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Antibacterial activity of *Hypericum* (Hypericaceae) species against Gram-positive bacterial isolates

B. Saleh*, M. Khawajkiah and A. Almariri

[1]*Department of Molecular Biology and Biotechnology PO Box 6091 AECS, Damascus. Syria*

Abstract

Antimicrobial activity of three *Hypericum* species widely distributed in Syria including *H. triquetrifolium* Turra, *H. thymifolium* Banks & Sol, and *H. perforatum* L. species has been evaluated using aqueous, methanol, chloroform and diethyl-ether solvents against eight Gram-positive bacterial isolates. Antibacterial test has been performed based on zone of inhibition (ZI), minimum inhibition concentration (MIC), minimal bactericidal concentration (MBC) determination and time-kill kinetic assays. Data revealed that the diethyl-ether *H. triquetrifolium* areal parts extracts showed the highest antibacterial activity by showing the highest ZI value of 16.3 mm and the lowest MIC & MBC values of 0.03 and 0.06 µg/mL against *Streptococcus faecalis.* Allover, diethyl-ether *H. triquetrifolium,* methanolic *H. thymifolium* and methanolic *H. perforatum* extracts were strongly active against all tested bacterial isolates. Whereas, aqueous *Hypericum* species extracts were non active against all tested bacterial isolates, regardless tested *Hypericum* species. Moreover, Time-kill kinetics test revealed that methanolic *H. thymifolium* extracts exhibited bacteriostatic action. Further performance studies regarding diethyl-ether *H. triquetrifolium,* methanolic *H. thymifolium* and methanolic *H. perforatum* extracts are requested.

Keywords: Antibacterial activity, *Hypericum* species, minimum inhibition concentration (MIC), minimal bactericidal concentration (MBC), zone of inhibition (ZI)

1.Introduction

The emergence of resistant bacterial pathogens caused antibacterial therapeutic failure worldwide. In this regards, *Staphylococcus aureus* and *Acinetobacter baumannii* and *Enterobacteriaceae* such as *Salmonella* species among multidrug resistance (MDR), were considered as global health problem in hospital intensive care units [1, 2].

Interest in the use of medicinal plants has increased in recent years for use them as a supplement to chemotherapy or conventional treatment with little side effects even as a future alternative. Plants are an important source of secondary metabolism (flavonoids, carbohydrates, tannins, alkaloids, amino acids, which makes them an effective candidate for use them in pharmacy and medicine. These plant species vary in their content from these active compounds by plant type, stages of growth and development, geographical distribution, soil type and altitude from the sea, and extraction methods also clearly affect their chemical content.

Plants can therefore be used as a basic pillar that can be adopted and used in the future cheaply in pharmaceutical studies. Of which *Hypericum* genus belongs to Hypericaceae family, includes approximately 500 species of flowering plants [3]. It is one of the 100 largest genera of flowering plants, comprise 22% of angiosperm diversity. Mouterde (1970) [4] reported the occurrence of 21 species belonged to this genus in Syria. Previously, Hyppocrats and Paracelsus since the ancient Greeks time used this genus for treatment and healing of wounds and as mild antidepressant [5].

Hypericum (Hypericaceae) species are one of the most attractive medicinal plants employed in pharmaceutical applications due to their richness in bioactive compounds either in their essential oil or different extracts. Thereby, many repots have been published in this section as an antibacterial agent; *e.g. H. triquetrifolium*, *H. perforatum* and *H. empertifolium* [6]; *H. perforatum* [7-10]; *H. triquetrifolium* [11]; *H. thymifolium* [12]; *H. humifusume* [13]; *H. roeperianum* [14]; *H.*

* Corresponding author: ascientific@aec.org.sy

triquetrifolium [11]; *H. ericoides* [15]; *H. mexicanum, H. juniperinum* and *H. myricariifolium* [16] and 6 *Hypericum* species [17, 18]. Moreover, Akgoz (2015) [19] reported *Hypericum* species antibacterial, antifungal, anticancer, antimalarial, antioxidant, antidepressant and antiviral properties. Indeed, Rouis et al. (2013) [11] reported cytotoxic, antibacterial, antifungal, and antiviral activities of *H. triquetrifolium.* More recently Kakouri et al. (2023) [20] reported nine *Hypericum* species antibacterial activity; similarly, Ilieva et al. (2023) [21] reported four *Hypericum* species antibacterial activity.Whereas, Sherif et al. (2023) [22] reported *H. perforatum* antibacterial activity.

Whereas, Agapouda et al. (2019) [23] reported the use of *Hypericum* genus as a food supplement worldwide, especially its effects on the central nervous system.

Thereby, the current study focused on evaluation of antibacterial activity of three *Hypericum* species widely distributed in Syria including *H. triquetrifolium* Turra, *H. thymifolium* Banks & Sol, and *H. perforatum* L. species against eight Gram-positive bacterial isolates.

2.Materials and Methods

2.1. Plant materials

Areal parts of three wild *Hypericum* species were harvested (10 plants bulked as representative for each *Hypericum* sp.) during flowering stage; wild grown in their natural habitat from West-Southern regions in Syria. Where, *H. triquetrifolium* Turra was collected from Damascus city. Whereas, *H. thymifolium* Banks & Sol and *H. perforatum* L. were collected from Lattakia city (Table 1).

Table 1. Original sites of *Hypericum* species collection

Hypericum spp.	Code	Original site	Sampling date	Altitude (m)	Annual rainfall (mm)
Hypericum triquetrifolium	HP1	Damascus	June	970	240
Hypericum thymifolium	HP2	Latakia	May	90	800
Hypericum perforatum	HP3	Latakia	May	450	850

2.2. Plant extracts preparation

Plant samples of the *H. triquetrifolium* Turra, *H. thymifolium* Banks & Sol and *H. perforatum* L. species were shade dried for two weeks. Dried materials were milled to fine powder by special electric mill and stored separately in glass bowls until extracts preparation process. One gram of powder for each species was subjected to extraction with 100 mL solvent (aqueous, methanol, chloroform and diethyl-ether solvents), until complete solubility. Then, the extracts were filtered with Whatman filter papers. Extracts were kept at laboratory temperature for 2 h to evaporate the solvent. All extracts were then kept in tightly fitting stopper bottles and stored in 4°C. The concentration of crude extract was considered 10 mg/mL.

2.3. Examined isolates and growth conditions

Eight pure Gram-positive bacterial clinical isolates (*Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis, Listeria monocytogenes, Streptococcus pyogenes, Streptococcus faecalis, Micrococcus luteus* and *Enterococcus faecalis*) were obtained from the Microbiology and Immunology division, Department of Molecular Biology and Biotechnology of Atomic Energy Commission of Syria (AECS) in Damascus - Syria. These bacterial were the most important microorganisms in clinical laboratories. Culture was maintained at 37°C on 2YT agar (peptone, 16 g/L; yeast extract, 10 g/L; NaCl, 5 g/L; agar, 13 g/L [Difco, BD, Spars, MD]); and incubated for 24-48 h. Prior to antibacterial sensitivity test, 0.2 mL of overnight culture of each organism was dispensed into 20 ml of sterile Mueller Hinton Broth (Hi-media Laboratory Pvt. Ltd., Mumbai, India) and then incubated for about 18-24 h. The bacterial microorganisms were suspended in a sterile phosphate-buffered saline (PBS). Bacterial abundance in PBS was screened by recording the optical density (OD) at 590 nm, to standardize the cultures to approximately 10^6 CFU/mL (Saleh et al., 2015) [2]. The exact counts were assessed retrospectively by viable counts on 2YT agar plates.

2.4. Antibacterial activity assay

2.4.1. The disc-diffusion test

The disc-diffusion test was carried out for monitoring three *Hypericum* species antibacterial inhibitory effect and Vancomycin (10 mg/mL) (Bayer, Istambul, Turkey) antibiotic was used as a standard drug control for *Hypericum* antibacterial effect [2, 24]. The sterilized discs filter paper (Whatman no.1 of 6 mm diameter) were inoculated with 100 µL of extract dilutions (10 mg/mL) and reconstituted in minimum amount of solvent were

applied over each of the culture plates previously cultivated with the 10^6 CFU/mL cultures of bacteria. Bacterial cultures were then incubated at 37°C for 18 h. Whereas, paper discs were inoculated with 20 µL of a solution of 10 mg/mL of Ciprofloxacin were used as standard antimicrobials for comparison. Negative control was achieved using solvents (final concentration of the solvent in the highest concentration of *Hypericum* extracts was tested). Antibacterial inhibitory effect was determined by measuring the zone of inhibition (mm) appeared around each paper disc. For each extract, duplicate trials were conducted against each microorganism.

2.4.2. Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Determination

Microdilution broth susceptibility assay was performed as reported by Ríos-Dueñas et al. (2011) [25]. Three replicates of serial dilutions of *Hypericum* extracts (50 mg/mL) or of antibiotic (128 mg/mL) were prepared in LB broth medium in 96-well microtiter plates, using a range of concentrations for aqueous and six solvents extracts of each examined *Hypericum* species from 0.166 to 40 µL per well. One hundred microlitres of freshly grown bacteria standardized at 10^6 CFU/mL in LB broth were added to each well. Positive control was prepared with the same conditions but without extract. As for negative control, it was also made with the same conditions but without adding the bacteria. The plate was then incubated with shaking for 24 h at 37°C. The lowest concentration that completely inhibited visual growth was recorded and interpreted as the MIC_{50}. Whereas, MBC was determined by plating 0.010 mL from the wells showing no visible growth on Mueller-Hinton agar plates (Oxoid) and incubating for 18–24 h at 37°C. The MBC was defined as the concentration at which there was a 99.9% reduction in CFU compared with the original inoculum.

2.4.3. Time-kill kinetics assay

Time-kill kinetics of methanolic *H. thymifolium* extracts against the studied 8 Gram-positive bacterial isolates was performed according to Olajuyigbe and Afolayan (2012) [26] protocol. Time-kill curve analyses were performed by determine the MIC values using microdilution method before the experiment, then the extracts were incorporated into 100 µL Mueller Hinton broth in 96 well plate at 1/2 MIC, 1 × MIC, 2 × MIC and 4 × MIC. Two controls were included, one Mueller Hinton broth without extract inoculated with test organisms and Mueller Hinton broth incorporated with the extract at the test concentrations without the test organisms, Inoculums density, approximately 0.5 macfarland was used to inoculate 100 µL volumes of plate. The plate was incubated at 37°C on an orbital shaker at 120 rpm. A 10 µL aliquot was removed from the culture medium at 0, 4, and 6 and 24 h for CFU/mL determination by the plate count technique by plating out 10 µL of each of the dilutions. Emergent bacterial colonies were counted after incubating at 37°C for 24 h, CFU/mL calculated and included in comparative study with the count of the culture control without the extract.

2.5. Statistical analysis

The data were analyzed using the Student's t-test. $p < 0.001$ was considered to be significant. Data were analyzed by one way ANOVA to test significance of differences among variables. All tests were performed in triplicates and mean values are presented as mean of three replicates.

3.Results and Discussion

Antimicrobial activity of three *Hypericum* species including *H. triquetrifolium*, *H. thymifolium* and *H. perforatum* species has been evaluated using aqueous, methanol, chloroform and diethyl-ether solvents against eight Gram-positive bacterial isolates.

Data showed that ZI ranged between 4.3 mm with chloroform *H. triquetrifolium* (against *B. subtilis*) & diethyl-ether *H. perforatum* (against *S. faecalis*) – 16.3 mm with diethyl-ether *H. triquetrifolium* (against *S. faecalis* and *E. faecalis*) followed by methanol *H. perforatum* (against *S. pyogenes*) and diethyl-ether *H. triquetrifolium* against *M. luteus* (15.7 mm) (Table 2). Whereas, all aqueous *Hypericum* species aerial parts extracts were non active against all the tested isolates regardless studied *Hypericum* species and tested solvent. Overall, ZI variance analysis was highly significantly different ($p < 0.001$) among the examined *Hypericum* species and solvents (Table 2).

Table 2. *Hypericum* species inhibitory effect using disc-diffusion method

Extract	*S. aureus*	*B. cereus*	*B. subtilis*	*L. monocytogeneses*	*S. pyogenes*	*S. faecalis*	*M. luteus*	*E. faecalis*
H1D	13.3	14.7	15.3	15.3	13.7	16.3	15.7	16.3
H2D	7.0	8.7	7.3	8.7	10.3	8.3	10.3	9.3
H3D	7.0	6.7	4.7	6.7	8.3	4.3	9.3	7.3
H1C	11.7	5.7	4.3	7.3	7.3	7.3	10.7	9.7
H2C	6.7	6.3	8.3	9.7	10.7	10.3	7.3	7.7
H3C	4.7	9.7	6.3	8.7	7.7	8.7	6.3	5.7
H1M	5.3	9.3	6.7	5.7	9.3	6.3	11.3	6.3
H2M	10.7	12.3	12.3	13.7	14.3	13.3	14.7	14.3
H3M	11.3	13.7	13.0	12.3	15.7	14.3	13.7	14.7
H1H2O	NA	NA	NA	NA	NA	NA	NA	NA
H2H2O	NA	NA	NA	NA	NA	NA	NA	NA
H3H2O	NA	NA	NA	NA	NA	NA	NA	NA
Vancomycin	18.0	22.3	20.3	18.3	20.7	19.7	20.7	18.3

- H1: *H. triquetrifolium* - H2: *H. thymifolium* and H3: *H. perforatum* species.
- D: diethyl-ether – C: chloroform and M: methanol solvents.
- NA: no activity

Table 3. Minimum inhibition concentration (MIC) (µg/mL) value of *Hypericum* species areal parts extracts against the studied bacterial isolates

Extract	*S. aureus*	*B. cereus*	*B. subtilis*	*L. monocytogeneses*	*S. pyogenes*	*S. faecalis*	*M. luteus*	*E. faecalis*
H1D	0.03	0.03	0.06	0.06	0.06	0.03	0.125	0.06
H2D	1	0.5	0.5	1	0.5	0.5	0.5	1
H3D	1	ND	ND	ND	ND	ND	ND	ND
H1C	ND	ND	ND	ND	ND	ND	ND	ND
H2C	1	1	1	0.5	1	1	0.5	1
H3C	ND	ND	ND	ND	ND	ND	ND	ND
H1M	ND	ND	ND	ND	ND	ND	ND	ND
H2M	0.06	0.06	0.125	0.06	0.25	0.125	0.125	0.06
H3M	0.06	0.125	0.06	0.125	0.25	0.125	0.125	0.125
H1H_2O	ND	ND	ND	ND	ND	ND	ND	ND
H2H_2O	ND	ND	ND	ND	ND	ND	ND	ND
H3H_2O	ND	ND	ND	ND	ND	ND	ND	ND
Vancomycin	1.25	0.6	0.3125	0.3	0.625	0.625	0.6	0.3

- H1: *H. triquetrifolium* - H2: *H. thymifolium* and H3: *H. perforatum* species.
- D: diethyl-ether – C: chloroform and M: methanol solvents.
- ND: not determined

Moreover, the lowest MIC value was recorded to be 0.03 µg/mL with diethyl-ether *H. triquetrifolium* against *S. aureus, B. cereus* and *S. faecalis* (Table 3). Whereas, chloroform *H. triquetrifolium* & chloroform and diethyl-ether *H. perforatum* were none active against all tested isolates except diethyl-ether *H. perforatum* extract against *S. aureus* (Table 3). Statistical variance test showed that the examined *Hypericum* species and solvents significantly ($p < 0.001$) affect MIC value.While, all aqueous *Hypericum* species aerial parts extracts were non active against all the tested isolates regardless studied *Hypericum* species and tested solvent (Table 3). As for MBC, the lowest MBC value was recorded to be 0.06 µg/mL with diethyl-ether *H. triquetrifolium* against *S. faecalis* (Table 4).

It worth noting that, only diethyl-ether *H. triquetrifolium* & methanolic and diethyl-ether *H. perforatum* extracts, among the tested extracts were active against all tested isolates. Whereas, the remaining extracts including organic and also aqueous once were non active against all tested isolates (Table 4). Statistical variance test showed that the examined *Hypericum* species and solvents significantly ($p < 0.001$) affect MBC value.

Overall, our data were in coherent with Mazandarani et al. (2007) [7] who reported that the aqueous *H. perforatum* aerial parts extract weakly affected bacterial isolates compared to ethanolic one.

Table 4. Minimal bactericidal concentration (MBC) (µg/mL) value of *Hypericum* species areal parts extracts against the studied bacterial isolates

Extract	*S. aureus*	*B. cereus*	*B. subtilis*	*L. monocytogeneses*	*S. pyogenes*	*S. faecalis*	*M. luteus*	*E. faecalis*
H1D	0.125	0.125	0.125	0.25	0.25	0.06	0.25	0.125
H2D	ND	ND	ND	ND	ND	ND	ND	ND
H3D	ND	ND	ND	ND	ND	ND	ND	ND
H1C	ND	ND	ND	ND	ND	ND	ND	ND
H2C	ND	ND	ND	ND	ND	ND	ND	ND
H3C	ND	ND	ND	ND	ND	ND	ND	ND
H1M	ND	ND	ND	ND	ND	ND	ND	ND
H2M	0.25	0.125	0.5	0.25	0.5	0.5	0.5	0.5
H3M	0.5	0.25	0.25	0.25	0.5	0.5	0.5	0.5
H1H_2O	ND	ND	ND	ND	ND	ND	ND	ND
H2H_2O	ND	ND	ND	ND	ND	ND	ND	ND
H3H_2O	ND	ND	ND	ND	ND	ND	ND	ND
Vancomycin	5	2.5	0.625	1.25	1.25	1.25	2.5	1.25

- H1: *H. triquetrifolium* - H2: *H. thymifolium* and H3: *H. perforatum* species.
- D: diethyl-ether – C: chloroform and M: methanol solvents.
- ND: not determined

However, Süntar et al. (2016) [10] reported ethanol *H. perforatum* and its sub-extracts including n-hexane, chloroform, ethyl acetate, n-butanol and water extracts against *S. mutans*, *S. sobrinus*, *L. plantarum* and *E. faecalis* bacterial isolates. They reported that water extract was the most potent against all the tested isolates by showing the lowest MIC value of 8, 8, 16 and 32 mg/mL against *S. mutans, S. sobrinus*, *L. plantarum* and *E. faecalis* bacterial isolates, respectively. Whereas, Dall'Agnola et al. (2003) [17] reported that methanolic *H. caprifoliatum* showed the highest activity against *S. aureus*, compared to the other 5 tested methanolic *Hypericum* species extracts. Indeed, among the six methanolic *Hypericum* species extracts, *H. polyanthemum* and *H. ternum* extracts showed activity only against *B. subtilis*. While, Meral and Karabay (2002) [6] reported that ZI was recorded to be 14, 15, 14; and 9, 10, 8 mm for *S. aureus* and *E. faecalis* with methanolic *H. triquetrifolium*, *H. perforatum* and *H. empertifolium* aerial parts extract, respectively. Whereas, Mazandarani et al. (2007) [7] reported that the ZI was recorded to be 13, 12, 26 and 12 mm against *S. aureus* for ethanolic *H. perforatum* fruit, flower, aerial part, and leaf, respectively. Whereas, they were 9, 9, 10 and 0 mm against *S. aureus* for ethanolic *H. androsaemum* fruit, flower, aerial part, and leaf, respectively.

Milosevic et al. (2007) [8] reported that ZI was recorded to be 2 and 1 mm with ethanolic *H. perforatum* extract (10 mg extract) against. *B. mycoides* and *B. subtilis* isolates, respectively, with MIC value of 3.5 mg/ml for the both isolates.

Whereas, Yousuf et al. (2012) [9] reported that ZI value was recorded to be 17, 19.33 and 18 mm against *S. epidermidis, B. subtilus* and *S. aureus* bacterial isolates, respectively with methanolic *H. perforatum* leaves extract. Moreover, Saddiqe et al. (2014) [15] reported that n-hexane fraction of *H. ericoides* aerial parts was the most active against methicillin-resistant *S. aureus* (MRSA) isolate with ZI of 12 mm at 1.875 mg/mL. Indeed, they reported MIC value of 2048, 1024 and 512 µg/mL against the same isolate using crude methanolic extract, n-hexane and dichloromethane fractions, respectively. Indeed, Süntar et al. (2016) [10] reported that the aqueous *H. perforatum* aerial parts extracts have a high antibacterial activity with MIC of 8 mg/mL against *Streptococcus sobrinus* and *Lactobacillus plantarum* positive-Gram, moderate effect at 32 and 16 mg/mL concentrations against *S. mutans* and *E. faecalis*, respectively. Moreover, ethyl acetate and n-butanol had an antibacterial activity against *L. plantarum* and ethyl acetate & aqueous extracts showed also activity against *E. faecalis* at the same concentrations (16 mg/mL).

Plazas (2017) [16] reported that ethanolic fractions of *H. mexicanum* and *H. myricariifolium* leaf extracts were potent against *S. aureus* and *S. epidermidis* isolates with ZI of above 15 mm. Moreover, the total extract and the hexane fraction of *H. mexicanum* exhibited the best antibacterial activity against *S. epidermidis* with MIC of 0.05 and 0.001 mg/mL, respectively. Whereas, Özkan et al. (2019) [18] reported MIC of 4.8, 4.8, 78, 78 and 78 µg/mL against *S. aureus* with methanol, acetone, chloroform, diethyl ether and petroleum ether *H. perforatum*, respectively.

Whereas, they were 156, 78, 156, 156 and 312 µg/mL against *Streptococcus epidermidis* with methanol, acetone, chloroform, diethyl ether and petroleum ether *H. perforatum*, respectively.
Hilan and Sfeir (2001) [12] reported *H. thymifolium* essential oil antimicrobial activity of aerial parts against 2 Gram-positive bacterial isolates (*S. aureus* and *Strepto fecalis*). Whereas, Yousuf et al. (2012) [9] reported that MIC value was recorded to be 0.78, 0.39, 1.56 and 0.78 mg/mL for *S. epidermidis, B. subtilus, P. vulgaris* and *S. aureus* bacterial isolates, respectively with methanolic leaves of *H. perforatum* extract. Furthermore, Toiu et al. (2016) [13] reported methanolic and ethanolic *H. humifusume* extracts antimicrobial activity against 3 Gram-positive (*S. aureus, B. cereus* and *L. monocytogenes*). They reported that MIC value was recorded to be 0.15, 0.62 and 0.15 mg/mL for methanolic and to be 0.078, 0.62 and 0.078 mg/mL for ethanolic extracts against *S. aureus, B. cereus, L. monocytogenes* bacterial isolates, respectively. Whereas, MBC value was recorded to be 0.3, 1.25 and 0.3 mg/mL for methanolic and to be 0.15, 1.25 and 0.15 mg/mL for ethanolic extracts against *S. aureus, B. cereus* and *L. monocytogenes* bacterial isolates, respectively.

More recently, Kakouri et al. (2023) [20] reported that the MIC and MBC values were recorded to be 0.06 and 0.51 mg/mL respectively against *S. aureus* and to be 0.13 and 0.51 mg/ml respectively against *E. faecalis* using *H. perforatum* extract (hydroalchoholic solution 70% v/v). Moreover, Sherif et al. (2023) [22] reported that ZI value of 23, NA, 22.3, 16.2 and 19.3 mm with methanol, n-Hexane, ethyl acetate, chloroform and aqueous aerial parts *H. perforatum* extracts (10 mg/mL), respectively against methicillin-resistant *Staphylococcus aureus* (MRSA). Whereas, it was 25.3, NA, 19.8, 20.9 and 18.4 mm with methanol, n-Hexane, ethyl acetate, chloroform and aqueous aerial parts *H. perforatum* extracts (10 mg/mL), respectively against *E. faecalis*. The later study reported that the MIC of methanolic aerial parts *H. perforatum* extracts (10 mg/mL) was recorded to be 15.63 and 3.9 µg/mL against MRSA and *E. faecalis*, respectively.

It has been demonstrated that the *Hypericum* antimicrobial inhibitory effect could be attributed notably to the presence of bioactive compounds especially Benzopyran of xanthones and flavonoids derived from them [19, 27].

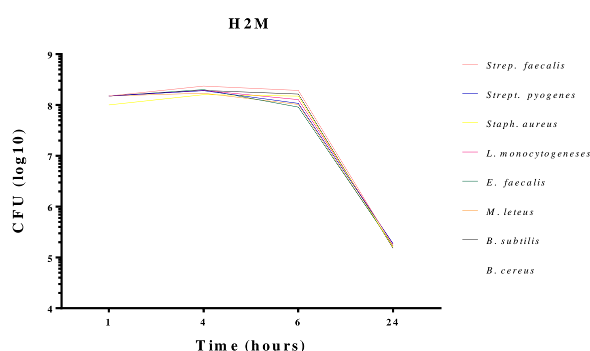
Our data regarding the MIC value against *S. aureus* pathogen obtained in the current study were comparable with that other those reported in literature (Table 5).

Table 5. A comparative study between the MIC value against *S. aureus* pathogene obtanied in the current study and other ones

Extract	MIC	Ref	Country
diethyl-ether *H. triquetrifolium*, *H. thymifolium* and *H. perforatum* extracts, respectively	0.03, 1 & 1 µg/mL, respectively	Current study	Syria
chloroform *H. triquetrifolium*, *H. thymifolium* and *H. perforatum* extracts, respectively	ND, 1 & ND µg/mL, respectively		
methanolic *H. triquetrifolium*, *H. thymifolium* and *H. perforatum* extracts, respectively	0.06, 0.06 & ND µg/mL, respectively		
methanolic *H. perforatum* leaves extract	0.78 mg/mL	[9]	India
methanolic extract, n-hexane and dichloromethane fractions *H. ericoides* aerial parts extracts, respectively	2048, 1024 and 512 µg/mL, respectively	[15]	Pakistan
methanolic and ethanolic *H. humifusume* extracts, respectively	0.15 and 0.078 mg/mL, respectively	[13]	Romania
methanol, acetone, chloroform, diethyl ether and petroleum ether *H. perforatum*extracts, respectively	4.8, 4.8, 78, 78 and 78 µg/mL, respectively	[18]	Turkey
H. perforatum extract (hydroalchoholic solution 70% v/v)	0.06 mg/mL	[20]	Greece
methanolic aerial parts *H. perforatum* extract	15.63 µg/mL	[22]	Egypt

Time-kill kinetics test has been also performed in order to accurate determine *Hypericum* sp. antibacterial activity. Of which, methanolic time-kill kinetics of *H. thymifolium* extract against the studied 8 Gram-positive bacterial isolates, has been evaluated (Figure 1). Time-kill kinetics test revealed that methanolic *H. thymifolium* extract exhibited bacteriostatic action (Figure 1). Time-kill kinetics of methanolic *H. thymifolium* extract profile against the selected isolates revealed that methanolic extract has no effect observed against selected bacterial isolates over the first 1 and 4 h. However, sharp killing ratio up 6th - 24th h has been recorded (Figure 1). Few studies reported the time-kill kinetics of mushrooms or plants extracts against bacterial isolates. In this regards, Appiah et al. (2017) [28] reported time-kill kinetics of methanolic Ghanaian mushrooms extract which has bacteriostatic action. Whereas, Kant et al. (2019) [29] reported that ethanolic *Arisaema tortuosum* leaf extract caused the maximum inhibitory growth of *Bacillus subtilis* and *Salmonella typhimurium* bacterial isolates after

~ 24 h compared to the other tested extracts. Moreover, Mordmuang et al. (2019) [30] reported that ethanolic *Rhodomyrtus tomentosa* leaf extract had a strong bactericidal activity against *S. aureus* isolate at time-kill (2 × MIC) of 4 h post-exposure.While, Kyahar et al. (2021) [31] reported complete kill ratio (100 %) has been recorded after 12 h exposure for *S. aureus* using chloroform *Adenodolichos Paniculatus* root extract. Whereas, Sowmya and Raveesha (2021) [32] reported time-kill (8 MIC) of 2-4 h for *S. aureus* isolate using acetonic *Terminalia catappa* L. leaf extract.



- H2M: methanolic *H. thymifolium* extracts

Figure 1. Time-kill kinetic of methanolic *H. thymifolium* against the selected bacterial isolates.

These differences in biological activity could be related to many factors; *e.g. Hypericum* species and their geographical distribution, extract concentration applied and solvent used.

In conclusion, antimicrobial effect of three *Hypericum* species has been investigated using aqueous, methanol, chloroform and diethyl-ether solvents against eight Gram-positive bacterial isolates. Antibacterial test revealed that aqueous *Hypericum* species extracts were non active against all tested bacterial isolates, regardless tested *Hypericum* species. Whereas, the diethyl-ether *H. triquetrifolium* areal parts extracts showed the highest antibacterial activity by showing the highest ZI value of 16.3 mm and the lowest MIC/MBC values of 0.03/0.06 µg/mL against *S. faecalis.* Allover, diethyl-ether *H. triquetrifolium,* methanolic *H. thymifolium* and methanolic *H. perforatum* extracts were strongly active against all tested bacterial isolates. Thereby, further studies regarding diethyl-ether *H. triquetrifolium,* methanolic *H. thymifolium* and methanolic *H. perforatum* extracts are needed.

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