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Evaluation of Anti-bacterial Activity of *Silybum marianum* against Pathogenic and Resistant Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MZ, TA, AA and ST designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AB, ST, RH, AY and MNM managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Our arsenal of antibiotics is running low due to overuse and misuse of these lifesaving drugs. Much research is needed on medicinal plants to look for drugs to tackle this issue
Methodology: so antibacterial activity of organic extracts of *Silybum marianum* was evaluated against Methicillin-resistant *Staphylococcus aureus* (MRSA), resistant *E. coli,* ATCC 12361, ATCC 12466 and ATCC 13581. Inhibition of bacterial growth was determined using agar well diffusion methods.
Results: Among all assayed organic extracts only Dimethylformamide presented highest activities

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against all tested strains except resistant *E. coli*. Dimethylformamide and Methanol showed better activity than cephradine against ACTT 13581. The Dichloromethane extract showed no antibacterial activity against any strains. Methanol and Isopropyl Alcohol showed moderate activities against all tested strains. *S. marianum* would be an interesting topic for further study and possibly for an alternative treatment for resistant and pathogenic bacterial infections.

Keywords: Silybum marianum; antibacterial; pathogenic and resistant bacteria.

1. INTRODUCTION

The natural compounds originated from plants are the cornerstone of today's era of synthetic drugs. The naturally occurring components obtained from plant kingdom, such as medicinal plants, vegetables, fruits that in addition to providing nutrients and fibers also act to protect against various diseases [1].

Phytochemicals are classified mainly in two categories, primary and secondary constituents. Sugars, amino acids, proteins and chlorophyll are classified into primary constituents, while alkaloids, tannins, phenolic compounds, saponins; flavonoids are classified into secondary constituents [2]. The therapeutic value of medicinal plants is based upon the phytochemicals constituents that cause a specific therapeutic response in the human body [3].

In the present decade, infectious diseases are classified as the most common causes of death all over the world, which accounts for almost one half of all deaths that takes place in tropical countries. Infectious diseases have also become a matter of worry in the developed countries. In America death due to infectious disease are 8% of total deaths [4]. So research on medicinal plants is needed so that new antibiotics can be developed to defeat infectious diseases.

S. marianum is native of southern Europe, mainly the Mediterranean regions, indigenous to North Africa, Asia Minor Southern Russian Federations. *S. marianum* is now naturalized throughout Europe, in North and South America, Australia and abundantly available in Khyber Pukhtoon Khwa and Punjab areas of Pakistan [5].

It grows 30 to 200 cm tall, having an overall conical shape with an approx. 160 cm maximum diameter base. The stem is grooved and more or less cottony. With the largest specimens the stem is hollow. The leaves are oblong to lanceolate. They are either lobate or pinnate, with spiny edges. They are hairless, shiny green, with milky-white veins. The flower heads are 4 to 12 cm long and wide, of red-purple color. They flower from June to August in the North or December to February in the Southern Hemisphere (summer through autumn). The bracts are hairless, with triangular, spine-edged appendages, tipped with a stout yellow spine. The achenes are black, with a simple long white pappus, surrounded by a yellow basal ring [6].

Fig. 1. Cypsela and leaves of milk thistle (*Silybum marianum)*

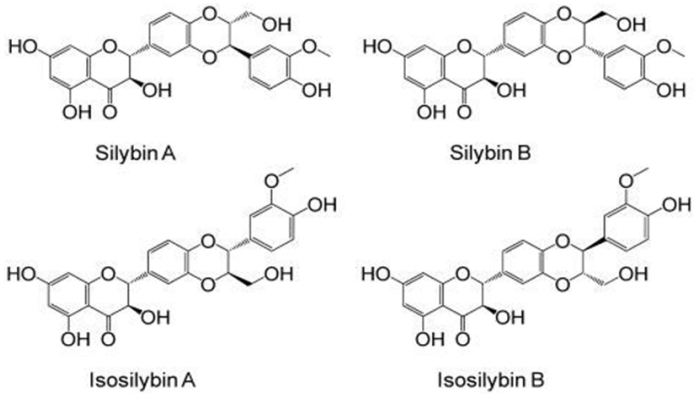

Fig. 2. Chemical structures of different polyphenolic compounds in silymarin.
[15]

The main active constituents of this plant are Flavonolignans collectively known as silymarin. It is used for the treatment of many liver disorders characterized by degenerative necrosis and functional impairment. In addition, it is capable to antagonize the toxin of Amanita phalloides [7,8], And provides hepatoprotection against poisoning by paladin [9], galactosamine [10], the thioacetamide [11], halothane [1] and carbon tetrachloride [12].

Traditional milk thistle extract is made from the seeds, which contain approximately 4–6% silymarin [2]. The extract consists of about 65–80% silymarin (a flavonolignan complex) and 20–35% fatty acids, including linoleic acid. Silymarin is a complex mixture of polyphenolic molecules, including seven closely related flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin) and one flavonoid (taxifolin) [3]. Silibinin, a semi purified fraction of silymarin, is primarily a mixture of 2 diastereoisomers, silybin A and silybin B, in a roughly 1: 1 ratio [13,14].

Silybum marianum extract has antifungal effects, preventing the growth of dermatophyte more than saprophyte fungi [16]. One pilot study showed that milk thistle may be as effective as fluoxetine in treatment of obsessive-compulsive disorder [17]. A 2007 study found that *Silybum* marianum blocked Hepatitis C virus (HCV) cell culture infection of human hepatoma cultures. A subsequent study in 2010 found that eight major compounds that comprise Silybum, including seven flavonolignans—silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and one flavonoid, taxifolin—are inhibitors of HCV RNA-dependent RNA polymerase [18,19]. *Silybum marianum* extract is known for tumor inhibition and has been shown to stimulate neurons in culture and increase lymphocyte proliferation [20]. Isosilybin A was described as the main component of *Silybum marianum* acting as partial agonist of the peroxisome proliferator-activated receptor type gamma, current pharmacological target in the metabolic syndrome and diabetes type 2 [21].

We plan to evaluate the antibacterial activity of this plant against resistant as well as pathogenic strains as now a day's bacteria are winning the war against humans due to misuse and overuse of antibiotics. Our arsenal of new antibiotics is running low and resistance is increasing so new research on the plant kingdom should be done as there are limitless possibilities and also because herbal plants have lesser side effects as compared to synthetic medicines. [22]

2. MATERIALS AND METHODS

2.1 Plant

The aerial parts of the plant were collected from Sector F10, Islamabad with the flowers. The plant was authenticated by Dr.Qasim Hayat, Plant biotechnology lab, National university of Science and Technology (NUST). Parts used were leaves, stems, flowers and seeds. The roots were not used.

2.2 Preparation of Extracts

After collection of the plant it was dried in the shade and grinded to a very fine powder in a blender. The extracts were prepared by percolating 100 gram of dried and powdered plant material in a percolator and then filtering it

through Whatman filter #1 and the volume was made up to 500 ml and it was packed in amber colored bottles. The solvents used for extraction were Methanol, Dimethylformamide, Dichloromethane and Isopropyl alcohol in respective order.

After the extracts were made they were put in a rotary evaporator to evaporate the solvents. The temperature was always set 10℃ lower than the boiling point of solvent then the material collected from the rotary evaporator was spread in Petri plates and left for drying after proper labeling.

2.3 Percentage Yield

The percentage yield was calculated by first drying the plant material in Petri plates and when it was completely dry, the powder was weighed and divided by the theoretical yield and multiplied by 100%. The percentage yield of Isopropyl Alcohol (IPA), Dimethylformamide (DMF), Dichloromethane (DCM), and Methanol is 0.1%, 0.714%, 4%, 6% respectively.

2.4 Sterilization of Materials

Nutrient Agar and Nutrient Broth were and all the materials including the nutrient Agar, nutrient Broth, Petri dishes, assay tubes containing nutrient broth and tips were all packed inside newspapers, taped and labeled. After that they were put inside the autoclave (HICLAVE HVA-85) and sterilized at 121*C for 15 minutes. After that they were kept in an incubator as steam caused some water droplets to form in the Petri plates and were left there till they were used for pouring and inoculating bacterial strains to maintain sterility.

2.5 Dilutions

The dilutions were made by dissolving 20mg of plant material in 4mL of Isopropyl Alcohol, Dimethylformamide, Dichloromethane, and Methanol separately in test tubes and labeled, 1ml contain 5mg of plant extract. DMSO was not used to make dilutions instead their own solvents were used and later used as controls as well.

2.6 Microorganisms and Preparation of Plates

The bacteria's used in this study are Methicillin resistant *Staphylococcus aureus* (MRSA), Resistant *E. coli,* ATCC 12361 (*Enterococcus faecalis)*, ATCC 12466 (*E. coli from contaminated water)* and ATCC 13581 (*Serratia marcescens)*. A fresh stock suspension was prepared of the bacteria in the assay tubes by transferring each bacterium from the master stock suspension through a wire loop which was sterilized on an open flame after each transfer and the tubes were kept overnight to check for turbidity. The stock suspension was turbid after 24hours indicating the presence of bacteria.

Before pouring the nutrient agar into the plates the laminar flow cabinet was cleaned with 70% ethanol and the U.V was turned on for 30 minutes prior to use. After that all our material the sterilized Petri plates, tips, nutrient agar, cork borer, the fresh stock suspension, the dilutions, the antibiotic dilution and the sterile swabs were placed in the cabinet and the plates were arranged in a row to make pouring easy. Then 25 mL agar is poured into the plates slowly and left to solidify and put in the incubator for 24 hours to see for any signs of growth. The next day the plates were clear and ready for inoculation with the bacteria.

2.7 Inoculation of Plates

Dip the sterile swab in the fresh inoculum of the first bacteria and make a lawn on the nutrient agar take special care to make sure that the lawn is uniform and that you don't leave any space untouched. Subsequently, the surface of the agar was punched with 4-mm-diameter wells using a cork borer. Five wells were made and they were labeled 1, 2,3,4,5 and 5*. Each well was filled with plant extract using micropipettes. Fixed volumes (0.1 ml) of the leaf extract were introduced into the wells in the plates. simultaneously; Cephradine was used as positive control. Control wells containing Methanol were made. After 24-hour incubation at 37ºC, all plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters.

All tests were performed in triplets and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

3. RESULTS

The antibacterial activity of *Silybum marianum* was assayed in vitro conditions by agar well diffusion method against *Methicillin resistant Staphylococcus aureus* (MRSA), resistant *E. coli, ATCC 12361, ATCC 12466* and *ATCC 13581*.

The inhibition of bacterial growth by four of *S. marianum* extracts is summarized in Table 1. The results showed that the Dimethylformamide extract was active against Methicillin resistant *Staphylococcus aureus*, the other extracts of IPA, methanol and Dichloromethane showed no activity. The isopropyl alcohol extract of the plant material showed minimal activity against resistant *E. coli*. The DMF extract showed good activity against ATCC 12466. Three of the extracts except DCM were active against ATCC 12361. An interesting result was seen with ATCC 13581 against which our control did not show any activity but DMF and methanol extracts showed better activity. In result we can see that DMF have good activity against all the strain except *E. coli*, but the result against ATCC 13581 were more important because against this strain DMF result are even better than the Control. Similarly methanolic extract all showed better activity than cephradine against ATCC 13581 but this activity is very less as compare to DMF. So this result is of great importance.

4. DISSCUSION

A standard milk thistle extract contains 70% silymarin, a mixture of the silibinin and flavonolignans. Silibinin has been reported as the most biologically active constituent according to *in vitro* assays**.** Silybin and Silymyrin-2 have been used as anti-carcinogenic because of their cytotoxic activity. So these phytochemicals may also be toxic to bacterial cell and may be responsible for the antibacterial activity of *Silybum marianum.* Silybin is thought to have more potent activity, than silymarin-2 [23]. Furthermore photochemistry of DMF should be done as its results were even better than the cephradine. The further phytochemical studies of of *Sylibum marianum* may reveal the active principle that may be responsible for antibacterial activity against ATCC 13581.Phytochemistry of methanolic extract may also be done to compare the phytochemical constituents of both DMF and methanolic extract because methanolic extract is also more active against ATCC13581 as compare to cephradine.

Table 1. Zone of inhibitions in mm of pathogenic and resistant strains

	Inhibition zone diameter (mm) of compounds					
Microorganisms	**1 DMF extract (5 mg/ml)**	**2 IPA extract (5 mg/ml)**	**3 Methanol extract (5 mg/ml)**	**4 DCM extract (5 mg/ml)**	**5 Positive Control**	**5* Control solvent**
Methicillin resistant *S. aureus*	7.6	0	0	0	15.8	0
E. coli resistant	0	4	0	0	25.5	0
ATCC 12361	8.6	4.25	6.8	0	28.3	0
ATCC 12466	8	0	0	0	16	0
ATCC 13581	5.8	0	2.4	0	0	0

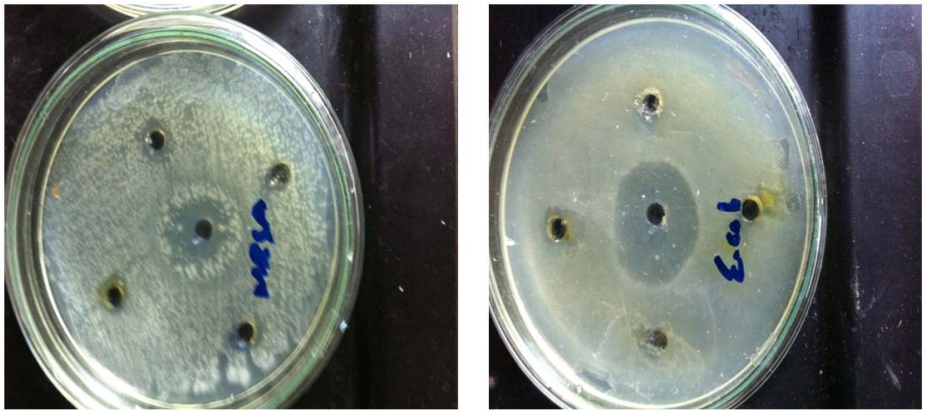

Fig. 3. Antimicrobial activity (zone of inhibition, mm) of various extracts of *Silybum marianum*

5. CONCLUSION

Resistant strains are causing a rise in health care costs and increasing mortality and morbidity that's why research has increased on finding new medicinal products for resistant restrains. In conclusion this plant is a good candidate for further research as it has shown good activity against resistant strains and is a viable candidate for future experiments. HPLC should be performed on the extracts of the plants to find which phytochemical constituents are present and then individual activities of those constituents should be carried out. If any one of them shows superior activity than the rest of the constituents then its structural modifications can be carried out and its activity evaluated against resistant strains. More work is needed be done on medicinal plants especially marine plants because that field is vast and largely untouched.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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