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RESEARCH ARTICLE

EVALUATION OF ANTIBACTERIAL ACTIVITY OF SOME MEDICINAL PLANTS BY BIOAUTOGRAPHY

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Abstract

Background *Acacia cyanophylla* is a medicinal plant of the Fabaceae family that is widely distributed in Australia and Asia, also it has many medicinal properties such as antibacterial and antioxidant activity. Thin layer chromatography (TLC) is wildly used in natural product extract analysis as a finger print.
Aim and objective: This study aimed to conducting a qualitative detection of the active compounds in *Acacia cyanophylla*, *Phlomis syriaca* and *Scolymus hispanicus* plants by thin layer chromatography (TLC) method and studying their antibacterial activity.
Methods: the qualitative detection of three plants was conducting using thin layer chromatography (TLC) method. Then, aqueous and ethanolic extracts of the aerial parts of the three plants were extracted using an Ultrasonic bath. The antibacterial activity on E. coli isolates for six extracts was evaluated using minimum inhibitory concentration (MIC) test. The active compounds that may be responsible for the antibacterial effect was isolated by direct bioautograph method.
Results: Performing Thin-layer chromatography TLC tests show that the three plant contain flavonoids, saponin, bitter principles and essential oils, and all extracts showed antibacterial activity on *E. coli* isolates, but the ethanolic extract of *Acacia cyanophylla* was the most effective as the MIC values ranged from 0.097to 3.125 mg/mL. Bioautography showed that *Escherichia coli* was inhibited by most of the separated flavonoids on the TLC plates where four inhibiting spots appeared in yellow color with *Acacia cyanophylla* and five spots with *Scolymus hispanicus*, while only one spot appeared with *Phlomis syriaca*.
Conclusion: *Acacia cyanophylla* extract has been considered as the best antibacterial properties among the selected plants due to the presence of flavonoids
Keywords: *Acacia cyanophylla*, antibacterial, bioautography, flavonoids, *Phlomis syriaca*, *Scolymus hispanicus.*

INTRODUCTION

The significance of medicinal plants cannot be overlooked. The natural products available either as pure compounds or as standard extracts, are considered the largest source for new drugs discoveries due to their content of various bioactive chemical compounds[1]. Nowadays, the health system is facing great difficulties in the treatment of bacterial diseases due to the development of antibiotics bacterial resistant strains which causes high rates of morbidity and mortality[2]. A wide variety of phytochemicals have been demonstrated to be potential antibacterial agents, including terpenoids, essential oils, alkalis, lectins, polypeptides, phenols and polyphenols[3]. Many plant extracts have plentiful phenolic compounds that may display anti-bacterial activity in addition to their antioxidant activity. The mechanisms of the antibacterial effect of phenolic compounds have not been fully known alteration 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 feature of phenolic compounds improves their antimicrobial activity. Flavonoids which are the base class of polypehols with general structure includes two phenyl rings (A and B) and a heterocyclic ring (C) may bind to soluble proteins found outside of cells and with the bacterial cell walls thus enhancing the formation of complexes. In addition, flavonoids may also act by inhibiting both energy metabolism and

nucleic acids synthesis[3,4]. Thin layer chromatography (TLC) has been generally used in analysis of plant extracts for active components because many samples can be analyzed in one development. If required, the affirmation can be performed using an automated technique like gas chromatography- mass spectrometry (GC-MS) or liquid chromatography- mass spectrometer (LC-MS). However, TLC continues the first-choice method due to simplicity and economical. The singularity 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]. Bioautography is efficacious technique for identifying a biologically active component with antibacterial activity from plant extracts[1]. There are three methods of bioautography; contact bioautography, direct bioautographic and overlay bioautography[6]. Direct Bioautography is the most widely applied of all bioautography methods[7]. The World Health Organization (WHO) has reported more than 21,000 plants which are utilized for many medicinal usesworldwide[8] and a large number of medicinal plants have been observed as useful sources of natural antimicrobial compounds[9]. The genus *Acacia* belongs to the *Fabaceae* family and involve an enormous number of species (about 1500), forming 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 *A. 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 hindered species including herbs, shrubs. This genus is distributed in the northern temperate regions, especially in Europe and Asia[14] that possess 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 perpetual herb with a circum-Mediterranean allocation which grows in Spain but it is scarce in the north of the country[16]. Although its leaf and root is usually used as a vegetable, it is also utilized in alternative medicine. *S. hispanicus* L. leaves stems and flowers are used as a "bitter" tonic to stimulate appetite[17]. It has depurative, diuretic, choleretic, digestive and lithiuretic effect[16]. The aerial parts contain bioactive compounds such as flavonoids, flavonoid glucosides and flavonol rutinosides[18] and this plant is rich in dietary fiber, total phenolic compounds, and showed elevated antioxidant capacity[17]. Among which *A. cyanophylla*, *P. syriaca* and *S. hispanicus* are known to be indigenous 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 growing in Syria. This study aimed to identify the various phytoconstituents components in the crude extracts of *A. cyanophylla*, *P. syriaca* and *S. hispanicus* that are responsible for antibacterial activity.

METHODS

Plant Material

Fresh aerial parts (leaves, flowers and stems) of *A. cyanophylla*, *P. syriaca* and *S. hispanicus* were collected between March\April 2020 from different regions of northern Syria. The plant were authenticated by Dr Ahmad Jaddouh, an expert at Faculty of Agriculture - University of Aleppo, Syria. The plant sample were cleaned with distilled water and dried at normal room temperature for 15 days[19,20]. The dried sample were ground to powder with the domestic blender and kept in glass container until use.[21]

Bacterial Strains

Fifteen *E. coli* isolates that included in the study was obtained from patients with urinary tract infections at Aleppo University Hospital laboratory. Isolates were identified 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 until use[8].

Plant Extraction

Fine powder of three plants (30g) was extracted with two different solvents (distilled water, ethanol 95%) for one hour in an 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 using the same procedure. 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].

Antibacterial Susceptibility

The antibiotic susceptibility of isolates was tested using 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 and Sulbactum (10\10 µg), Cefepime (30 µg), Nalidixic acid (30 µg), Ceftazidime (30 µg), Cefpodoxime (10 µg), Cifuroxim (30 µg), Ceftriaxone (30 µg) and Nitrofurantoin (300 µg). (IVD Group, Benex Limitted, USA)

Minimum Inhibitory Concentration (MIC) Test

The antibacterial activity of the extracts was determined by establishing the minimum inhibitory concentration (MIC) 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 Mueller- Hinton broth (MHB) and 50 μL of bacterial suspension at a concentration of 0.5 McFarland ($1.5x10^8$ CFU/mL) 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 μL are taken from the mixture for the fourth well and so on, the concentrations of tested extracts ranged between (0.097 to100) 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; 2, 3, 5-triphenyltetrazolium chloride (TTC) into the microtiter plate wells and incubated for 30 min at 37°C and observed any color change to red color which indicates bacterial growth[2,23]. TTC is colorless in the oxidized form and red in reduced form. The dehydrogenase enzyme of living bacteria reduces tetrazolium salt in the indicator into intensely pinkish-red formazan compound[7]. When the solutions color of microtiter plate wells remains unchanged, this indicates that the growth of bacteria is inhibited[2,7]

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 detection 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 inspection of alkaloids.

Extract$_3$: A methanol extract was prepared as in extract 1. After evaporation, 1 mL of filtrate was mixed with 0.5 mL water, followed by 3 mL butanol for detection of saponins

Extract$_4$: Powdered plant (1 g) was extracted by heating under reflux for 15 min with 10 mL Dichloromethane. The filtrate was evaporated to dryness, and the residue was dissolved in 1 mL toluene for inspection of essential oils.

From each extract, 20 µL of extract was applied to a TLC silica gel plate (60F254, 10 cm x 10 cm). Chromatography was conducted in one of these 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 permitted to pass 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 component by spraying with an suitable 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].

Table 1: Susceptibility of *E. coli* isolates against studied antibiotics.

Isolates number	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 according to (CLSI, 2017)

Bioautograph Method

Flavonoids were selected for our microbiological tests in the presented study using bioautography method. Direct bioautography was performed with *E. coli* isolate that exhibit good sensitivity to the ethanolic extract of the 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 dried for removal of the solvents[25] and dipped in mixture of Muller–Hinton (MH) broth and MH agar in the ratio of 90:10 containing bacterial suspension at a concentration of 0.5 McFarland. The TLC plates were then incubated at 37°C for 24 h under humid condition. After 24 h of

incubation, tetrazolium salts are used for visualization of microbial growth. These salts are sprayed onto the plates and re-incubated at 37°C for 1 h. Clear white zones against a purple background on the TLC plate point to antimicrobial activity of the sample. Then, these zones of inhibition were compared with the RF of the related spots on the reference TLC plate[6].

RESULTS AND DISCUSSION

Antibacterial Susceptibility:

The results of antibacterial susceptibility of *E. 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]. As the resistance rate to chephalosporine antibiotics group was ranged from 48.5% to 77.4% and the nitofurantoin resistance rate was 35.8%.

MIC Assay

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 *E. coli* isolates. The MIC value was determined as the lowest concentration of the extract that inhibiting any bacterial growth and this is detected after adding tetrazolium salts and observed color change.

Table 2: The MIC values for the aqueous and ethanolic extracts from *A. cyanophylla, P. syriaca* and *S. hispanicus* against *E. coli* isolates.

Isolates number	*A. cyanophylla*		*P. syriaca*		*S. hispanicus*	
	Aqueous mg/mL	Ethanolic mg/mL	Aqueous mg/mL	Ethanolic mg/mL	Aqueous mg/mL	Ethanolic mg/mL
1	3.125	0.190	3.125	3.125	6.25	6.25
2	12.50	0.190	6.250	3.125	12.50	12.5
3	6.250	0.390	3.125	3.125	12.50	12.5
4	3.125	0.390	12.50	3.125	25.00	25.0
5	6.250	0.780	6.250	6.250	25.00	25.0
6	12.50	0.390	12.50	0.780	12.50	6.25
7	6.250	0.780	6.250	1.560	12.50	12.5
8	12.50	3.125	12.50	6.250	25.00	25.0
9	6.250	0.390	3.125	1.560	12.50	12.5
10	6.250	0.780	6.250	0.780	12.50	12.5
11	6.250	3.125	12.50	1.560	25.00	25.0
12	12.50	0.390	6.250	3.125	12.50	12.5
13	6.250	0.780	6.250	3.125	12.50	12.5
14	3.125	0.097	6.250	6.250	12.50	12.5
15	3.125	0.190	1.560	1.560	12.50	12.5

In general, the ethanolic extracts were more effective than the aqueous extracts for the same plant. However, the ethanolic *A. cyanophylla* extract was the most effective against *E. coli* isolates as its MIC values ranged from (0.097 to 3.125) mg\mL. The aqueous extract MIC values ranged between (3.125 to12.5) mg \mL. The ethanolic extracts of *P. syriaca* activity on *E. coli* isolates 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 from 12.5 to 3.125 mg/mL for the studied isolate. Both the ethanolic and aqueous extracts of *S. hispanicus* showed similar efficacy against *E. coli* isolates, where the efficacy ranged between (25-6.25) mg/mL. The highest antibacterial efficacy of *A. cyanophylla* ethanolic extracts may be is attributed to the richness in phenols and flavonoids, as documented in previous study[28]. This is in agreement with a (SADIQ *et al.*,) study on *A. nilotica* where MIC values for *Acacia* leaf extracts were 1.56 to 3.12 mg/mL, while fruit and bark extracts showed somewhat higher values of 3.12 to 6.25 mg/mL on *E. coli* and *Salmonella*.[29]

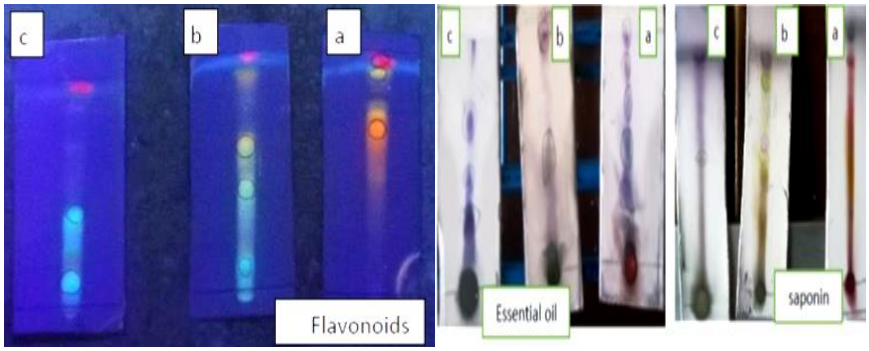

Figure 1: Separated compounds of ethanolic extract of *A. cyanophylla, P. syriaca* and *S. hispanicus* aerial part on TLC.

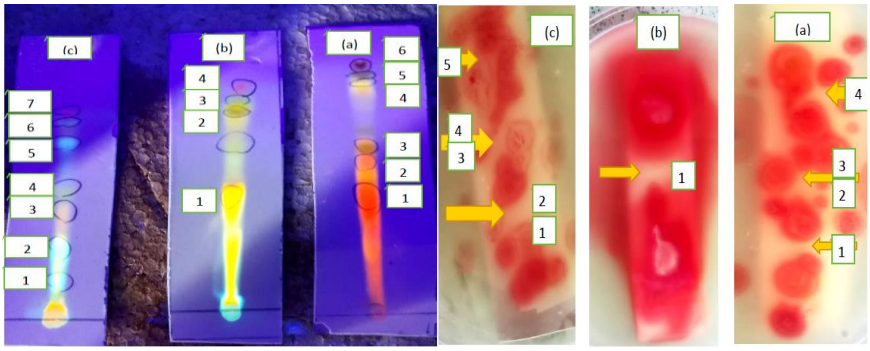

Figure 2: Antibacterial activity of separated flavonoids from ethanolic extracts of (a). *A. cyanophylla* (b). *P. syriaca* and (c). *S. hispanicus* aerial part by bioautography on *E. coli.*

It also converges with (Marmouzi *et al*.,) study, where *S. hispanicus* extract was tested against five types of bacteria (*E. Coli, S. aureus, B. subtilus, S. enteric* and *P. aeruginosa*), and the results showed that *S. hispanicus* roots had the strongest antibacterial activity against *E. coli* bacteria at MIC value of 1.56 mg/mL[30]. These differences in values can be accounted for the fact that the flavonoids and phenols content are affected by different factors such as place and time of harvest, geographical conditions, climate, time and procedure of extraction, solubility and degree maturation of the plant[31].

Table 3: Separated compounds of ethanolic extract of *A. cyanophylla, P.syriaca* and *S. hispanicus* 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
Phlomis syriaca	1	Yellow	0.48
	2	Green	0.85
	3	Blue	0.9
	4	Red	0.96
S. hispanicus	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

Thin Layer Chromatography (TLC)

Qualitative phytochemical screening is a basic step to prove presence of particular secondary metabolites in the plant extract of clinical significance. The presence of any important bioactive natural product indicates the necessity of separation of the compounds through appropriate chromatographic techniques[32]. In the present study, the all extracts were checked by thin layer chromatography. the Greenish and orange florescent spots observed after spraying with NP-PEG indicates the presence of flavonoids whereas a violet bluish spots after spraying with vanillin-sulphuric acid and heating indicates bitter principles, saponins and essential oils presence. Red-brown spots were observed using dragendorff reagent indicates the presence of alkaloids[24]. Hence, it has been proven that the three plants contain variable classes of bioactive compounds such like flavonoids, saponin, bitter principles and essential oils. Alkaloids were present in *A. cyanophylla* while it was absent in *P. syriaca* and *S. hispanicus* (Figure 1). These results are in agreement with the literature review on the studied plants[11,15-18]. These constituents are responsible for various pharmacological properties as antibacterial and antioxidants[12,15-17].

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 and Figure 1. 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 *A. cyanophylla* six spots of different colors with Rf values0.42, 0.55, 0.6, 0.83, 0.88, 0.92, respectively. While it appeared with *P. syriaca*, four color spots with Rf values 0.48, 0.85, 0.90, 0.96, respectively. Also featured with *S. hispanicus* are seven spots 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[33]. Also the appearance of fluorescence orange indicates the possibility of presence flavones such as Luteolin and their glycosides[33]. After spraying with tetrazolium salts, the antibacterial activity of some separated flavonoids compounds were revealed as yellow inhibition zones around *E. coli* Figure 2.

Figure 2 shows that the antibacterial activity with *A. cyanophylla* was shown in spots (1, 2, 3, 4) with R_f values of 0.42, 0.55, 0.6, 0.83, While in *P. syriaca* it appeared in only one spot (1) with R_f values (0.48) and in *S. hispanicus*, the anti-bacterial activity appeared in the spots 1, 2, 3, 4, 5 with R_f values of 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 restricted peptidogly can synthesis in amoxicillin-resistant *E. coli*. The inhibition activity of different types of lactamases produced by bacteria by flavonoids was also documented[3]. It was found that *A. cyanophylla*, *P. syriaca* and *S. 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], Which reflects the antibacterial activity of these plants.

CONCLUSIONS

In this study, TLC results showed that the three plants contain flavonoids, saponins, bitter principles and essential oils. The ethanolic extract of *A. cyanophylla* has strong antibacterial activity against *E. coli* compared to the other two studied plants, as MIC values were ranged 0.097 to 3.125 mg/ml. The results indicate that most flavonoids in the ethanolic extract of the three plants have antibacterial activity, as shown by the direct Bioautography technique

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AUTHOR'S CONTRIBUTION

Al-Kayali R: study design, writing original draft. **Jalab J:** literature survey, critical review. **Kitaz A:** data interpretation. **Abdelwahed W:** methodology, formal analysis. The final manuscript was read and approved by all authors.

DATA AVAILABILITY

The data supporting the findings of this study are not currently available in a public repository but can be made available upon request to the corresponding author.

CONFLICT OF INTEREST

None to declare.

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