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Antioxidant and Antimicrobial Activities of Cynara scolymus L. Rhizomes

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Abstract

The natural forests and meadows of the Green Mountain of Libya are classified as the richest floristic region of the country. Among various species *Cynara scolymus* L. (Asteraceae), is the most used medicinal plant. *C. scolymus* L., has long been regarded as noncrop food and also as an effective medicinal plant. In the current study, total phenols as well as flavanoids were determined in methanolic and aqueous extracts the of *C. scolymus* L. rhizomes in order to assess their contribution to the antioxidant activity. Free radical scavenging activity of the extracts was investigated and compared with ascorbic acid. Methanolic extract exhibited a higher total phenols (45.11 mg GAE/g DW) and flavonoids (37.00 mg Rutin/g), that results in a high antioxidant activity with IC_{50} (17.77 µg/ml). In contrast, aqueous extract showed a lower total phenolic and flavonoid contents (37.79 mg GAE/g DW and 15.51 mg Rutin/g DW, respectively) and IC_{50} (66.3 µg/ml). Isolated flavonoids showed the highest free radical scavenging activities (IC_{50} = 13.33 µg/ml). The *in vitro* antibacterial activity of *C. scolymus* L. rhizomes was investigated against various strains of bacteria by hole-plate diffusion method. The minimum inhibitory concentrations (MICs) of the crude methanol, flavonoids and alkaloids extracts found to be between 0.03 and 200 mg/ml for the susceptible organisms, with that of MRSA being the least.

Keywords: medicinal plants, flavonoids, free radical scavenging capacity, antioxidant, phenolics, antibacterial

1. Introduction

Medicinal plants are considered as a vital portion of the vascular flora distributed all over Libya especially in the Green Mountain, Ghadames, Gharian, Awbari and Tarhona regions. Generally they are used in folk medicine against common colds, asthma, kidney problems, skin inflammations, liver diseases and hypertension (Rateeb et al., 1996). One of the most frequently used medicinal plants of Libya is artichoke (*Cynara scolymus* L.). This plant is well-known for its nutritional and curative properties due to some bioactive components that have antioxidant and antibiotic activities. In addition, it shows interesting tendency of protection against degenerative diseases such as cancer. In folk medicine, many parts of *C. scolymus* L. have been widely used as astringent, blood cleanser, cardiotonic, detoxifier, digestive stimulant, diuretic and hypoglycaemic, hypocholesterolemic as well as medicine for liver complaints (Lattanzio et al., 2009). Moreover, artichoke leaf extracts was proved to have hepatoprotective, anticarcinogenic, antioxidative, anti-inflammatory, antibacterial, anti-HIV, bile-expelling, and urinative activities as well as the ability to inhibit cholesterol biosynthesis and LDL oxidation (Martino et al., 1999; Lattanzio et al., 2009; Bundy et al., 2009). These variable therapeutic functions cannot be attributed to a single active compound; however it could be due to the presence of several bioactive components which generate synergistic pharmacologic effects.

Many evidences are indicating that the interaction between various phytochemicals, especially phenolic and flavonoid compounds is involved in reducing the risk of various degenerative diseases (Ness & Powles, 1997; Riboli & Norat, 2003). A prominent property of flavonoids is their antioxidant capacity which could afford some protection against oxidative stress. The antioxidant activity of flavonoids and other polyphenols appears to play a major role, due to their capacity of scavenging reactive oxygen and nitrogen species (Frei & Higdon, 2003). Flavonoids induce enzymes of detoxification and inhibit platelet aggregation. They have an inhibitory effect against LDL-oxidation (Lattanzio et al., 2009; Ceccarelli et al., 2010). However, inhibitory effects of these

compounds on prooxidant enzymes such as xanthine oxidase, myeloperoxidase and lipoxygenases may additionally contribute to the potential beneficial activity of flavonoids and polyphenols (Sadik et al., 2003).

The most active components found in artichoke species consists of flavones, their glycosides, coumarins, sterols caffeoylquinic acids and triterpenoid saponins (Willett et al., 1995; Martino et al., 1999; Visioli et al., 2004; Pinelli et al., 2007) which are associated with their relative activity against microorganisms. The mechanisms of phenolic toxicity against microorganism might be related to their reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason & Wasserman, 1987).

The high prevalence of the multidrug resistant bacteria which is still uncertain to wait the discovery of effective antimicrobial drugs represents a pressing demand on the pharmaceutical industry (Gowri & Vasantha, 2010). Therefore, there is a need to evaluate plants as a source of potential chemotherapeutic agents, antimicrobial agents and their ethno-medicinal uses (Prashanth et al., 2006).

Despite of many studies revealed that the leaf and head of *Cynara scolymus* L. extracts have remarkable antioxidant and antibacterial activities (Wang et al., 2003; Zhu et al., 2004; Brat et al., 2006; Vamanu et al., 2011), in Libya people pay more attention to under-ground parts as a good source of bioactive compounds than green parts due to the spiny green parts that artichoke has and to their availability for short period (end of February till the beginning of April) as well as it is not cultivated. Therefore, the aim of the present study was to determine total polyphenols, flavonoids and alkaloids contents in *C. scolymus* L. rhizomes and evaluate their antibacterial activities against some human pathogenic bacteria. The antioxidant capacity was also evaluated by measuring reducing power and DPPH radical scavenging potential.

2. Material and Methods

2.1 Chemicals and Test Bacteria

Chemicals including 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), potassium persulphate, sodium carbonate, monobasic sodium phosphate, dibasic sodium phosphate, trichloroacetic acid, potassium ferric cyanide [$K_3Fe(CN)_6$] and Rutin were obtained from Sigma Aldrich (Gillingham, Dorset, UK). Other chemicals were of acceptable laboratory grade.

The test bacteria (*Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli, Methicillin-resistant Staphylococcus aureus* (*MRSA*), *Salmonella typhi* and *Pseudomonas aeruginosa*) were kindly provided by the Microbiology Laboratory, Faculty of Pharmacy, Tripoli University, and Biotechnology Research Center (Twaisha), for cultivation and bacterial growth, nutrient broth and Mueller-Hinton agar media (Difco, England) were used.

2.2 Plant Material

The rhizomes of artichoke (*Cynara scolymus* L.) were collected during spring of year 2009 from the Green Mountain (east of Libya). The plant was authenticated by the Botany Department, Faculty of Science, Tripoli University, Tripoli, Libya. Fresh rhizomes were allowed to dry at room temperature, ground into a powder, passed through a suitable mesh sieve and the dried rhizomes powder was then stored at room temperature (25°C±2) until analysis.

2.3 Preparation of Plant Crude Extracts

The dried rhizomes powder (20 g) was soaked for 6 hours in 100 ml of distilled water in tightly sealed vessels at room temperature. The same weights of plant materials were extracted with boiling water for 10-15 min. The methanolic extracts were prepared by extracting the dry rhizomes powder (20 g) three times each with 200 ml of methanol. All crude extracts were filtered through Whatman filter paper (No. 1) and kept at 4°C until tested.

2.4 Phytochemical Analyses

The fresh methanolic crude extracts were qualitatively screened for the following constituents: flavonoids, coumarines, hydrolysable tannins, alkaloids, terpenes, anthraquinones and saponins according to Harborne (1992). The qualitative results have been rated from (+ve) for faint to (++++ve) for dense turbidity or colour.

2.5 Alkaloids Extraction

The extraction was carried out according to Hadi and Bremner (2001). Methanol extraction was continued until the powdered plant material gave a negative result for alkaloids (Mayer's test). The final extract was filtered and the filtrate was concentrated under vacuum. The crude alkaloid mixture was then separated from neutral, acidic materials, and water soluble ingredients by extraction with aqueous acetic acid (5%) followed by

dichloro-methane extraction, then basification of the aqueous solution using sodium carbonate (5%, w/v) and re-extracted with dichloromethane. The alkaloid extract was tested for antibacterial activity.

2.6 Flavonoids Extraction by Soxhlet Method

The extraction was carried out using powdered plant material and petroleum ether in a Soxhlet apparatus for 12 h. The petroleum ether extract was discarded and the residue was mixed with methanol and refluxed again. The final extract was collected and the *p*H was adjusted to *p*H 5 using diluted glacial acetic acid. The flavonoid residue was kept for further analysis.

2.7 Determination of Total Phenolics Content

The content of total phenolics in plant samples was determined using the Folin-Ciocalteu method (Singleton et al., 1999). Absorbance was measured at 765 nm after 1 h. Gallic acid was used as a standard.

2.8 Determination of Total Flavonoids Content

Flavonoid content of the crude extracts was estimated according to the modified method of Marinova et al. (2005). The diluted standard solutions or plant extract (0.5 ml) were separately mixed with 1.5 ml of 80% ethanol, 0.1 ml of 10% aluminum chloride (Merck, Germany), 0.1 ml of 1M potassium acetate (Kemika, Croatia) and 2.8 ml of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. Rutin was used for calibration curve (25, 50 and 100 μg ml^{-1} in 80% ethanol).

2.9 Determination of Antioxidant Activity

2.9.1 Reducing Power

The antioxidant activity was investigated using the ferric reducing antioxidant power (FRAP) assay for plant extracts according to the method of Oyaizu (1986). Plant extract of 1, 10, 50 or 100 mg in 1 ml of ethanol was mixed with 2.5 ml of phosphate buffer (2 M, *p*H 6.6) and 2.5 ml of potassium ferricyanide (10g/L) then the mixture was incubated at 50^0C for 20 min., then 1.5 ml of trichloroacetic acid (100 g/L) was added to the mixture, which was centrifuged at 3000 rpm for 10 min. Finally, 0.5 ml of the supernatant solution was mixed with 1.0 ml of distilled water and 0.5 ml of $FeCl_3$ (1.0 g/L) and the absorbance was measured at 665 nm. Increased absorbance of the reaction mixture indicates increasing of reducing power of plant ingredients.

2.9.2 DPPH Radical Scavenging Activity

The free radical scavenging activity was quantitatively tested using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) based on Wong et al. (2006) method. The initial absorbance of DPPH in methanol was measured at 515 nm until the absorbance remains constant. A volume of 40 μl of extract was added to 3 ml of 0.1 mM methalonic DPPH solution. The mixture was incubated at room temperature for 30 min. After that the absorbance at 515 nm was measured. The percentage of inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = [(A_{515} \text{ of control} - A_{515} \text{ of sample})/A_{515} \text{ of control}] \times 100$$

The IC_{50} was calculated as the amount of antioxidants present in the sample necessary to reduce the initial DPPH concentration by 50%. All determinations were performed in triplicates.

2.10 Antibacterial and Minimum Inhibitory Concentration (MIC) Assessment of Plant Extracts

The antimicrobial activity of the crude and alkaloid extracts was determined by the "hole-plate diffusion" method (Daud et al., 2005). Each test organism was maintained on nutrient agar slant and was recovered for testing by growth in nutrient broth (Biolab, Difco) for 14 h at 37°C before streaking. Cultures were routinely adjusted to a suspension of 1×10^6 to 2×10^6CFU/ml using pre-made calibration curve representing viable cell count ($X \times10^6$) against OD_{660} nm (Y). 150μl aliquots of the extract were placed into wells of 8mm diameter. The plates were kept for 1h at 4°C for allowing better diffusion of the extract into the agar. Subsequently, plates were incubated at 37°C for 18h. Vancomycin and Tetracycline (30μg each) were used as positive control while sterile water or 70% methanol was used as negative control. Diameters of inhibition zones (DIZ) were measured in mm and the results were recorded as the mean of triplicate experiments. The MIC was carried out using agar two fold serial dilution assay (Irith et al., 2008). The concentrations (mg/ml) of extracts were prepared; reference antibiotics and the solvents were also assayed. The MIC was recorded as the lowest concentration of the sample that inhibits visible growth giving clear zones of inhibition after 24 h incubation.

2.11 Bioautography

10 μL of solutions corresponding to 1000 μg of alkaloids and Flavonoids extracts were applied to precoated Silica-gel TLC plates, developed with ether: ethylacetate (3:1, v/v) for both extracts, and evaporated to complete dryness. The dried plates were overlaid with nutrient agar medium seeded with *B. subtilis* (10^6-10^7 CFU/ml) and

then incubated for overnight at 37°C. For comparative purposes, an additional plate was run alongside under the same conditions and sprayed for visualization of alkaloids and flavonoids (Krebs et al., 1969).

2.12 Statistical Analysis

Statistical Analysis: All the grouped data were statistically evaluated with Microsoft Excel 2007 Data Analysis software. Hypothesis testing methods included Student's "t" test. *P* values of less than 0.05 were considered statistically significance. Values are presented as the mean ± S. D. of each triplicate in each experiment.

3. Results and discussion

Although the chemical components of artichoke shoot have been studied extensively and found to be a rich source of polyphenolic compounds (Lattanzio et al., 2005; Orlovskaya et al., 2007), few studies have been mentioned regarding rhizomes of *C. scolymus* L. The rhizomes of *C. scolymus* L. are widely used as a folk medicine in Libya. It is used as anti-hypertension, antidiabitic as well as hypercholesterolemia. Therefore, in the present study, we investigated the most important bioactive constituents of the rhizomes with special attention to the antibacterial and antioxidant activities.

The phytochemical constituents of *C. scolymus* L. rhizomes methanolic extract (Table 1) proved to be rich in tannins (++++ve), alkaloids (+++ve), flavonoids (+++ve) as well as terpenes (++ve), anthraquinones (++ve) , but poor in saponins (+ve) with no coumarines (-ve), these results are in agreement with a previous study (Wagenbreth et al., 1996).

Table 1. Phytochemical screening of the methanolic crude extract of *C. scolymus* L. rhizomes

Phytochemical Compounds	*Intensity*
Alkaloids	+++ve
Saponins	+ve
Anthraquinones	++ve
Tannins	++++ve
Terpenes	++ve
Flavonoids	+++ve
Coumarines	-ve

3.1 Flavonoids and Phenols Content of the Extracts

There is a growing interest in research and food industry in many plant extracts that have dual antioxidants and antimicrobial activity, due to their possible use as natural additives instead of synthetic antioxidants and as safer preservative sources (Deba et al., 2008).

In general, the results revealed that total phenolic (PC) and flavonoid contents (FC) were higher in methanol than those in water extracts (Pourmorad et al., 2006) (Figure 1). Flavonoids content in terms of Rutin equivalent in methanol extract (37.00 ± 2.84 mg/g DW) was significantly higher ($P<0.05$) compared with water extract (15.51 ± 1.94 DW). Figure 1 also shows the contents of total phenols that were measured by Folin Ciocalteu reagent and calculated as Gallic acid equivalent. The phenols content in the methanolic extract (45.11±1.22 mg/ g DW) was slightly higher than that in water extract (37.79 ± 1.12 mg/ g DW). Previous findings indicated that the heads of artichoke have higher polyphenolic content than the leaves. Our results using the *C. scolymus* L. rhizomes showed higher polyphenolic content compared with both head and leaves, as reported by Brat et al. (2006).

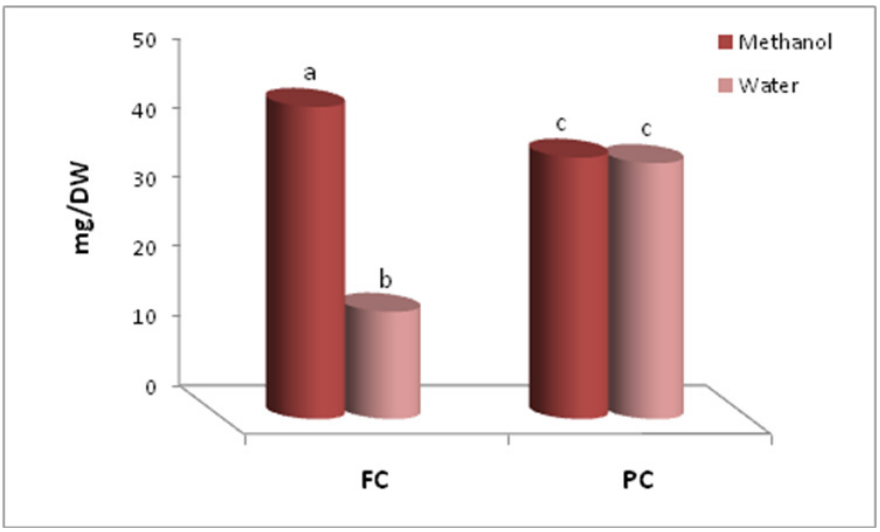

Figure 1. Total phenolic content (PC) and flavonoid content (FC) of methanolic and water extracts of *C. scolymus* L., (mean± SD). mg/g DW: milligram galic acid equivalent per gram dry weight (PC); mg Rutin/g DW: milligram Rutin equivalent per gram dry weight (FC). Arithmatic Means with the same letter are not significantly different at $P < 0.05$

3.2 Antioxidant Activity

3.2.1 Reducing Power

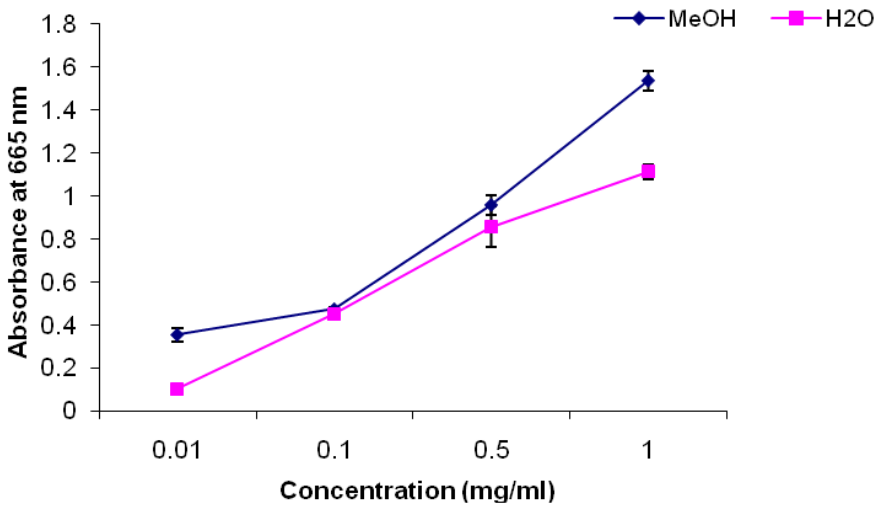

Figure 2. Reducing ability of aqueous and methanolic extracts of *C. scolymus* L.

Figure 2 shows the level of reducing power of the *C. scolymus* L. rhizome extracts, as a function of their concentrations. The reducing power of methanol and water extracts were found to be very potent and increased with increasing concentration of reducers (Duh et al., 1999) in which the highest reducing power was reached at the highest concentration tested (1mg/ml). The reducing power capacity for water extract was noticeably ($P<$ 0.05) less than that recorded for methanolic extracts especially at highest concentration (1 mg/ml). The high reducing power of methanol extract indicated their potential as electron donors to scavenge free radicals efficiently (Schimada et al., 1992).

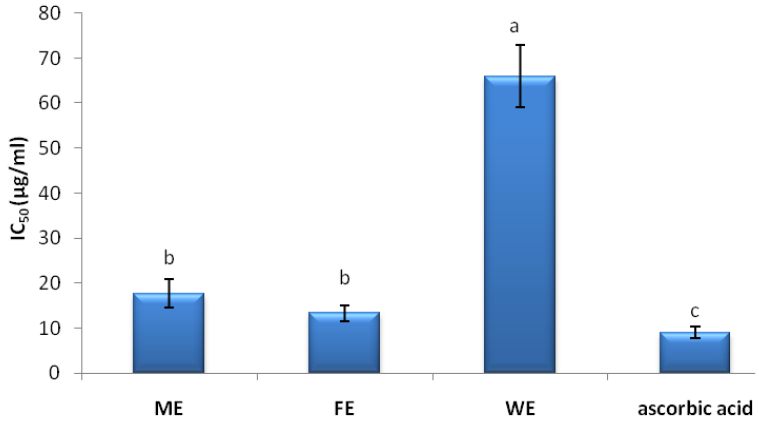

Figure 3. Free radical scavenging activity of methanolic (ME), flavonoids (FE) and water (WE) extracts from *C. scolymus* L. Ascorbic acid is a positive control. Values are means±SD of three replications. Arithmatic Means with the same letter are not significantly different at $P < 0.05$

Plants contain a wide variety of free radical scavenging molecules, such as flavonoids, anthocyanins, vitamins and endogenous metabolites which are natural products with antioxidant activities (Willett et al., 1995; Visioli et al., 2004). Free radical scavenging capacities of the extracts, measured by DPPH assay are shown in Figure 3. Methanol and flavanoids extracts had stronger activity than the aqueous extracts due to their high phenolic content Figure 1 (Sadik et al., 2003; Naili et al., 2010). The activity of the extracts is proportional to the concentrations and the lower IC_{50} value reveals better protective action. The methanol and flavonoid extracts of C. *scolymus* L. rizhomes were able to reduce the stable free radical DPPH with an IC_{50} of 17.7 and 13.3 µg/ml respectively, whereas the aqueous extract was found to be less efficient in radical scavenging with an IC_{50} value 66 µg/ml. In addition, the result demonstrated that antioxidant activity of this plant extracts by DPPH assay had similar trend with reducing power assay.

3.3 Antimicrobial Activity

In this study crude methanol, flavonoids and alkaloids extracts are found to be very effective in inhibiting the growth of all the tested bacteria giving a range of 16-40 mm inhibition zone diameter (Table 2). The maximum inhibitory effect of flavonoid extract was observed on *Methicillin-resistant Staphylococcus aureus* (MRSA), *Escherichia coli*, *Salmonella typhi* meanwhile, it was moderate against *Bacillus subtilis, Pseudomonas aeruginosa and Saphylococcus aureus*. Alkaloids extract exhibited strong antibacterial effect against MRSA and *B. subtilis*, moderate antibacterial effect against *S. aureus* with mild effect against *E. coli*, *P. aeruginosa* and *S. typhi*. Crude Methanol extract showed maximum inhibitory effect on MRSA, *E. coli*, and *P. aeruginosa* but moderate antibacterial effect against *S. aureus*, *B. subtilis* and *S. typhi*. Crude water extract demonstrated maximum inhibitory effect on MRSA, *S. aureus* and *B. subtilis* and moderate antibacterial effect on *S. typhi* but mild inhibitory effect against *P. aeruginosa* and *E. coli*.

According to MIC results (Table 3), flavonoids extract was the most effective one against bacteria (with MICs of 0.03-0.4 mg/ml), followed by the alkaloids, and methanolic extracts. The *P. aeruginosa* was found to be the most resistant species, with MICs of 0.312-200 mg/ml. Unexpectedly; MRSA was the most sensitive, with MICs of 0.03-6.25 mg/ml. In some cases, commercial Tetracycline showed higher antibacterial potency than did the extracts tested except against MRSA and *P. aeruginosa* (Table 2).

The antibacterial activity of *C. scolymus* L. rhizomes extracts can be related to their content of flavonoid and phenols which have been found effective antimicrobial substances against a wide array of microorganisms *in vitro* (Srinivasan et al., 2001). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, (Tsuchiya et al., 1996). Some alkaloids are also found to have antimicrobial properties (Omulokoli et al., 1997; Alghazeer et al., 2012).

Table 2. Antibacterial activities of the extracts of Rhizomes of *C. scolymus* L.

Test bacteria	Aqueous, Methanolic, Alkalaoids and Flavonoids extract from Rhizomes of *C. scolymus* L.					
	Zone of inhibition (mm)[a]					
	CW	CM	A. Extract	F. Extract	Vancomycin	Tetracyclin
B. subtilis	24±0.3b	21±0.2b	29±1.9a	16±0.2c	15	24
S. aureus	28±0.4a	18±0.7b	21±0.9b	12±0.3c	10	26
E. coli	11±0.7d	30±1.9a	19±0.1d	21±0.6c	24	27
S. typhi	18±0.2c	23±0.2b	12±0.2d	25±0.4a	21	25
MRSA	34±1.9a	35±2.1a	30±1.6b	40±2.5a	-	-
P. aeruginosa	14±0.4c	28±0.7a	12±0.4c	17±0.2b	-	-

Values are means of three replications ± SD. Means with the same letter are not significantly different at $P< 0.05$; a: Diameter of hole 8 mm; CW: crude water extract (30 mg/hole); CM: crude methanol extract (30 mg/hole); A: Alkaloids(1.5 mg/hole); F: Falavnoids (0.12 mg/hole).

Table 3. The *in vitro* MIC (mg/ml) values of methanolic crude, flavonoids, and alkaloids extracts of rhizomes of *C. scolymus* L.

Test Organism	*Methanol*	*Flavonoids*	*Alkaloids*
B. subtilis	12.5	0.20	0.625
S. aureus	12.5	0.20	0.312
E. coli	12.5	0.40	2.5
P. aeruginosa	200	0.20	5.0
S. typhi	50	0.20	0.625
MRSA	6.25	0.03	0.312

More investigation for antimicrobial properties of alkaloids and flavonoids extracts was carried out using bioautography method (Nostro et al., 2000) to specify the biologically active separated spots (against *B. sub*) on thin layer chromatograms from alkaloids and flavonoids extracts (Table 4). Thin Layer-Bioautographic application showed the presence of four biologically active spots (S1-S4) with R_f values of 0.12-0.79 from alkaloids extract as detected by alkaloid spray reagent. Flavanoids extract was also subjected to bioautography and revealed four biologically active spots as allocated with R_f values of 0.13-0.77. This could be preliminary orientation results concerning how many possible bioactive plant components need separation and identification.

Table 4. Bioautographic profile (with R_f values) of alkaloid and Flavonoid extracts of *C. scolymus* L. rhizomes

	Alkaloids extract				Flavonoids extract			
Spot No	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
R_f	0.79	0.63	0.56	0.12	0.13	0.22	0.39	0.77

All spots separated were active against the most sensitive test bacterium (*Bacillus subtilis*). Best separation was accomplished using Chloroform: Methanol: (25%) Ammonia solution (8:2:0.5 v/v) for alkaloids, Benzene: Methanol: Ethyl acetate: Chloroform (5:1:2:2 v/v) for flavonoids.

4. Conclusion

The most important biologically active components including flavonoids and alkaloids of *C. scolymus* L. were extracted and investigated. The methanolic, aqueous and flavonoids extracts were screened for their capacity to scavenge the DPPH radical. It can be concluded that most extracts of rhizomes especially methanolic and flavonoids extracts exhibited reliable free radical scavenging activity. Methanolic extract consisted of a mixture of polar compounds. Most of these compounds especially polyphenols which have been recognized for their strong antioxidant potential were also responsible for the high free radical scavenging activity. Antibacterial properties of methanolic, alkaloids and flavonoids extracts were also assessed. All extracts showed very good

activities that are comparable with the commercial antibiotics. Therefore, *C. scolymus* L. rhizomes extracts can be used in the treatment of infectious diseases and as a promising source of some compounds that could be used to formulate new antimicrobial drugs of natural origin.

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