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Research article

Antibacterial Effect of Flavonoids Extracted from Seeds of *Silybum marianum* against Common Pathogenic Bacteria

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ABSTRACT

This research was carried out to evaluate the antibacterial effect of *Silybum marianum* flavonoids extracted from seeds on pathogenic bacteria (*Staphylococcus saprophyticus*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*) and detect the bioactive compounds found in the seed extract. Different methods were used to tech the aims of the study. Agar well diffusion method and mixed up the extract with agar media were used to evaluate the antibacterial activity of the flavonoids extracted from seeds of *S. marianum*. The chemical analysis to seeds showed that it contained several antibicrobial compounds such as terpenoids, flavonoids and tannins. The cold alcoholic extract of milk thistle did not appear significant antibacterial activity against the bacterial isolates when agar well diffusion method was used, while mixing the seed extracts with agar media showed antibacterial activity (1500-2900 µg/ml) against *S. sprophyticus*, *E. coli, K. pneumoniae* and *S. aureus*.

Keywords: Bioactive compounds, Flavonoids, *Silybum marianum*.

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INTRODUCTION

Microbial infections have become one of the major problems of public health in the world and the development of resistance to the available antibiotics has lead researchers to investigate the antimicrobial activity of medicinal plants [1]. Medicinal plants were used for centuries as remedies for human diseases because they contain components of therapeutic value, there is an increasing interest in phytochemicals as new sources of natural antioxidant and antimicrobial agents [2]. Phytochemicals are naturally occurring compounds of plant kingdom, such as medicinal plants, vegetables, fruits, that work with nutrients and fibbers to act against diseases or more specifically, provides protection against diseases [3].

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Silybum marianum (L.) (milk thistle) is a serious weed in many areas of north and south America, Africa, Australia, and the Middle East. Milk thistle is grown commercially as a medicinal plant in Europe, Egypt, China, and Argentina [4]. Flavonoids are a group of natural compounds known to have various pharmacological actions such us antioxydative, anti-inflammatory and diuretic [5]. The extracts of the flowers and leaves of *S. marianum* have been used for centuries to treat liver, spleen, and gallbladder disorders [6]. In the 1960s, a flavonolignan mixture named Silymarin isolated from seed and fruit extracts that most of the clinical studies were carried out. The main constituents are Silibinin, Isosilibinin, Silicristin, and Silidianin [7].

In the present study aims to evaluate the antibacterial activity of *S. marianum* flavonoids extracted from seeds against number of pathogenic bacteria and detect some phytochemicals in the seed extract.

MATERIALS AND METHODS

Sample collection and processing

Plant sample (seeds) of *S. marianum* was collected from Baghdad. The seeds were grinded and stored at room temperature until use.

Estimation of bioactive compounds in *S. marianum* seeds

Flavonoids

Five gram of seed powder was mixed with 10 ml of absolute ethanol (ethanolic extract) (BDH, England). Equal amounts of 5% ethanol and 50% NaOH (BDH, England) were mixed and used for detection of flavonoieds in seed extract the yellow color indicating the presence of flavonoieds [8].

Alkaloids

Ethanolic extract was warmed at 60 ^{o}C with 2% H_2SO_4 (BDH, England) for two minutes then treated with Mayer's reagent (3.5 g of HgCl2 and 50 g of KI). The presence of creamy white colored precipitation indicating the presence of alkaloids [9].

Glycosides

Five milliliter of diluted sulfuric acid was added into the seed extract in a test tube. The mixture was boiled for 15 min in a water bath. 20% potassium hydroxide was added for neutralizing the pH of solution. A Fehling's solution (Maknur Laboratories/ Canada) were added and boiled for 5 min. A red precipitate indicates the presence of glycosides [10].

Tannins

A small quantity (0.5 ml) of the extract was boiled with 5 ml of 45% solution ethanol for 5 min. The mixture was cooled and filtered. A cream gelatinous precipitation indicated positive test for tannins when treated with 1% of lead acetate (BDH, England), while greenish to black color appeared when treated with ferric chloride (BDH, England) [10].

Coumarin

Ethanolic extract was covered by filter paper moistened with a dilute solution of NaOH, boiled for 3 min then set to UV light to observe the yellow greenish color that indicates the positive result [11].

Volatile oil

Filter paper was saturated with extract and set to UV light to see the bright pink color due to the presence of volatile oil [12].

Terpenoids

The extract was mixed with 2 ml of chloroform (BDH, England) and 3 ml of concentrated H_2SO_4 (BDH, England). A reddish brown color is formed to show the positive result of presence of terprnoids [10].

Steroids

Two milliliter of acetic anhydride (BDH, England) was mixed with seed extract and 2 ml of H_2SO_4. The color changed from violet to blue or green indicating the presence of steroids [10].

Preparing of seed Flavonoids extract

According to Harborn [9] method, 100 g of seed powder was added to the 200 ml of hydrochloric acid (2N), covered and boiled in a water bath for 45 min, then cooled at room temperature (21 ^{o}C) and filtrated under vacuum. Carotenoids, chlorophyll and wax eliminated by using the petroleum ether four times (25 ml each time). The flavonoids compounds were extracted from solvent layer using Ethyle acetate (BDH, England) four times with 25 ml each time. The solution layer that contain flavonoids was separated and evaporated by using a rotary evaporator under vacuum condition at 40ºC, the yielded product was weighted and estimated for presence of flavonoids.

The antimicrobial activity of flavonoids seed extract

Preparation of bacterial suspension

Four clinical isolates of *S. auerus, S. sprophyticus, E. coli* and *K. pneumoniae* were used in this study. Bacterial suspensions were made in nutrient broth to concentration 10^8 cell\ml using counting plate method.

Agar well diffusion method

The agar well diffusion method was used to determine the antimicrobial activity of the flavonoids seed extract. 100 µl of 10^8 cell\ml of each bacterial isolate was spreaded onto nutrient agar plate. The plates were left for 30 min. five well 10 mm in diameter was made in each of plates using sterile cork borer No. 8. The wells

were filled with 0.4 ml of extract (10^3, 10^4, 2.5×10^4 and 5×10^4 µg/ml) and one well was filled with solvent and considered as control. The plates were incubated at 37°C for 24 h. Three replicates were also performed for each concentration against each of the bacterial isolate. After incubation the diameter of the clear zone was measured, the averages were calculated [13].

Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

The minimum inhibitory concentrations (MICs) were determined for flavonoids by mixing up the seed flavonoids extract with the Agar. The concentrations of flavonoids extract were prepared between 10^3-10^4 µg/ml that added to the melted and cooled nutrient agar medium. 100 µl of 10^8 cell\ml bacterial suspensions was inoculated into nutrient agar plates then incubated at 37 ^oC for 18 h. The MIC value of the extract was determined as the lowest concentration that completely inhibited bacterial growth. For the determination of MBC, 5 µl from each concentration that exhibited no growth were taken and then incubating at 37 ^oC for 24 h. The lowest concentration that showed no visible bacterial growth after sub-culturing was taken as MBC. Positive and negative cultures were prepared [14].

RESULTS

The results of bioactive compound detected that the seeds of *S. marianum* contained the major groups of these compounds such as flavonoids, tannins and terpenoids (**table 1**).

Table 1. Bioactive compounds detected in seeds of *S. marianum* extract. -, absence of compound; +, presence of compound.

Bioactive compound	Results
Flavonoids	+
Alkaloides	-
Glycosides	-
Tannins	+
Coumarins	+
Volatile oil	+
Terpenoids	+
Steroids	-

Moreover, the percentage of flavonoids in seeds extract was 1.97%. The cold alcoholic extract of milk thistle seeds showed no antibacterial activity against all bacterial isolates when used agar well diffusion method. While mixing the extract with agar was showed antibacterial activity at concentrations ranged from 1500 to 2900 µg/ml. The MIC and MBC values of the flavonoids extract against *S. sprophyticus, E. coli, K. pneumoniae* and *S. aureus,* was shown in **table 2**. The present results showed that most susceptible bacteria to flavonoids seeds extract was *E. coli*, the lowest MIC and MBC was found in this isolate. However, the highest MIC and MBC were found in case of *K. pneumoniae*.

DISCUSSION

Bioactive compounds are playing an important role for the treatment of different diseases. From the finding results, the *S. marianum* seeds contained major bioactive compound such as flavonoids tannins and terpenoids that explain the importance of *S. marianum* as medicinal plant. It has been reported that extracts of plants that rich with flavonoids possess antimicrobial activity [15]. This findings agreement with previous study [3,16] were confirmed the presence of flavonoids tannins and terpenoids in this plant. Previous studies flavonoids and phenolic compounds were extracted from leaves and seeds of *Brassica oleracea* var. capitata [17,18] in these studies the fine chemical compositions and their structures were evaluated.

Table 2. MIC and MBC of *S. marianum* flavonoids seeds extract against several clinical bacterial isolates.

Bacterial isolates	Average MIC µg/ml	Average MBC µg/ml
E. coli	1800	2000
S. sprophyticus	2600	2750
S. aureus	2700	2800
K. pneumoniae	2800	2900

The antibacterial activity of flavonoids extract not observed against all tested bacteria by using agar well diffusion method even at the high concentrations. This may be largely belonged to the flavonoids found in the seeds of milk thistle composed a high proportion of Silymarin that has lower melt in water [19], therefore not diffused in media when used agar well diffusion method in comparison to mixing up the seed flavonoids extract with the agar media.

In current study, it was observed clearly that the *S. marianum* seeds extract was effective against all bacterial species, although its effectiveness against *E. coli* was higher than others. In another studies, Gram-positive bacteria as well as Gram-negative bacteria showed susceptibility to Silymarin and Silibinin [20].

The antibacterial activity of flavonoids can be explained by the toxicity of this compound towards non specific interactions in showed susceptibility, such as the establishment of hydrogen bonds with the cell walls proteins or enzymes, the chelation of metal ions, inhibition of bacterial metabolism, sequestration of substances necessary for the growth of bacteria. Also, the β ring of flavonoids is important in the intercalation with nucleic acids, thus inhibits DNA and RNA synthesis. It can also inhibit the DNA gyrase of *E. coli* [21,22].

The resistance of *K. pneumoniae* to the extract of seed due to the presence of the capsule that may hinder the flavonoids to enter across the walls thereby plays important role of virulence of this bacterium. Indeed, Gram negative have an additional layer to the outer membrane based on phospholipids, proteins and lipopolysaccharides forming an impermeable barrier to most hydrophobic molecules [23]. From the study it can

be concluded that the *S. marianum* seeds extract have several bioactive compounds and the extracted flavonoids have antibacterial activity against all clinical bacterial isolates that used in this study.

Conflict of interest

The authors declare that they have no conflict of interests.

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