



Original Article

Activity of *Silybum Marianum* in Oral Candidiasis of Albino Rats

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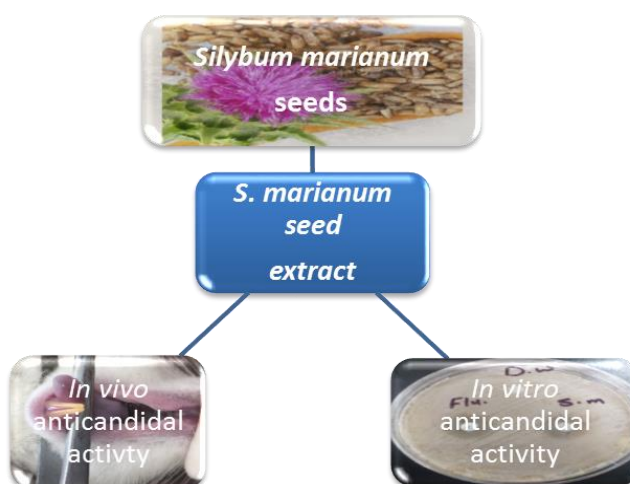
Fluconazole

Silybum marianum

ABSTRACT

Silybum marianum is a primitive compound mixture that has pharmaceutical and pharmacological activities. Studies have highlighted the role of *Silybum marianum* in treating candidiasis. The aim of the study was to evaluate the *in vivo* anticandidal and ulcer healing capacity of *Silybum marianum*-extract. *Silybum marianum* was prepared in aqueous extract which showed a positive result for the presence of four phytochemical compounds. *In vitro* anticandidal effect was reported for the extract against *Candida albicans* ATCC 7012. Animals were partitioned into three; group I treated with distilled water (negative control), group II treated with oral *Silybum marianum*-extract and group III treated with oral fluconazole (positive control). Treatment protocol was carried out for 7 days, then rats of three groups were sacrificed for histology and microbiology assessment. *Silybum marianum*-extract demonstrated a beneficial eradicating activity on *C. albicans* comparable to that of fluconazole and in enhancing ulcer healing rate (ulcer length: 810 μ m vs 381.77 μ m of negative control group and extract-treated group, respectively). *Silybum marianum* may be suggested as an alternative or an adjuvant therapy to standard existing drugs used for treatment of oral candidiasis.

GRAPHICAL ABSTRACT



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Introduction

Day by day medicinal plants act as a promising treatment source for various remedies with less side effects than synthetics medications. *Silybum marianum* (milk thistle) is a primitive compounds mixture that have pharmaceutical and pharmacological activity, in addition to its antioxidant and antimicrobial activity [1].

Different parts of the plant *Silybum marianum* were used anciently. However, effectiveness has been attributed to the activity of flavonoids such as silymarin [2]. Extraction process was a very important step to increase product quality therefore, the desired molecules diffuse into solvent phase from the bulk herb by using an appropriate solvent [3].

Candidiasis caused by different species of the genus *Candida*, a harmless dimorphic fungus with infection ranged from superficial to invasive. *Candida* invasiveness occurs when there is a disturbance in the normal flora or debilitation in host immune system [4]. Many species of *Candida* were recovered from the oral cavity in case of oral candidiasis, but the most common encountered species of clinical concern was *Candida albicans*. This species has been shown to account for around 80% of all *Candida* isolates [5]. Using experimental rats models has been long a useful technique of deeply understanding the pathogenesis of *C. albicans* infection *in vivo* [6].

Studying oral candidiasis in experimental rats models was useful for the understanding pathogenesis of *C. albicans* [6].

Fluconazole was the most commonly used member belonging to triazole group of antifungal drugs, which showed a good activity against most of *Candida* species especially *C. albicans* in local and systemic infection [7, 8]. Fluconazole is considered as the first choice in the treatment of candidiasis, their efficiency ranged from 50%-90% in treating such infections. *In vitro* susceptibility test showed a good activity of fluconazole, while *in vivo* activity in animal models with fungal infection fluconazole has shown to improve the survival of animals inoculated with *Candida* spp. Fluconazole doses of 4 -12 mg/kg/day was used for systemic fungal

infection, while doses of 1.0-2.5mg/kg/day was shown effective to cure 50 to 100% of animals with oral and vaginal candidiasis [8, 9].

This study aimed to prepare an aqueous extract of *Silybum marianum* with assessing the phytochemical activity. In addition, this project meant to study the anticandidal activity of *Silybum marianum* versus fluconazole *in vitro* using disc diffusion method and *in vivo* employing an albino rat-model via microbiological and histopathological assessment.

Materials and Methods

Extraction method

Seeds of *Silybum marianum*, washed several times with tap water, dried under shade for several days and then powdered manually. One hundred grams of powdered seeds were weighed and macerated by soaking in distilled water (DW) with continuous stirring using magnetic stirrer. After maceration the resultant aqueous extract was filtered and air dried, then the residue was weighted. Every 100 g of *Silybum marianum* yielded 6.2 g (6.2%) of the aqueous extract.

Phytochemical detection test

Five tests for the detection of the phytochemical compounds were applied to *Silybum marianum*-aqueous extract according to previous reports [9, 10].

Alkaloids: 1 mL of Mayer's reagent was mixed with 1 mL of *Silybum marianum*-aqueous extract. Yellow creamy precipitate formation indicated a positive result.

Terpenoids: 2 drops of chloroform with 1mL of concentrated hydrochloric acid were added to 1 mL of *Silybum marianum*-aqueous extract, followed by heating for 2 minutes. The formation of red to brown color specified a positive results.

Tannins: 2-3 drops of 10% neutral lead acetate were added to 1 mL of *Silybum marianum*-aqueous extract. yellow to white precipitate designated that extract contains tannins.

Phenols: 2-3 drops of ferric chloride were added to 1 ml of *Silybum marianum*-aqueous extract. Blue to black color formation indicated the presence of phenols.

Flavonoids: 2-3 drops of concentrated H_2SO_4 were added to 1 mL of *Silybum marianum*-aqueous extract. Orange color indicated the presence of flavonoids.

The isolate

A fresh subculture on Sabouraud's dextrose agar was obtained from a stock *C. albicans* control isolate ATCC 7012 as a reference strain, then incubated aerobically for 2-3 days at 37 °C. At the end of the incubation period, a suspension was prepared by mixing a colony of *C. albicans* with Sabouraud's dextrose broth by vortex to produce a turbid suspension, which followed by serial dilutions until obtaining a suspension turbidity equal to 0.5 MacFarland equivalent to 10^5 colony forming unit (CFU) per mL [11].

Disc agar diffusion method

In vitro assessment of the anticandidal activity of *Silybum marianum*-extract was conducted using disc agar diffusion technique. Briefly, discs of filter paper of 6 mm diameter were prepared. 100 discs were then soaked in 5 mL of 50 mg/mL *Silybum marianum*-extract for 24 hr. The same number of discs were soaked in DW (negative control). The prepared suspension of 10^5 CFU/mL *C. albicans* was swabbed on Sabouraud's dextrose agar. After that, corresponding discs were placed carefully on the swabbed agar. Discs of fluconazole (25 µg) was used as the positive control, antimicrobial activity assessment was carried in triplicate to ensure the accuracy of procedure.

Experimental animals

Thirty adult male albino rats weighed between 250-350 g and aged between 14-16 weeks were selected for this study. The animals were housed in separate cages under optimal experimental conditions according to the guidelines of the Institutional Animal Care and Use Committee UM.VET.2022.01 provided by the College of Veterinary Medicine, University of Mosul. They were fed on ground barely and supplied with water. Acclimatization rats for one week, followed that dividing them into three groups

equally with 10 rats for each group. All animals in the study were subjected to induce ulcer.

Procedure to induce ulcer in rats

Prior to creation an oral ulcer, the animals were anaesthetized by intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (25 mg/kg [12]. Filter papers of diameter 6 mm were saturated with 10 mL of 50% acetic acid for 24 hr. which were used to induce the oral ulcer. The acid-soaked filter paper was pressed onto the hard palate soft tissue of each rat for 60 seconds [13]. After that *C. albicans* reference strain of a concentration equivalent to 10^5 CFU/mL were inoculated into the experimental rats ulcer area by swabbing the ulcer with cotton swab.

Animal groups

Animals enrolled in this study were divided into three groups according to the treatment materials which were given orally via a specialized gastric tube. Group I (negative control group): rats (n=10) of this group were given a daily oral dose of 10 ml/kg body weight of DW [14]. Group II (treatment group): rats (n=10) in this group were given a daily oral dose of *Silybum marianum*-extract (50 mg/kg) after dissolving in 1 ml DW, dose was chosen according to previous studies [14]. Group III (positive control group): rats (n=10) enrolled in this group were given a daily oral dose of fluconazole (10 mg/kg) after dissolving in 1 ml DW [11].

Treatment protocol

Four days following inoculation with *C. albicans*, swab was taken from each rat oral ulcer from the three groups. Swabs were immediately carried to the microbiology laboratory, College of Pharmacy to be cultured on Sabouraud's dextrose agar and incubated aerobically for 2-3 days at 37 °C until visible yeast colonies were grown. After that, animals of each group were treated with the corresponding materials as explained above for 7 days. An oral swab was taken daily from each induced ulcer then cultured on Sabouraud's dextrose agar and incubated aerobically for 2-3 days at 37 °C. After incubation, colonies on

Sabouraud's dextrose agar were examined macroscopically as pasty white color colonies, then followed by identification as *C. albicans* by Gram positive budding yeast cells, morphology of germ tube test, growth and characteristic of chlamydospore on corn meal agar with Tween 80 [15]. After 7 days of treatment, rats were sacrificed and ulcer area was taken for histopathology study [11, 13].

Histopathology study

Specimens were immediately fixed in 10% neutral formalin for 72 h. then demineralized using a 10% EDTA solution for 21 days. The demineralized specimens were then washed with tap water and embedded in paraffin. A histological section of 5 µm thickness was prepared from each specimen and stained with hematoxylin and eosin stain. Light microscope was used with 100X, magnification to examine the stained sections [16].

Results and Discussion

Maceration with DW was the method used in this study to extract *Silybum marianum*. Every 100 g

of dry powder seeds yielded 6.2 g (6.2%) as shown in Table 1.

Unfortunately, *C. albicans* resistance to antifungal therapy was the most common problem associated with candidiasis. Scientists have therefore shifted their way toward using medicinal plants for the treatment of various infectious diseases. Exploiting its content of bioactive compounds, *Silybum marianum* dried seeds extracts were used anciently in the treatment of many diseases especially diseases associated with liver and bile, in addition to its antimicrobial and anti-inflammatory activity. Extract's yield depends on many factors such as the type of solvent used, harvesting time, storage conditions, and geographic climate [17-20].

Phytochemical detection test

Screening of phytochemicals which showed a positive result for the presence of flavonoids, phenols, terpenoids, and tannins. However, alkaloids showed a negative result as shown in Table 2.

Table 1: Percentage yielded of *Silybum marianum* seeds extract

Weight of <i>Silybum marianum</i> dry powder (g)	Weight of aqueous extract (g)
100	6.2

Table 2: Phytochemical screening test of *Silybum marianum* aqueous extract

Phytochemicals	Result
Alkaloids	-
Flavonoids	+
Phenols	+
Terpenoids	+
Tannins	+

Phytochemical detection has been first reported in the beginning of the 2000s Shah and coworkers (2011) in their study on *Silybum marianum* phytochemicals have reported the presence of flavonoids, phenols and tannins with no alkaloids and saponin, however the presence flavonoids and phenols accounts for the multiple biological activities of plants which make it an effective treatment for various disorders [21].

In vitro study

Anticandidal activity was tested by using fluconazole disc (25 µg) as the positive control, *Silybum marianum*-extract 0.5 mg/ disc and DW as the negative solvent control against control isolate of *C. albicans*. Inhibition zone diameter caused by fluconazole was found to be 19 mm, while inhibition zone of *Silybum marianum*-extract was measured as 15 mm with no anticandidal activity detected for DW disc as shown in Figure 1.

In vivo study

Viable cell count

Viable counts of *C. albicans* colonies (Figure 2) demonstrated a trend of decline in yeast counts in response to treatment with fluconazole and

Silybum marianum from day 1 when treatment started until day 7 where no viable cells were cultured (Figure 3). However, treatment with DW did not show any effect on *C. albicans* growth throughout the treatment period (Figure 3).



Figure 1: In vitro anticandidal activity of fluconazole, Silybum marianum-extract on *C. albicans* control isolate

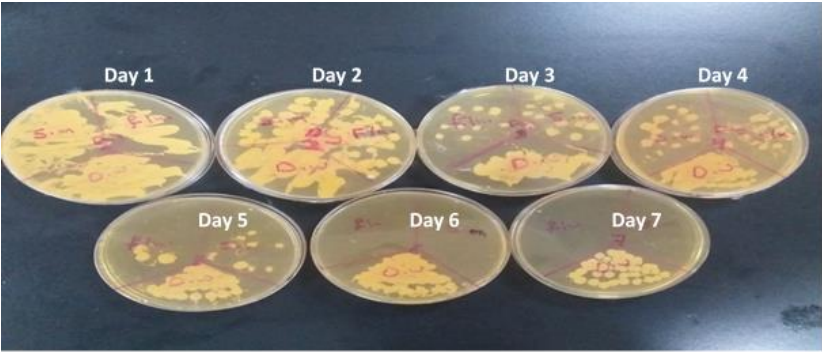


Figure 2: Representative images of *C. albicans* growth reduction during the treatment period from day 1 until day 7. A trend of growth decline is shown in response to treatment with fluconazole and *Silybum marianum* extract

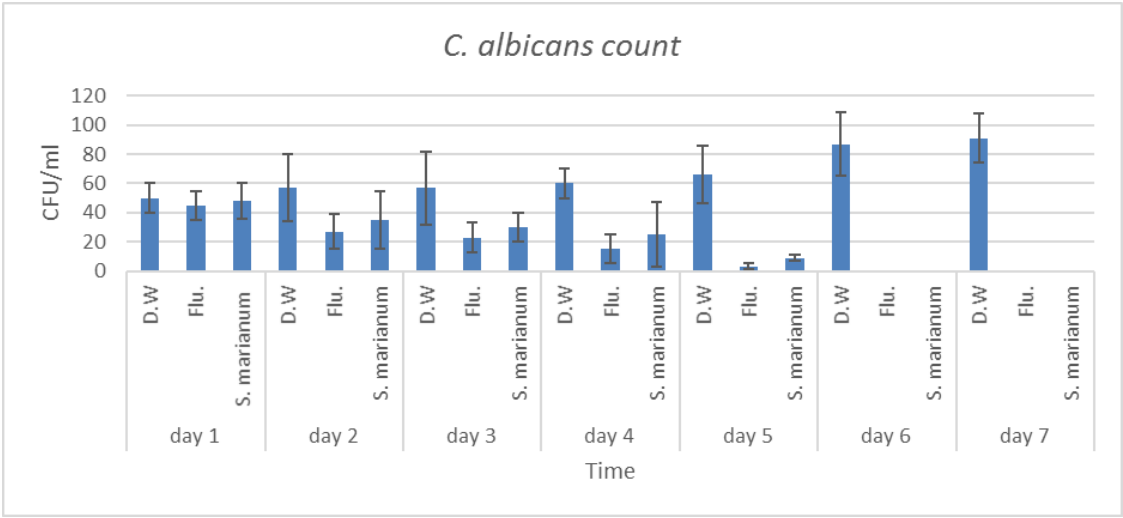


Figure 3: *C. albicans* count during 7 days of treatment in three study groups. DW; distilled water, Flu.; fluconazole, S. marianum; Silybum marianum. Error bar represents mean \pm SD

Histopathology study result

Histopathology sections of the three study groups were prepared to study the anticandidal and ulcer healing activity of *Silybum marianum*-extract versus the standard treatment fluconazole. Figure 4a and b illustrates the hard palate histology section of group I. A clear ulcerative lesion (Figure 4 (↔)) with tissue necrosis and mucosal sloughing was demonstrated. Budding yeast cells at the periphery of the ulcer site with necrosis were found (Figure 4a). No re-epithelization was monitored (Figure 4b). Budding yeast cell at the periphery of ulcer site (Figure 4b).

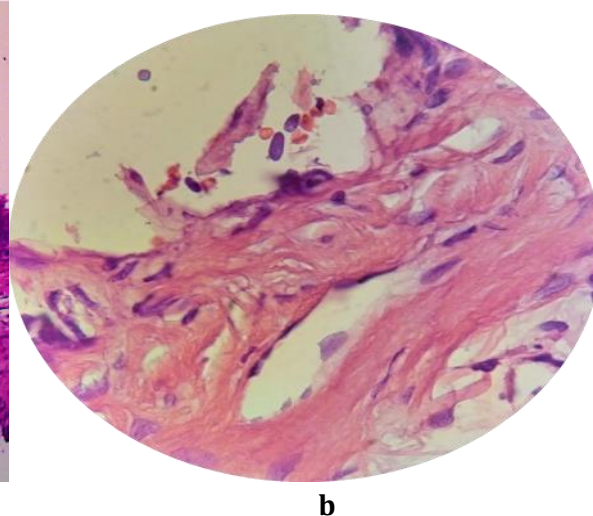
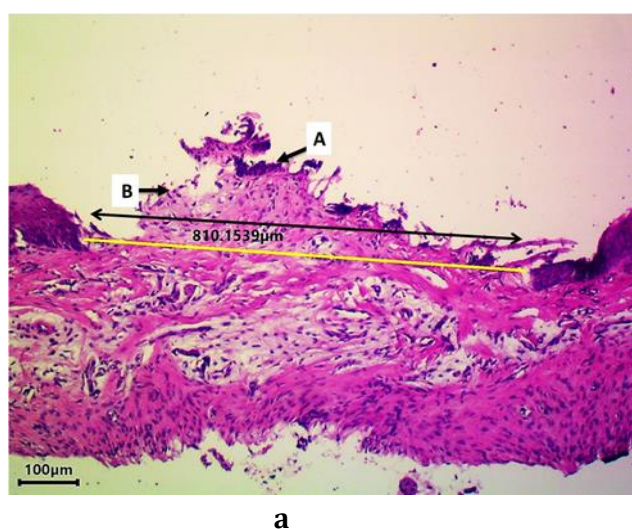


Figure 4: Photomicrograph of rat hard palat of negative control group (GI). Ulcer size (↔) measures 810 μm. Necrosis, budding yeast cells at the periphery (A) and no reepithelization (B) was monitored (100X). b: Magified (400X) budding yeast cells at the periphery of ulcer site. Sections were stained with H&E stain

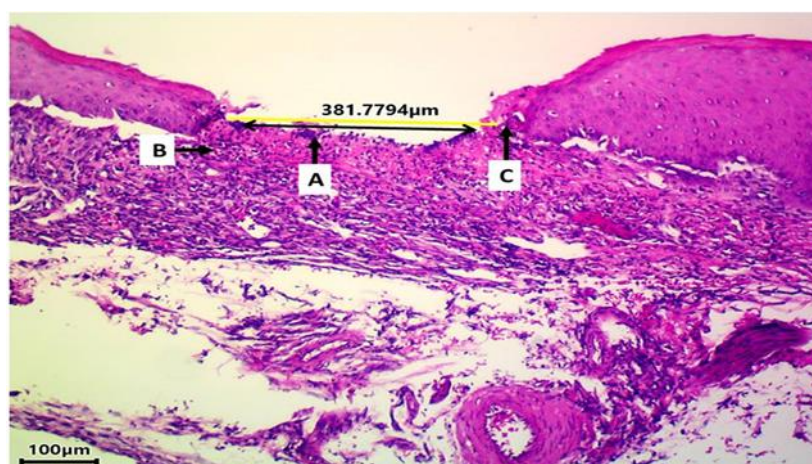


Figure 5: Photomicrograph (100X) of rat hard palat of II (group treated with *Silybum marianum*) shows the site of ulcer with mild necrosis and sloughing mucosa (↔) (ulcer size is 381.77 μm in length), (A) epithelial cell syncytia at the periphery of the ulcer site, (B) inflammatory cells (C) well developed re-epithelialization. Sections were stained with H and E stain

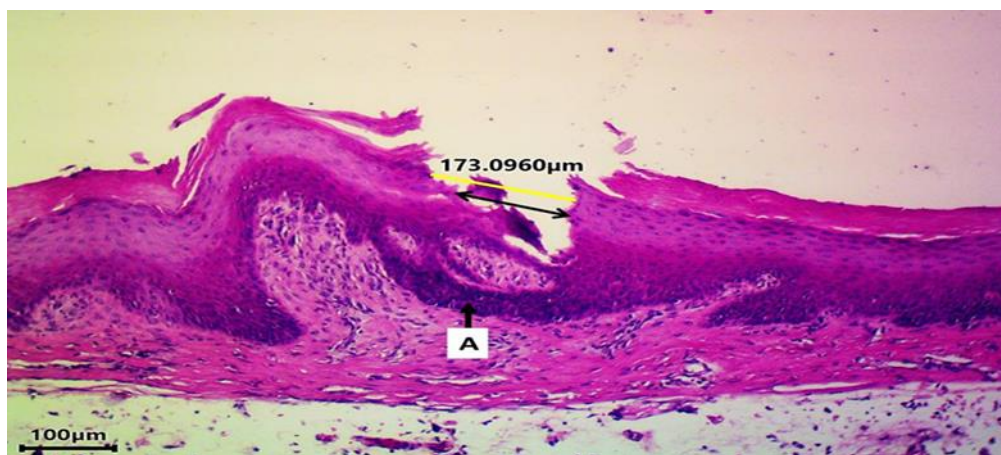


Figure 6: Photomicrograph (100X) of rat hard palate of group III (treated with fluconazole) shows the site of ulcer with mild necrosis and sloughing mucosa (\leftrightarrow) (173.09 μ m in length), (A) with well-developed re-epithelialization as bridge formation. Sections were stained with H and E stain

In vivo and *in vitro* studies were a very important diagnostic procedure to detect the activity of any medicinal extract versus control drug used in treatment any diseases [22]. Current *in vitro* antimicrobial assay showed that the zone of inhibition of fluconazole was 19 mm, while *Silybum marianum*-extract showed a zone of inhibition of 15 mm, with no anticandidal activity of the negative DW control. This significant growth suppression effect reported for *Silybum marianum* may be due to the action of the natural antifungal proteins with a defensin domain, with other protective proteins that have been found in *Silybum marianum* [23]. This result came in accordance with the *in vivo* finding of viable microbial cells culturing and enumeration on the selective medium. The decline in ulcer yeast load following treatment with *Silybum marianum* confirms the reported *in vitro* anticandidal effect. *In vivo* rats models with *C. albicans* infection it turned out to be the best way for testing the antifungal effectiveness of the *Silybum marianum* before use in humans. *Silybum marianum* had an excellent activity inferior to that of fluconazole against *C. albicans* in rat animal model [22]. Oral ulcer with candidiasis is a complex problem making treatment procedure more complicated, although effective treatment requires regulation of the inflammatory process with antifungal therapy. *Silybum marianum* was reported to promote healing process by augmenting the anti-inflammatory activity of cytokines associated with natural antifungal activity *Silybum*

marianum proteins [24]. In day 7 of treatment procedure for the two treated groups (group II & group III), histology examination revealed a site of healing ulcer with re-epithelization and mild ulcer necrosis with no budding yeast in comparison with the negative control of DW (group I). Results of this study indicated that *Silybum marianum* had a beneficial eradicating activity on *C. albicans* and in enhancing ulcer healing process, since *Silybum marianum* had no health side effect when use within the therapeutic dose [25-28].

Conclusion

This study concluded that *Silybum marianum* had a good anticandidal therapy that may be used as an alternative or adjuvant therapy to the standard existing medicines used for the treatment of oral candidiasis. Information can be provided from experiment rat model to discover the potential antifungal agents as *Silybum marianum* extract to be used in anticandidal pharmaceutical herbal oral product.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

The author declared that they have no conflict of interest.

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