Original Research Article

TLC Screening and Evaluation of Antibacterial Activity of Some Medicinal plants by Bioautography Method

ABSTRACT

This study aimed to conduct a qualitative detection of the active compounds in *Acacia cyanophylla*, *Phlomis syriaca* and *Scolymus hispanicus* plants by TLC method. The antibacterial activity for of the aqueous and ethanolic extracts of the aerial parts of the three plants was evaluated using MIC test. The active compounds that may be responsible for the antibacterial effect was isolated by direct bioautograph method. The results showed that the three plants contained flavonoids, saponin, bitter principles and essential oils by TLC method, and all extracts showed antibacterial activity, but the ethanolic extract of *Acacia cyanophylla* was the most effective as the MIC values ranged from (0.097-3.125) mg/ml. Bioautography showed that *Escherichia coli* was inhibited by most of the separated flavonoids on the TLC plates. We concluded that the *Acacia cyanophylla* plant has strong antibacterial properties among the selected plants and the flavonoids isolated from this plant play a great role in this.

Key words: Bioautography, Antimicrobial, Flavonoids, *Acacia cyanophylla, Phlomis syriaca, Scolymus hispanicus*.

INTRODUCTION

Using herbal medicine in Asia has a long history of human interactions with the environment. Natural products, such as plant extracts, are available either as pure compounds or as standard extracts provide great opportunities for new drug discoveries due to the unparalleled availability of chemicals diversity [1]. At present, the world faces great challenges in modern healthcare services because many antimicrobial agents have lost their effectiveness in treating infectious diseases due to the development of microbial resistance [2]. Epidemiological studies revealed high morbidity and mortality rates from infectious diseases due to antibiotics resistance. Currently, a wide variety of phytochemicals have been proven to be potential antibacterial agents, including terpenoids, essential oils, alkalis, lectins, polypeptides, phenols and polyphenols [3]. Many plant extracts are rich in phenolic compounds that may exhibit anti-bacterial activity in addition to their antioxidant activity. The mechanisms of the antibacterial effect of phenolic compounds have not been fully explored. Modification of the permeability of cell membranes, loss of functions within cells due to hydrogen binding of phenolic compounds to enzymes and modifying of cell wall stiffness with loss of integration have been documented [3]. The elevated lipophilic character of phenolic compounds enhances their antimicrobial activity. Flavonoids which are the base class of polypehols with C6-C3-C6 Skeleton, may bind to soluble proteins found outside of cells and with the bacterial cell walls thus promoting the formation of complexes. Also, Flavonoids may act by inhibiting both energy metabolism and DNA and RNA synthesis [3,4]. Thin layer chromatography (TLC) has been widely used in analysis of plant extracts for active components for a long time, as many samples can be analyzed in one development. If required, the confirmation can be done by means of automated techniques such as gas chromatography- mass spectrometry (GC - MS) or liquid chromatography- mass spectrometer (LC - MS). However, TLC continues the first-choice method due to its simplicity and low cost. The unique feature of TLC is that it can provide information about biological properties of the

sample, for example, antioxidants and antimicrobial when combined with direct bioautography [5]. Bioautography is planar chromatographic analysis hyphenated with the biological detection method [6], it is simple, economical, time-saving, do not require advanced equipment and more sensitive than any other methods [7]. The World Health Organization (WHO) has listed more than 21,000 plants which are used for many medicinal purposes worldwide[8] and a large number of medicinal plants have been recognized as valuable sources of natural antimicrobial compounds [9]. The genus *Acacia* belongs to the Fabaceae family and contains a large number of species (about 1500), making it the largest genus within the previous family. It is widespread in Australia, Asia, Africa and the Americas [10]. *Acacia* species have been reported to contain secondary metabolites including amines, alkaloids, fatty acids, amino acids, terpenes (including essential oils, diterpene and triterpene), hydrolyzed tannins, flavonoids, and condensed tannins [11]. Several studies have reported that *Acacia cyanophylla* has strong potential antimicrobial effects [12]. It also has important antioxidant and anti-acetylcholinesterase activity [13]. The genus *Phlomis* L. is one of the largest genera of the Lamiaceae family, with more than 100 species including herbs, shrubs. This genus is distributed in the northern temperate regions, especially in Europe and Asia [14]. it has found many evidence supporting the different activities of some types of *Phlomis*, including anti-inflammatory, immunosuppressive, antioxidant and antimicrobial effects. Various classes of glycosides, consisting mainly of diterpenoids, iridiodes, phenylpropanoid, phenylthanoid, flavonoids essential oils have been identified [15]. *Scolymus hispanicus* L. is a thistle-like plant in the family Asteraceae, a prickly perennial herb with a circum-Mediterranean distribution which grows all over Spain but it is scarce in the north of the country [16]. Although its leaf and root is usually consumed as a vegetable, it is also used in alternative medicine. *Scolymus hispanicus* L. leaves, stems and flowers are traditionally used as a "bitter" tonic to stimulate appetite [17]. It has many medicinal properties such as diuretic, depurative, digestive, choleretic and lithiuretic [16]. The aerial parts contain bioactive compounds such as flavonoids, flavonoid glucosides and flavonol rutinosides [18] and this plant was rich in dietary fiber, total phenolic compounds, and also it had a high antioxidant capacity [17]. Among which *Acacia cyanophylla, Phlomis* syriaca and *Scolymus hispanicus* are known to be native in the flora of Syria. In particular, despite widespread of these plants, the literature contains few reports of antibacterial activity and chemical composition of these plants. This study aimed to identify the various phyto-constituents components in the crude extracts of *Acacia cyanophylla, Phlomis syriaca* and *Scolymus hispanicus* that are responsible for antibacterial activity.

SUBJECTS AND METHODS

Plant material: fresh aerial parts (leaves, flowers and stems) of *Acacia cyanophylla*, *Phlomis syriaca* and *Scolymus hispanicus* were collected between March\April 2020 from different regions of northern Syria. The plant materials were authenticated by Dr Ahmad Jaddouh, an expert at Faculty of Agriculture - University of Aleppo, Syria. The plant materials cleaned with distilled water and dry at normal room temperature [19,20] for 15 days and grind to make it powder with the domestic blender and kept in airtight glass container until use. [21]

Bacterial Strains: Fifteen *Escherichia coli* isolates include was obtained from urinary tract infections from at Aleppo University Hospital laboratory. Isolates were defined by Gram stain, their microscopically appearance and their growth on the differential media. Isolates are kept in the liquid nutrient medium with 30% glycerol, at -20 C^{o} until use [8].

Methods

1. Extraction Procedures:

The fine powder of three plants (30g) was extracted with two different solvents (distilled water, ethanol 95%) for one hour in ultrasonic bath ultrasonic bath (POWERSONIC 405 (Hwashin Technology Co, Korea). The temperature was maintained at 50°C. The plant: solvent ratio was 1:10 (w/v). The extract solutions were filtered through Whatman No. 1 filter papers, and the residual material was re-extracted twice times using the same procedure. After that, the combined extracts were evaporated to dryness in a rotary evaporator (Rotary evaporator, Heidolph Instruments, Germany) at 50°C and under reduced pressure to remove the solvent [20]. The obtained crude extracts were stored in dark glass bottles and refrigerated at -4°C until use [21].

2- Antimicrobial susceptibility:

The antibiotic susceptibility of isolates was tested using the Disk diffusion method (Kirby Bauer) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [22] to eight antibiotics belonging to four main groups: Ampicillin+Sulbactum, Cefepime, Nalidixic acid, Ceftazidime, Cefpodoxime, Cifuroxim, Ceftriaxone and Nitrofurantoin.

3- Determination of antibacterial Activity using microdilution method:

The antimicrobial activities of extracts were determined by establishing the minimum inhibitory concentration (MIC) of the extracts within microtiter plates using microdilution method. For this purpose, stock solutions of examined extracts and Mueller-Hinton broth were prepared. The stock solution of each extract was prepared by dissolving 200 mg/mL of extract in dimethyl sulfoxide (DMSO). First, adding 50 μL of Muellur Hingtom broth (MHB) and 50 μL of bacterial suspension at a concentration of 0.5 McFarland to the first well as a negative control. Then, adding 50 μL of the selected plant extract at the highest concentration to the second well and 50 μL of bacterial suspension as a positive control. Second, 50 μL of the selected plant extract at the highest concentration to the third well and it is diluted by adding 50 μL of MHB, then 50 microliters are taken from the mixture for the fourth well and so on, the concentrations of tested extracts ranged between (0.097-100) mg\ml. then 50 μL of bacterial suspension are added for each wells. The microtiter plate was sealed with parafilm and incubated at 37°C for 24 h. the MIC of each tested isolate was detected by adding 20 μL of 0.2 mg/mL indicator dye of 2, 3, 5-triphenyltetrazolium chloride (TTC) into the microtiter plate wells and incubated for 30 min at 37°C and observed any color change [2, 23]. TTC is colorless in the oxidized form and red when reduced. The dehydrogenase of living bacteria reduces tetrazolium salt into intensely pinkish-red formazan. When solutions of microtiter plate wells remain without color change, we infer that the bacterial were inhibited. [2,7]

4- Thin Layer Chromatography TLC:

Three types of extracts were prepared:

$Extract_1$: Powdered plant (1 g) is extracted by heating on a water bath for 15 min with 5 ml methanol then filtrated. (For analysis of bitter principles and flavonoids)

$Extract_2$: Powdered plant (1 g) is moistened with 1 ml of 10% ammonia solution, and then extracted by shaking for 15 min at 60^0C with 5 ml methanol. (For analysis of alkaloids.)

$Extract_3$: A methanol extract is prepared as in 1. (above). This is evaporated and then 1 ml mixed with 0.5 ml water, then extracted with 3 ml butanol. (For analysis of saponins)

$Extract_4$: Powdered plant (1 g) is extracted by heating under reflux for 15 min with 10 ml Dichloromethane. The filtrate is evaporated to dryness, and the residue is dissolved in 1 ml toluene. (For analysis of essential oils)

From each extract, 20 uL applied to a TLC silica gel plate (60F254, 10 cm x 10 cm). Chromatography is performed in one of the following solvent systems:

1) Ethyl acetate-methanol-water (100: 13.5: 10): For the analysis of bitter principles, flavonoids, alkaloids and saponins.

2) Toluene-ethyl acetate (93: 7): For the analysis of essential oils.

Both solvents are allowed to run for a distance of 8 cm. After inspection in UV- 254 nm and UV-365 nm, each chromatogram is analyzed for the presence of one of the groups of plant constituents by spraying with an appropriate reagent such as:

1-Bornträger reagent (10% ethanolic KOH): for detection anthraquinones.

2-Dragendorff reagent: for detection alkaloids.

3-Natural products-polyethyleneglycol reagent (NP/PEG): for detection flavonoids.

4-Vanillin-sulphuric acid reagent: for detection Bitter principles and saponins. [24]

5. Detection of antibacterial Activity by direct bioautograph method:

Many of the widely distributed plant substances, including alkaloids, organosulfur compounds, phenolic acids, flavonoids, terpenes, tannins, and many primary metabolites exhibit antibacterial properties. Among them, flavonoids are a promising group of biologically active substances with low systemic toxicity and show strong antioxidant activity [23]. Therefore, flavonoids were selected for our microbiological tests in the presented study using bioautography method. Direct bioautography was performed with a culture of *Escherichia coli* (isolate 10) which showed a good sensitivity to the ethaolic extract of three plants. TLC plates of ethanolic extract of three plant were prepared in ethyl acetate-methanol-water (100: 13.5: 10) as mobile phase to separate flavonoids. TLC plates were carefully dried for complete removal of the solvents [25] and sprayed with or dipped into bacterial suspension at a concentration of 0.5 McFarland. Incorporated a new medium for direct TLC bioautography which is fluid enough to disperse microorganisms and viscous enough to adhere to the TLC plates; according to them a mixture of Muller–Hinton (MH) broth and MH agar in the ratio of 90:10 fulfills this requirement. Then, the TLC plates are incubated at 37°C for 24h under humid condition. After 24 h of incubation, for visualization of microbial growth, tetrazolium salts are used. These salts are sprayed onto the plates and are re-incubated at 37°C for 1 h. Clear white zones against a purple background on the TLC plate indicate antimicrobial activity of the sample. Then, this zones of inhibition were compared with the RF of the related spots on the reference TLC plate [6].

RESULTS AND DISCUSSION

1. Antimicrobial susceptibility:

The results of antimicrobial susceptibility of *Escherichia coli* isolates against selective antimicrobial agents are listed in Table (1). The results showed complete sensitivity to nitrofurantoin and prevalence of resistance with different percentage to other different antibiotics ranged from 20% to 86.67%. The emergence of antimicrobial resistance among uropathogenic *E. coli* is well documented in our country [26].

Table (1): Susceptibility of *Escherichia coli* isolates against studied antibiotics

Isolates NO.	SAM 10/10 µg	FEP 30 µg	NAL 30 µg	CAZ 30 µg	CPD 10 µg	CXM 30 µg	CRO 30 µg	NIF 300 µg
1	S	S	R	R	R	R	R	S
2	S	S	R	S	S	S	S	S
3	S	S	R	S	R	R	R	S
4	S	S	R	S	S	S	S	S
5	I	R	R	R	R	R	R	S
6	S	S	R	S	R	R	R	S
7	S	S	R	R	R	R	R	S
8	R	R	S	R	R	R	R	S
9	R	S	R	R	R	R	R	S
10	S	S	R	S	R	R	R	S
11	S	R	R	R	R	R	R	S
12	S	S	S	R	R	R	R	S
13	R	I	R	R	R	R	R	S
14	R	S	S	R	R	R	R	S
15	R	S	R	R	S	S	R	S
Resistance	%40	%26	%80	%60	%80	%80	%86.7	%0

SAM: ampicillin-sulbactam, FEP: Cefepime, NAL: nalidixic acid, CAZ: Ceftazidime, CPD: Cefpodoxime, CXM :Cefuroxim, CRO: Ceftriaxone, NIF: Nitrofurantoin , R: Resistant, S: Sensitive, I: Intermediate

2. Determination of antibacterial activity using microdilution method

Finding healing powers in plants is an ancient idea. With the increasing prevalence of antibiotic resistance, the search for natural alternatives was an imperative to eradicate resistant strains. [27]

Table (2) shows the MIC values for the six extracts from the three plants against *Escherichia coli* isolates. The MIC value was taken as the lowest concentration of the extract that inhibiting any visible bacterial growth and this is detected after adding tetrazolium salts and observed color change.

In general, the ethanolic extracts were more effective than the aqueous extracts for the same plant. However, the ethanolic *Acacia cyanophylla* extract was the most effective against *E. coli* isolates as its MIC values ranged from (0.097-3.125) mg\ml. The aqueous extract also showed good efficacy on *E. coli* isolates, as its MIC ranged between (3.125-12.5) mg \ml. the ethanolic extracts of *Phlomis syriaca* showed good efficacy on *E. coli* isolates, as the activity started at a concentration of 6.25 mg / ml for some isolates, while the highest activity was reached at a concentration of 0.78 mg / ml. The aqueous extracts MICs ranged between (12.5-3.125) mg / ml for the studied isolate.

The ethanolic and aqueous extracts of *Scolymus hispanicus* showed similar efficacy against *E. coli* isolates, where the efficacy ranged between (25-6.25) mg / ml. The highest antibacterial efficacy of *Acacia cyanophylla* ethanolic extracts maybe be attributed to the richness in phenols and flavonoids, as documented in previous study where the total phenol content (TPC) recorded 98.39 ±4.755 mg GAE g/ g of dry extract and total flavonoids content (TFC) recorded 121.64 ±6.469 mg RE/g of dry extract [28]. This is in agreement with a (SADIQ et al., 2017) study on

Acacia nilotica plant extracts where MIC values for *acacia* leaf extracts were 1.56--3.12 mg / mL, while fruit and bark extracts showed somewhat higher values of 3.12--6.25 mg / mL on *E. coli* and *Salmonella*. [29] It also converges with Marmouzi et al., study, where *Scolymus hispanicus* extract was tested against five types of bacteria, and the results showed that *Scolymus hispanicus* roots had the strongest antibacterial activity against *E. coli* bacteria at (MIC) value of 1.56 mg/ml [30]. This variation in values can be explained by the fact that the flavonoids and phenols content are influenced by different parameters such as time and place of harvest, climate, geographical conditions, method and time of extraction, solubility and degree maturation of the plan [31].

Table (2): The MIC values for the aqueous and ethanolic extracts from *Acacia cyanophylla, Phlomis syriaca* and *Scolymus hispanicus* against *Escherichia coli* isolates.

Isolates number	*Acacia cyanophylla*		*Phlomis syriaca*		*Scolymus hispanicus*	
	Aqueous mg/ml	Ethanolic mg/ml	Aqueous mg/ml	Ethanolic mg/ml	Aqueous mg/ml	Ethanolic mg/ml
1	3.125	0.19	3.125	3.125	6.25	6.25
2	12.5	0.19	6.25	3.125	12.5	12.5
3	6.25	0.39	3.125	3.125	12.5	12.5
4	3.125	0.39	12.5	3.125	25	25
5	6.25	0.78	6.25	6.25	25	25
6	12.5	0.39	12.5	0.78	12.5	6.25
7	6.25	0.78	6.25	1.56	12.5	12.5
8	12.5	3.125	12.5	6.25	25	25
9	6.25	0.39	3.125	1.56	12.5	12.5
10	6.25	0.78	6.25	0.78	12.5	12.5
11	6.25	3.125	12.5	1.56	25	25
12	12.5	0.39	6.25	3.125	12.5	12.5
13	6.25	0.78	6.25	3.125	12.5	12.5
14	3.125	0.097	6.25	6.25	12.5	12.5
15	3.125	0.19	1.56	1.56	12.5	12.5

3. Thin Layer Chromatography TLC:

Qualitative phytochemical screening is an essential step towards the discovery of new drugs as it provides the information regarding the presence of a particular secondary metabolite in the plant extract of clinical significance. The presence of any significant bioactive natural product indicates the necessity of separation of the compounds through suitable chromatographic techniques [32]. In the present study, forth extracts i.e. methanolic, butanolic, methanolic with ammonia and dichloromethane were checked by thin layer chromatography. After dealing with spray reagents the greenish and orange florescent after NP-PEG indicates the presence of flavonoids whereas the detected violet bluish spots after dealing with vanillin-sulphuric acid and heating indicates bitter principles, saponins and essential oils presence. Also, the red-brown spots after dragendorff reagent indicates the presence of alkaloids [24]. Hence, it has been proven that the three plants contain diverse classes of bioactive compounds such as flavonoids, saponin, bitter principles and essential oils. Also, Alkaloids were present in *Acacia cyanophylla* while it was absent in *Phlomis syriaca* and *Scolymus hispanicus* (Figure 1). These results are in agreement

with the literature review on the studied plants [11, 15-18]. These constituents are responsible for the various pharmacological properties of these plants, as many reports are available on the antibacterial and antioxidant properties.[12,15-17].

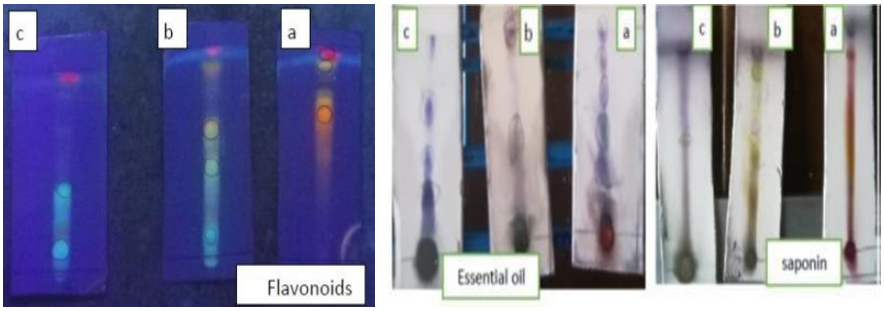

Figure (1): Separated compounds of ethanolic extract of *Acacia cyanophylla, Phlomissyriaca* and *Scolymushispanicus* aerial part on TLC

4- Detection of antibacterial activity by Bioautography:

The chromatograms of ethanolic extract of three plants were developed in order to calculate Rf values of the spots Table (3) & Figure (1). The results in Table (2) showed that the three plants showed several flavonoids spots of different colors after spraying with the NP / PEG reagent where it was observed with *Acacia cyanophylla* six spots (1,2,3,4,5,6) of different colors with Rf values (0.42, 0.55, 0.6, 0.83, 0.88, 0.92), respectively. While it appeared with *Phlomis syriaca*, four color spots (1,2,3,4) with Rf values (0.48, 0.85, 0.90, 0.96), respectively. Also featured with *Scolymus hispanicus* are seven spots (1,2,3,4,5,6,7) in different colors with RF values (0.16, 0.30, 0.48, 0.58, 0.78, 0.86, 0.92), respectively. Previous studies indicate that when the fluorescence appears orange- yellow colors, the compound may be flavonols while yellow-green fluorescence indicates possible presence Kaempferol, isorhamnetin and their glycosides and Apigenin and their glycosides. Also the appearance of fluorescence orange indicates the possibility of presence flavones such as Luteolin and their glycosides [33].

After spraying with tetrazolium salts some separated flavonoids compounds showed antibacterial activity by revealing yellow inhibition zones on *Escherichia coli* Figure (2).

Figure (2) shows that the antibacterial activity with *Acacia cyanophylla* was shown in spots (1, 2, 3, 4) with R_f values (0.42, 0.55, 0.6, 0.83), While in *Phlomis syriaca* it appeared in only one spot (1) with R_f values (0.48) and in *Scolymus hispanicus*, the anti-bacterial activity appeared in the spots (1, 2, 3, 4, 5) with R_f values (0.16, 0.3, 0.48, 0.58, 0.87) .This is consistent with previous studies where it was found in vitro that the antibacterial activity of flavonoids it can be practiced in three ways: direct and synergistic killing of bacteria the antibiotics activate and weaken the bacteria pathogenicity. Also, the flavonoids showed inhibitory activity against the flow pump bacterium, and it also restricted peptidoglycan synthesis and ribosome in amoxicillin-resistant Escherichia cells *coli* (AREC). They also showed inhibition activity against different types of lactamases produced by bacteria; they are the main enzymes that inactivate the common antibiotics [3]. The reports also documented that *Acacia cyanophylla*, *Phlomis syriaca* and *Scolymus hispanicus* have a total phenolic content in ethanolic extract of aerial part reached to 98.39, 46.73, 14.48 mg GAE g/ g extract respectively and total flavonoids contents reached to 121.64, 52.49, 35.59 mg of RE/g extract respectively [28].

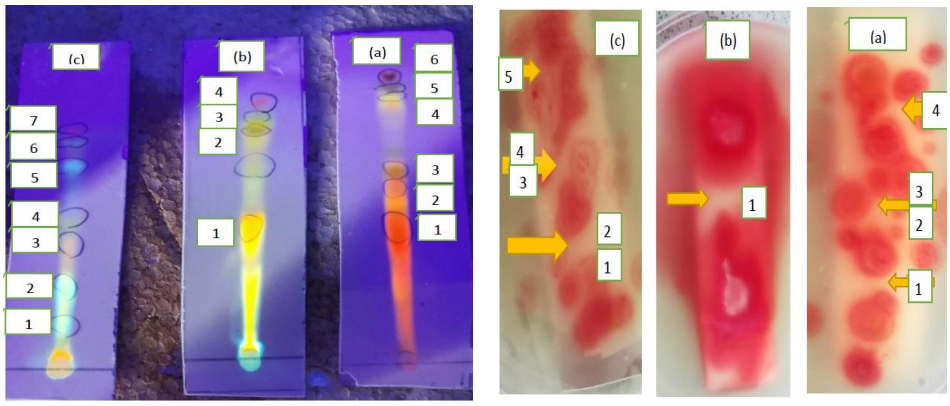

Figure (2) Antibacterial activity of separated flavonoids from ethanolic extracts of *(a) Acacia cyanophylla (b) Phlomissyriaca* and (c) *Scolymushispanicus* aerial part by bioautography on *Escherichia coli*

Table (3) Separated compounds of ethanolic extract of *Acacia cyanophylla, Phlomissyriaca* and *Scolymushispanicus* aerial part on TLC and their R_F values.

Ethanolic plant extract	**spots**	UV /NP/PEG	**Rf values**
Acacia cyanophylla	1	Red	0.42
	2	Orange	0.55
	3	Green	0.6
	4	Yellow	0.83
	5	Green	0.88
	6	Red	0.92
Phlomissyriaca	1	Yellow	0.48
	2	Green	0.85
	3	Blue	0.9
	4	Red	0.96
Scolymushispanicus	1	Blue	0.16
	2	Blue	0.3
	3	Orange	0.48
	4	Green	0.58
	5	Blue	0.78
	6	Blue	0.86
	7	Red	0.92

CONCLUSION

In this study, TLC results showed that the three plants contain flavonoids, saponins, bitter principles and essential oils. The ethanolic extract of *A. cyanophylla* have strong antibacterial activity compared to the other two plants studied.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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