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Antimicrobial activity of leaf and fruit extracts of Jordanian *Rubus sanguineus* Friv. (Rosaceae)

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***In vitro* antimicrobial activity of ethanolic and methanolic extracts of the leaf and fruit of *Rubus sanguineus* were investigated against pathogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans* using agar well diffusion and microdilution broth assays. This study showed that leaf ethanolic extract exhibited the best antimicrobial activity with zone of inhibition ranging from 20 to 22 mm. The minimum inhibitory concentration (MIC) of the ethanolic leaf extract range was 1.56 and 12.5 mg/mL while that of methanolic extract range was 0.78 to 12.5 mg/mL. The ethanolic leaf extract exhibited appreciable activity against *Candida albicans* with zone of inhibition of 20 mm. The anticandida activity was support by MIC tests. In conclusion, the methanolic and ethanolic leaf and fruit extracts of *Rubus sanguineus* have a significant activity against Gram positive bacteria and Candida but have not shown any significant activity against Gram negative bacteria investigated in this work. Results showed that there is a basis for the traditional use of this plant as a healthy remedy in Jordanian culture.**

Key words: Antimicrobial activity, Gram positive bacteria, Gram negative bacteria, *Candida albicans*, *Rubus saguineus*, Jordan.

INTRODUCTION

Plants have been playing an important role in alternative medicine since ancient times (Oran and Al-Eisawi, 1998). Many of these plants are used as chemical feed stocks or as raw material for many scientific investigations, also they are commercially important especially in pharmaceutical industry (Joy et al., 1998). *Rubus* species belong to the family Rosacea and in use as alternative medicine to cure diarrhea, intestinal disorders and its fresh juice is used for treating tuberculosis (Oran and Al-Eisawi, 1998). *Rubus*, latin name for holy bramble or blackberry and *sanguineus* is blood colored. This plant is a wild shrub with edible fruits found near river banks, by .prings and swamps (Zohary, 1972). *Rubus sanguineus* disperse their seeds via frugivores, change fruit color from green to red with very sour taste while still unripe and then to black or dark blue upon ripening. The antimicrobial effect of this plant has not been extensively studied, and there is little information about the medicinal uses of different species of *Rubus*. However; few studies showed that some species of *Rubus* have antimicrobial capacities and they have been investigated using different techniques (Panizzi et al., 2001; Thiem and Goślińska, 2004).

In Jordan, this plant is reputed traditionally for its use to

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treat different infections. This necessitated our attention to investigate the role of ethanol and methanol extracts of *R. sanguineus* leaf and fruit, which is found in the upper and lower Jordan valley, for its antibacterial activity against human pathogenic Gram positive bacteria that is, *Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) and Gram negative bacteria that is, *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and also to determine the antifungal activity of these extracts against *Candida albicans*.

MATERIALS AND METHODS

Plant materials

The plants leaves and fruits of *R. sanguineus* were collected from different areas along Jordan valley and later identified by Sawsan Oran (Professor of plant biosystematics at the University of Jordan, Faculty of Science, Department of Biological Sciences, Amman, Jordan).

Microorganisms and growth conditions

Microorganisms were obtained from American Type Culture Collection. Pathogenic organisms were two Gram positive bacteria*, S. aureus ATCC 29213* and *B. cereus ATCC14579*, and two Gram-negative bacteria, *E. coli ATCC35218* and *P. aeruginosa ATCC10145,* and the yeast *C. albicans ATCC 90028*. The bacterial cultures were maintained on Mueller Hinton Agar (MHA) (Oxoid). Overnight cultures were prepared by inoculating 5 mL of Mueller Hinton Broth (MHB, Oxoid) with 5 colonies of each microorganism taken from MHA. Broths were incubated overnight at 37°C. However, *Candida* strain was maintained on Sabouraud Dextrose Agar (SDA, Oxoid). Suspensions were prepared using Sabouraud Dextrose broth. Bacterial and yeast suspensions were prepared by diluting overnight cultures in PBS to 0.5 *McFarland* standard. These suspensions were further diluted with PBS as required.

Preparation of plant extracts

Collected plant materials (leaves and fruits separated) were air dried for approximately two weeks. Dried plant samples were grinded using a grinder (Ambar, Liban) and then 50 g of the dried powdered plant were soaked separately in 1 L of ethanol and methanol. After soaking for two weeks they were filtered using Whattman no. 1 filter paper. All filtrates were evaporated using rotary evaporator (Janke and Kunkel, Germany) and left to dry at room temperature for 24 h and weighed. The air dried stock extracts were then reconstituted in 25% dimethylsulphoxide (DMSO) solution to get 25 mg/ml concentrations and sterilized by filtration (mini pore filter 0.22 µm) and stored in refrigerator at 4°C prior to determination of antimicrobial activities of the extracts (Othman et al., 2011; Rawani et al., 2011).

Microbiological screening

Antimicrobial activities of different extracts were evaluated by the agar well-diffusion method, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (Murray et al., 1995). MIC of each extract was expressed as the lowest dilution level of the extract needed to inhibit bacterial growth, while MBC is the lowest dilution level which completely kills the bacteria.

Determination of antimicrobial activity of the extracts

The antimicrobial activity was determined by the well diffusion method according to NCCLS M2-A5 (NCCLS, 1993). Mueller-Hinton and Sabouraud Dextrose plates were inoculated by streaking the swab over the entire agar surface using bacterial suspensions containing 10^8 CFU/mL and yeast suspensions containing 10^7 CFU/mL. The plates were allowed to dry at room temperature. Using a sterile agar cutter, 6 mm diameter wells were bored in the agar. The antimicrobial activity of the extracts was checked by introducing 50 µL of 25 mg/mL concentrations into triplicate wells. An additional well in each plate was filled with the solvent DMSO 25% v/v as a control. Commercially prepared gentamicin susceptibility discs 5 µg from Oxoid and 50 µg fluconazole (Sigma) discs were prepared by pipetting 12.5 µl volumes of stock fluconazole (4 mg/mL) onto sterile blank discs were used as positive controls. The culture plates were allowed to stand on the bench for 30 min at room temperature and were incubated at 35°C for 24 h. After 24 h, the antimicrobial activity of the extracts and the antibiotics were determined. Zones of the inhibition around each of the extracts and the antibiotics were measured to the nearest millimeter (Lino and Deogracios, 2006; Wendakoon et al., 2012). The experiment was repeated at least three times for each microorganism.

Determination of minimum inhibitory concentration (MIC)

The MIC test was carried out on the plant extract which showed inhibition zones in the antimicrobial screening. The MIC of the extracts were determined for each microorgansim in triplicates by double fold serial microdilution assay using 96-well microliter plates (Nunc) according to NCCLS M7-A5 guidelines (NCCLS, 2000). The different plant extracts were taken (25 mg/mL) and serially diluted with Mueller-Hinton broth for bacterial culture and Sabouraud Dextrose broth for yeast with their respective inocula were used (Sarker et al., 2007). The final concentrations ranged from 12.5 to 0.097 mg/mL when reconstituted with bacterial and yeast suspension. The wells were inoculated with 5×10^5 CFU/mL of the test bacterial strain according to NCCLS M7-A5 and with 1×10^5 CFU/mL candida strain according to NCCLS M-27 (NCCLS, 1997). The microplates were incubated for 24 h at 35°C. One of the 12 columns served as growth control (bacterial suspension or yeast without plant extract) and another one for the sterility control with only broths in them. The lowest concentration without visible growth was defined as MIC. The readings were compared with gentamicin for flucanazole for bacteria and *Candida* respectively used as control.

Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The MBCs were determined by sub-cultivation of loopful (0.001 mL) the test dilution from 96 well plates used to determine MIC previously onto Mueller –Hinton and Sabouraud Dextrose agar plates. The plates were incubated at 35°C overnight and the lowest concentration, with no visible growth, was defined as MBC or MFC (Minimum Fungicidal Concentration), indicating 99.5% killing of the original inoculum (Wendakoon et al., 2012).

Table 1. Antimicrobial activity of methanol and ethanol extracts of *R. sanguineus* fruit and leaf by well diffusion method.

Organism	Antibiotic/antifungal	Ethanol extract		Methanol extract	
		Leaf	Fruit	Leaf	Fruit
	Zone of Inhibition (mm)				
Yeast	Flucanazole 25 µg				
C. albicans	20 ± 2*	20 ± 1	15 ± 1	18 ± 1	16 ± 1
Gram Positive bacteria	Gentamicin 5 µg				
S. aureus	20 ±1	20 ± 0.5	18 ± 0.5	18 ± 0.5	18 ± 0.5
B. cereus	12 ± 1	22 ± 0.5	19 ± 1	19 ± 1	20 ± 0.5
Gram Negative Bacteria	Gentamicin 5 µg				
E. coli	10 ± 1	7 ± 0.5	7 ± 0.5	7 ± 0.5	7 ± 0.5
P. aeruginosa	7 ± 2	7± 0.5	7 ± 0.5	7 ± 0.5	7 ± 0.5

Values indicate average zone of inhibition in (mm). * Standard errors for three experiments.

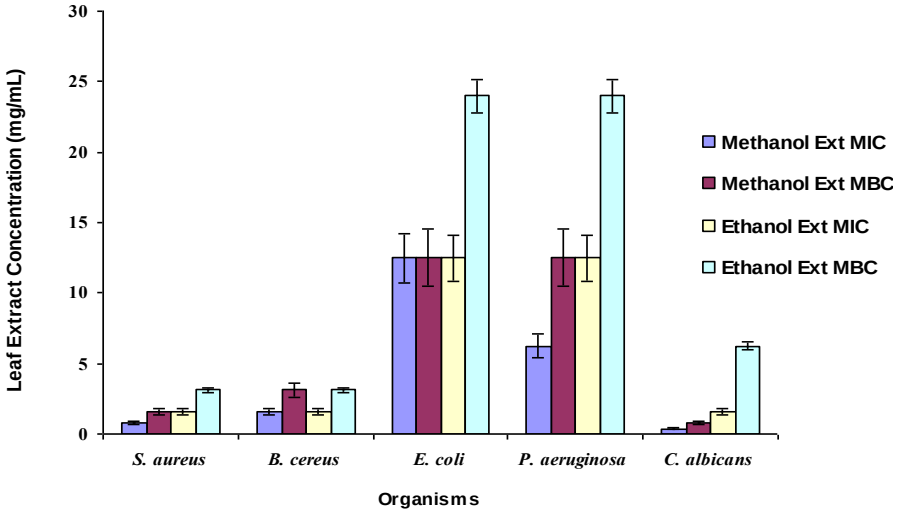

Figure 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of leaf ethanol and methanol extracts of *Rubus sanguineus* against the test organisms.

RESULTS

The results obtained on methanol and ethanol extracts of *R. sanguineus* fruit and leaf by agar well diffusion method are presented in Table 1. Both extracts showed antimicrobial activity against Gram positive bacteria and *C. albicans.* The eaf ethanol extract exerted highest activity on bacteria and *Candida* tested when compared with others. The leaf ethanol extract (1.25 mg) in 6 mm well, 22 mm was recorded as diameter of the zone of inhibition against *B. cereus,* this was followed 20 mm zone of inhibition against *C. albicans* and *S. aureus*. Methanolic fruit extract exerted the highest activity against *B. cereus* (20 mm) when compared with the other extracts. The lowest antimicrobial activity was for ethanolic fruit extract against *C. albicans* (15 mm). The lowest activities (7 mm zone of inhibition) were recorded by DMSO (25 v/v %) used as a solvents, and leaf/fruit ethanol/methanol extracts against *E. coli* and *P. aeruginosa*.

The antimicrobial activity and the potency of the extracts were quantitatively assessed by MIC and MBC as given in Figures 1 and 2. Wherever low MIC and MBC values observed against the test organism means that the plant has the potential to treat any ailments associated

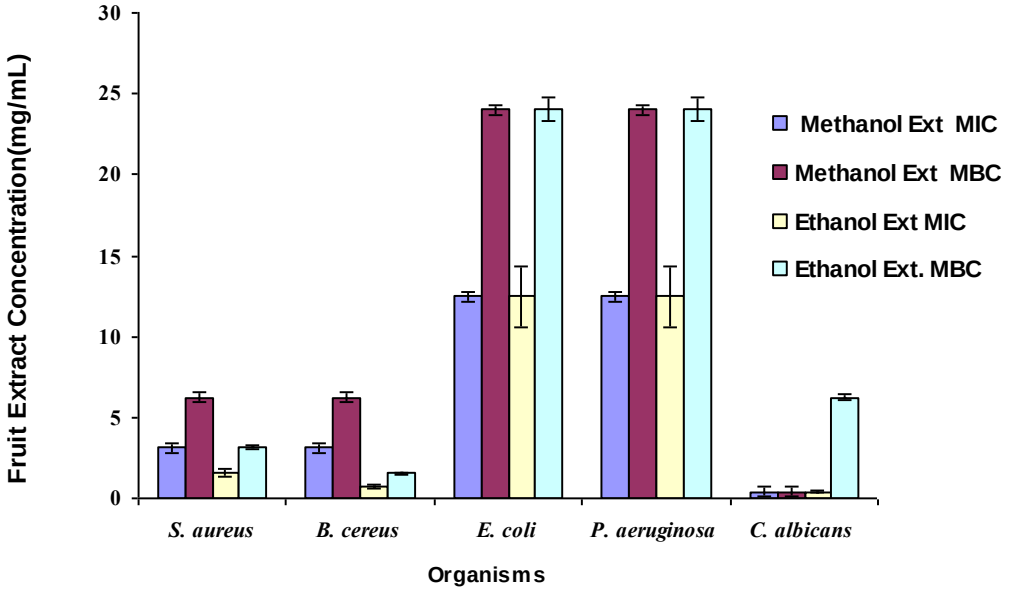

Figure 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fruit ethanol and methanol extracts of *Rubus sanguineus* against the test organisms.

with these pathogens effectively. At the same time high MIC and MBC values however, is an indication of lack of efficacy of the plant extracts against the test organism.

The lowest MIC and MBC values were recorded for methanol leaf extracts with 0.37 and 0.78 mg/mL, respectively against *C. albicans.* Low MIC and MBC values were read when the methanol leaf extracts were tested against Gram positive bacteria (*S. aureus* and *B. cereus).* The MIC readings were in range of (0.78 to 1.56 mg/mL) and MBC readings were in range of (1.56 to 3.13 mg/mL). Whereas high MIC and MBC values were recorded when the extracts were tested against Gram negative bacteria (*E. coli* and *P. aeruginosa*). This result is in accordance with the poor activity noticed in agar diffusion tests. However, the MIC and MBC for the ethanol leaf extracts showed the best activity against Gram positive bacteria and the values were 1.56 and 3.13 mg/mL, respectively (Figure 1).

In Figure 2 the MIC and MBC of fruit ethanol and methanol extracts of *R. sanguineus* are presented against the test microorganisms. Based on the results showed in Figure 2, the MIC value of 0.39 mg/mL is equal to MBC value of the methanol fruit extracts when tested against *C. albicans*. Interestingly, the same MIC value was recorded for the ethanol fruit extract against the yeast; however the MBC was 6.25 mg/mL. Whereas the ethanol fruit extract showed good activity against *B. cereus* with MIC and MBC values 0.78 and 1.56 mg/ml, respectively. Furthermore, MIC and MBC were of 1.56 and 3.13 mg/mL, respectively when the extract was screened for its activity against *S. aureus*.

DISCUSSION

Plant extracts are considered to be valuable source of biologically active compounds showing significant antimicrobial in several cases. In this study, the antimicrobial activity of *R. sanguineus* leaf and fruit ethanol and methanol extracts was assessed against different bacteria and *C. albicans*. These organisms are associated with different types of infections including urinary tract infections, wound infections, gastroenteritis, food poisoning, pneumonia and meningitis (Jawetz et al., 2010).

Results recorded by agar well diffusion method indicated that, the strongest antibacterial activity was obtained for the ethanol extract of *R. sanguineus* leaf against *B. cereus* (zone of 22 mm), followed by methanol extract of the fruit (zone of 20 mm) and by both the ethanol fruit and methanol leaf extracts (zone of 18 mm).

The leaf ethanol extract of *R. sanguineus* showed the highest antibacterial activity against *S. aureus* (zone of 20 mm) and the other extracts had the same activity of 18 mm. Whereas the Gram negative bacteria had showed resistance to all the studied extracts and that was manifested with no inhibition zones in agar well diffusion experiments. The extracts' activities were recorded same as DMSO (25 v/v %) activity which was used as a solvent control in the study.

Among the leaf and fruit methanol and ethanol extracts of *R. sanguineus*, the ethanolic extract of the leaf showed high zone of inhibition with diameter of 20 mm against *C. albicans*. This result was not in full support according to microdilution broth experiments. The lowest MIC value

was recorded for the ethanol and methanol fruit extracts and methanol leaf extracts (MIC = 0.39 mg/mL), this was followed by the ethanol leaf extract activity with MIC equals to 1.56 mg/mL against *C. albicans* . The differences in the observed activity of the various extracts may be caused by the varying solubilities of the active ingredients in the primary solvents (ethanol and methanol) and the secondary solvent which was DMSO (25 v/v %). It is well known that different solvents have diverse solubilities capacities for different phytoconstituents (Marjoriue, 1999). The other explanation might be due to the presence/ absence of one or more ingredient(s) different in dextrose sabouraud broth and dextrose sabouraud agar which influence or blocks the active phytoconstituents.

Results from microdilution experiments showed that the MIC and MBC values of the ethanol extract of *R. sanguineus* leaf against *B. cereus, and S. aureus* were 1.56 and 3.13 mg/mL, respectively. While the MIC and MBC values of the fruit methanol extract were 3.13 and 6.25 mg/mL, respectively. At the same time high MIC and MBC values for the extracts against *P. aeruginosa,* and *E. coli* is an indication of lack of efficacy of the plant extracts against the test bacteria and/or the possibility that the bacteria may possess the capacity to develop resistance against the plant extracts. However, the observed low MIC and MBC values against candida and Gram positive bacteria means that the plant has the potential to treat any ailments associated with these pathogens effectively.

Conclusion

Demonstrating the antimicrobial activity of leaf and fruit extracts of *R. sanguineus* against some pathogenic Gram positive bacteria and *C. albicans* is an indication that this plant might be considered as an alternative therapy to antibiotics for developing novel antimicrobial agents.

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