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Full Length Research Paper

Silymarin Natural Antimicrobiol Agent Extracted from Silybum Marianum

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ABSTRACT

The goal of this work is the study and the valorisation of a medicinal plant *Silybum marianum*, widely responded in Mediterranean region, particularly in Algeria. The chloroform and butanolic solvents extracts of *Silybum marianum* were screened for antibiocidal and phytochemical properties. Flavonoids were detected in both extracts. These extracts were active against *Staphylococcus aureus*, *Staphylococcus albus*, *Candida albicans* and *Saccharomyces cerevisiea* with a diameter exceeding (15mm). Flavonoides were separated and identified by a thin layer chromatography (TLC) on silica gel. The TLC results allows to identify 3 different spots S1, S2 and S3.The thermostability essays revealed their resistance at low (-5°C, 4°C) and high temperatures (40°C, 60°C) during 30 min and inactivated at 100°C. These results prove antibiocidal effects of flavonoids extracted from *silybum marianum*, which enlarge the therapeutic properties of this plant.

Key words: silymarin, flavonoids, antibiocidal activity, TLC, CMB/CMI

1. INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Nostro et al., 2000).

Flavonoids are a group of natural compounds known to have various pharmacological actions such us antioxydative, anti-inflammatory and diuretic (Havsteen, 2002).

The development of resistance to the available antibiotics has lead researchers to investigate the antimicrobial activity of medicinal plants. *Silybum marianum* commonly called blessed milk thistle is a small trees belonging to *Asteracea* family with up to 1meter high, widely spread in Mediterranean region notably in Algeria.

Flavonoids are naturally occurring substances that possess various pharmacological actions and therapeutic applications. Some, due to their phenolic structures, have antioxidant effect and inhibit free-radical mediated processes (Montvale; 2000).

The extracts of the flowers and leaves of *Silybum marianum* (St. Mary's thistle, milk thistle) have been used for centuries to treat liver, spleen, and gallbladder disorders (Rainone, 2005). In the 1960s the biologically active principles of the seed and fruit extracts were isolated, and the chemical structures were elucidated. The isolation led first to a mixture that was named *Silymarin*, and it was with this flavonolignan mixture that most of the clinical studies were carried out. The main constituents are *Silibinin*,

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Isosilibinin, *Silicristin*, and *Silidianin* (Sonnenbichler and al., 1999).

One of the important issues about *Silymarin* is that it may be accepted as a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designed therapeutic dosages (Montvale, 2000). In this study, the chloroformic acetate of ethyl and butanolic extracts of *Silybum marianum* were investigated for antimicrobial and antifungal activity. The phytochemical components were also investigated as a scientific assessment for the claim of therapeutic potency.

The study aimed investigating the antimicrobial activity of the plant by preliminary in-vitro bioassay screening using aqueous and petroleum ether as well as chloroform extracts.

2. MATERIALS AND METHODS

2.1. Plant Material

Flowers of *Silybum marianum* were collected and seeds than pulverized into small coarse powder stored until required for use.

2.2. Microorganisms

Microorganisms denoted with ATCC included in this study (*Staphylococcus aureus*, *Staphylococcus albus*, *Pseudomonas* sp, *Escherichia coli*, *Serratia* sp; *Aspergillus* sp, *Penicillum* sp, *Candida albicans* and *Saccharomyces cereviseae*) were provided from the medical institute of microbiology (Constantine; Algeria). The microorganisms were maintained on nutrient agar slants at 4°C, reidentified by biochemical tests and sub-cultured in nutrient broth for 24h prior to testing.

2.3.Extraction and Fraction Procedure

Fractionation of the extracts was fractionated using ethanol-water 80/20 v/v for 24h during three days and different organic solvents (petroleum-ether, chloroform, acetate of ethyl and n-butanol). The powdered extract of the plant 100g was overnight fractionated with ethanol-water (800 ml) at room temperature (Isaac and Chinwe, 2001). The extract was filtered and then partitioned into petroleum-ether than chloroform, acetate of ethyl and butanolic solvents. The different alcoholic extracts were evaporated in Rotavapor at 40-50°C. Finally reconstituted in 6 ml of methanol as a contributory antimicrobial effect of the organic fractions. (Markham, 1982).

2.4. TLC Analysis

Merck silica gel plates Kieselgel F254 (Merck, Germany) and the following mobile phases were used as eluent for TLC:

S1: toluene/butanol/ethanol/petroleum ether 20/10/10/20 v/v.

S2: chloroform/acetone/formic acid 75/16,5/8,5 v/v.

S3: ethyl acetate /methanol/water 50/20/10 v/v.

The chromatogram was evaluated under light after spraying the plate with godin reagent (1% ethanolic solution of vanillin, following by 3% of perchloric acid solution).

2.5. Antimicrobial Assays

Pure culture of the organisms were inoculated onto Muller-Hinton nutrient broth (Oxoid, England), incubated for 24h at 37°C. Diluted with sterile nutrient broth to a density of 9×10^8 cfu/ml equivalent to *McFarland* test. The suspension was used to streak for confluent growth on the surface of *Muller-Hinton* agar on *Petri* dishes with sterile swab. Using a sterile 6mm disk contained methanol as positive control. The *Petri* dishes were placed in the incubator overnight at 37°C. The

antimicrobial activity was recorded if the zone of inhibition was greater than 9mm (Hassan et al; 2006).

Antimicrobial activity was investigated by the disk diffusion method and the broth two fold macro dilution methods. Results of the diffusion method were expressed as the diameter of the inhibition zone around the hole filled with investigated solution.

Dilution method results were recorded as the minimum inhibitory concentration (MIC) and minimum microbiocidal concentration (MMC). Details of both methods are described else-where.

The determination of the minimum inhibitory concentration MIC for butanol and chloroform extracts showed significant activity (d > 9mm) and were chosen for MIC assay. MIC was determined by the standard method (Kamagate and al; 2002) in there nutrient broth was prepared and sterilized. 5 ml of the prepared broth was dispensed in to the test tubes.

Serial dilutions of plant extract (chloroform and butanol) were undertaken to test the growth capacity of the different microorganisms. 200 µl of each extract dilution were transferred into each tube with exception of control tubes and incubated 24-48 h at 37°C.

2.6. Effect of Temperature

Chloroform and butanolic extracts were treated at: 4°C, 40°C, 60°C and 100°C during 30min. 100 µl of the extracts were added to suspension of nutrient broth and *Yeast* extract glucose medium (YG) inoculated by *Staphylococcus albus* and *Candida albicans*. The suspension was used to streak for confluent growth on the surface of *Muller-Hinton* agar on *Petri* dishes with sterile swab. Using a sterile disk of 6mm diameter, tow disk contained methanol were used as reference or positive control. The Petri dishes were placed in the incubator at 37°C overnight.

2.7. Effect of Variation

400µl of extract (chloroform, ethyl acetate) were distributed in curved *Eppendorf* tubes then dried by evaporation and treated (NaOH/HCl) 1N/1N, fitted by means of a PH-meter to the following PH values: 1.16; 3.01; 6.5; 8.6; 9.57; 11.9; 12.8; 13.15 and finally left reacting for 30min.

3. RESULTS AND DISCUSSION

3.1. TLC

TLC was employed to determine the composition of flavonoids in each fraction. Flavonoids of *S. marianum* were separated in three main fractions, which were evaporated and redisoleved in 98% methanol. The results lead to different spots S1, S2, S3 with an RF (Rf1 = 0.36cm; Rf2 = 0.40cm; Rf3 = 0.53cm) subsequently corresponding to *Silydianine*, *Silychristine*, and *Silybine*, the active constituent of *Silymarine* (Fig. 1).

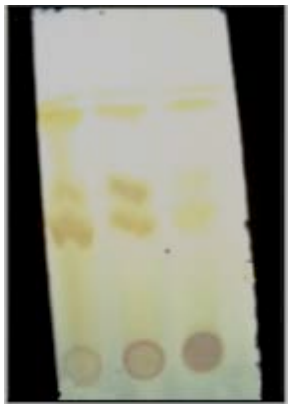

Figure 1 TLC results of the different extracts (1: chloroform; 2: ethyl acetate; 3: butanol).

3.1. Antimicrobial Results

98% methanol showed no inhibition zones. Both flavonoid fractions inhibited the growth of most of the microbial strains tested. The only exceptions were gram negatives and *Mycelium fungi*. The

majority of yeasts were sensitive to both flavonoids fractions. *Candida albicans* and *Saccharomyces cereviseae* were the only yeast inhibited by both extracts. Among *Mycelium fungi* were resistant.
The size of all inhibition zones was between 9 and 16mm for fungi. Average size of inhibition zones was around 11mm for chloroform and ethyl acetate fractions.
Bacteria most susceptible to both fractions were gram-positive bacteria: *Staphylococcus aureus*, and *Staphylococcus albus* with a diameter of 17 and 18mm.

Dilution phase	C0	C1/2	C1/4	C1/8	C1/16	C1/32
chloroforme	---	---	---	+++	+++	+++
Butanol	---	---	---	+++	+++	+++

(+) resutlts; (-): results

Table 1 Liquid dilution method results against *S. albus* using chloroformic exacts.

Phase Dilution	C0	C 1/2	C 1/4	C1/8	C1/16	C1/32
Chloroforme	---	---	---	+++	+++	+++
Butanol	---	---	---	+++	+++	+++

(+) results; (-): results

Table 2 Liquid dilution method results against *C. albicans* using chloroformic exacts.

3.2. MIC and CBM Results

98% methanol showed no inhibition zones. Both flavonoid fractions inhibited the growth of most of the microbial strains tested. The only exceptions were gram negatives and *Mycelium fungi*. The majority of yeasts were sensitive to both flavonoids fractions. *Candida albicans* and *Saccharomyces cereviseae* were the only yeast inhibited by both extracts, *however mycelium fungi* were resistant.

	CMI (mg/ml)	CMB (mg/ ml)	CMI (mg/ml)	CMB (mg/ml)	Chloroforme Extract	Butanolic Extract
	chloroformic Extract		butanolic Extract		CMB / CMI ratio	
S. albus	10 ,25	41	7	28	4	4
C. albicans	20,5	41	14	28	4	2

Table 3 Results of CMI and CMB; CMI/CMB of actives extracts against *Staphylococcus albus* and *Candida albicans*.

The MIC of chloroform and butanolic extracts ranged from 10, 25 - 20, 5 mg/ml and 7- 14 mg/ml respectively (Table 3). The chloroform extract has the lowest MIC compared to butanolic extract.
The CMB of the extracts ranged from 41 and 28 mg/ ml respectively. The CMB / CMI ratio ranged from 4 and 2 mg/ml subsequently indicated a bacteriostatic action of flavonoids (Archambaud, 2001).
The antimicrobial properties of this plant probably explain its traditional use for treating bacterial diseases. In 1996 Freiburghans indicate that different solvent extracts of some plant may exhibit pharmacological properties.
The mechanism of action of constituents of *S. marianum* may be difficult to speculate; however, many antibacterial agents may exhibit their action through inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (Palanichamy et al., 1990). It is probable that the antimicrobial agents in the extracts act via some of the above cited mechanisms. Further studies for in-*vitro* activity, isolation and structural elucidation of the active components of the plant extracts are recommended.

3.3. Effect of Temperature

Chloroform and butanolic extracts were treated at: 4°C; 40°C; 60°C; 100°C during 30min.
100µl of the extracts were added to suspension of nutrient broth and YG inoculated by *Staphylococcus albus* and *Candida albicans*.

The suspension was used to streak for confluent growth on the surface of *Muller-Hinton* agar on *Petri* dishes with sterile swab. Using a sterile disk of 6mm diameter, tow disk contained methanol were used as reference or positive control. The Petri dishes were placed in the incubator overnight at 37°C. The antimicrobial activity recorded if the zone of inhibition was greater than 9mm (Hassan et al; 2oo6).

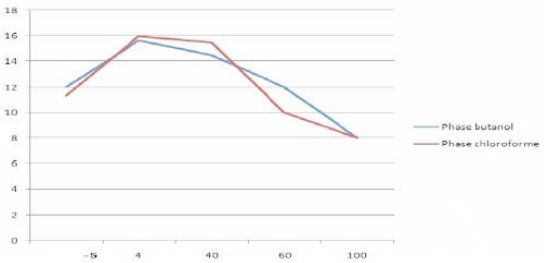

Figure 2 Inhibition of microbial growth via butanolic and chloroformic extracts treated at different temperatures.

These results indicate that flavonoids of *Silybum marianum* conserves their activity at moderate temperature [-5; 4; 40; 60°C]; and inactivated at 100°C, this results are relatively different comparative at those obtained in 2006 by Daughari. The optimum activity of biological molecules was located at 40°C and 60°C.

3.4. Effect of Ph Variation

According to the face (Fig. 3) three intervals of Ph appear: from 1, 16 to 3, 01.

The value of the diameter of inhibition increases slightly; it is situated between 10mm and 11mm for the extract of chloroform, and of 15mm in 17mm for ethyl acetate extract. From 3,01 to 9,75: the diameter of inhibition increases and remains relatively constant in value, it is situated between 15mm and 16mm for the chloroform extract and 9mm and 12mm for the ethyl acetate extract with a decrease of diameter of inhibition. Slightly until reach15 mm for the extract of ethyl acetate (Laleh et al., 2006).

From9, 75 to 13, 16: the value of the diameter of inhibition decreases until it reaches the 10mm value for the extract of chloroform and then increases. The PH influences the activity of flavonoids by substitution of the groupings (OH) which surmount the structure three-dimensional of these compounds what is confirmed by the statistical study: Analysis of the variance (ANOVA): Fobs > F 1, 14, 5%; (6,68 >2, 4):

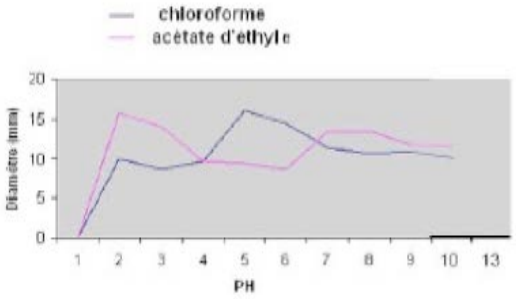

Figure 3 Influence of PH variation.

4. CONCLUSION

Both flavonoid fractions inhibited the growth of most of the microbial strains tested: Gram-positive bacteria and Yeast. The CMB/CMI ratio ranged from 2 and 4 mg/ml subsequently indicated a bacteriostatic action of flavonoids. TLC results led to obtaining different spots S1, S2, S3 with an RF of Rf1=0.36cm; Rf2=0.40cm; Rf3=0.53cm subsequently corresponding to *Silydianine*, *Silychristine* and *Silybine* the active constituent of *Silymarine*.

The optimum activity of biological molecules was located at 40°C and 60°C and at moderate Ph [6,5 - 8,5].

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