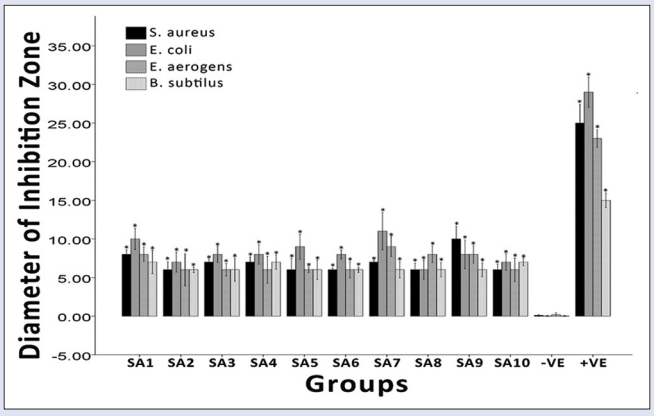

Figure 2: Antibacterial effect of the fractions of *Silybum marianum* seed extract. *The statistical difference between the treatment and the control groups at $P < 0.05$

lowest LD_{50} was found in fraction SA9 (49.99 µg/mL) followed by fraction SA5 (68.55 µg/mL). LD_{50} values were found highest in case of fraction SA6 (211.74 µg/mL). Our findings are in line with those of another study report on cytotoxic activity that showed significant results.[29] The findings of our study were presented as % cytotoxicity as depicted in Figure 4.

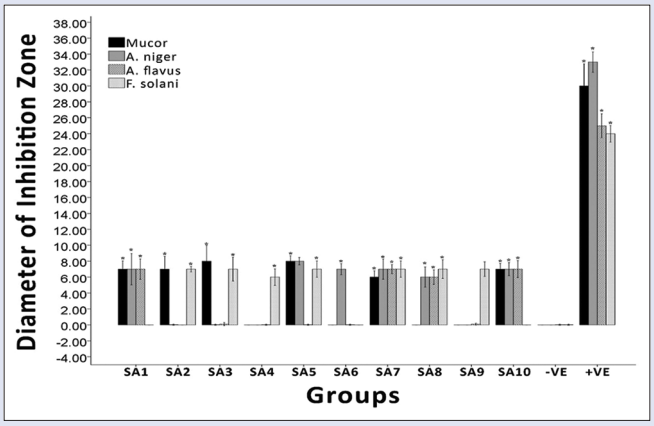

Figure 3: Antifungal potential of the fractions of *Silybum marianum* seed extract. *The statistical difference between the treatment and the control groups at $P < 0.05$

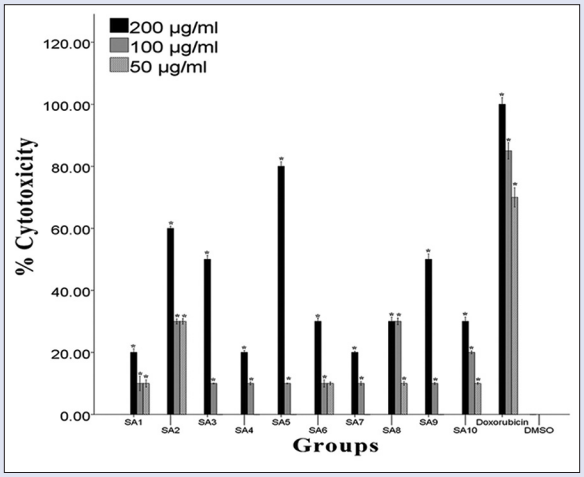

Figure 4: Brine shrimp lethality bioassay of the fractions of *Silybum marianum* seed extract. *The statistical difference between the treatment and the control groups at $P < 0.05$

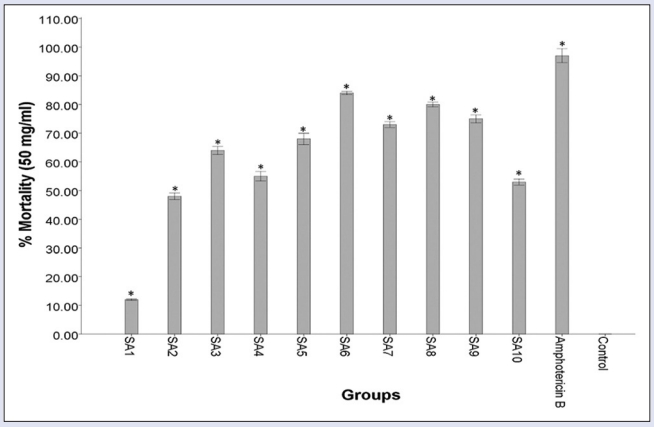

Figure 5: Antileishmanial activity of the fractions of *Silybum marianum* seed extract. *The statistical difference between the treatment and the control groups at $P < 0.05$

In-vitro antileishmanial assay

In case of antileishmanial activity assay, the crude extract exhibited statistically significant ($P < 0.05$) activity with 68% inhibition. However, the fraction SA6 was found more significant and mortality rate was 84%, followed by fractions SA8 (80%), SA9 (75%), and SA7 (73%). The least mortality rate was found for fraction SA1 (12%). These findings correlated with those of a previous report that methanolic extract of *Sonchous cornatus* showed significant % mortality against antileishmanial parasite.[30] The tested sample results are depicted in Figure 5.

CONCLUSION

In this study, phytochemical and pharmacological investigation of SM seed extract was undertaken. The results demonstrated an interesting phenomenon between the crude extract and its constituent fractions. In general, the extract has lower effect in the tested biological assays than compared to the fractions. In all the biological tests, the fractions were more pronounced than the extract. In addition, the results of the present study demonstrated that fractions of MESM seeds, prepared using column chromatography, demonstrated abundant levels of total phenols and flavonoids. This could probably be attributed to the potential antioxidant property of the extract. However, due to the difference in the polarity, different fractions revealed differential antioxidant properties. Moreover, the fractions exhibited remarkable antibacterial and cytotoxic activities. Generally, all the tested fractions display strong antileishmanial effect. The results revealed that all the tested fractions showed differential potentials in the biological assays tested, which indicates the presence of different types of bioactive phytoconstituents in extracts. With this background, it can be concluded that the extract of SM seeds can be a good source of therapeutic agents. However, further study is required to confirm the effect of the extract using suitable preclinical and clinical models.

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Conflicts of interest

There are no conflicts of interest.

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