Phytochemical screening and in vitro antibacterial activity of *Cissus subaphylla* and *Euphorbia spiralis* endemic in Socotra Island

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Abstract

Cissus subaphylla and *Euphorbia spiralis,* endemic in Socotra Island, were screened for their chemical constituents and antibacterial activity. The phytochemical screening of the chloroform and the 70% ethanol extracts of both plant stems indicated the presence of sterols, triterpenoids, cardiac glycosides, anthraquinons, flavonoids, tannins and carbohydrates as chemical constituents. Testing the antibacterial activity of chloroform and the 70% ethanol extracts (5 and 10mg) of both plants against *Staphylococcus aureus* (305-864-669), *Staphylococcus epidermidis* (505-864-689), *Pseudomonas aeruginosa*(909-825-1793) and *Escherichia coli*(909-825-1793), using agar well diffusion assay, demonstrated that all tested extracts displayed a significant antibacterial activity with activity index (AI) above 0.5 against test microorganisms, except the chloroform extract of *C. subaphylla*, which was found inactive against *S. aureus*. The 70% ethanol extract (10mg)of *E. spiralis* showed the highest antibacterial activity (AI=0.80-0.94) against *E. coli*.

Key words: *Cissus subaphylla*, *Euphorbia spiralis*, Phytochemical screening, Antibacterial activity, Socotra Island

Introduction

The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries despite the development and spread of modern medicine. Approximately, 60-80% of the world population still relies on traditional medicines, especially on the use of medicinal plants for the treatment of common diseases (41, 43).

Yemeni traditional medicine is still prevalent, especially in rural areas and to some extent in urban areas, for the treatment of a number of diseases (4,10, 16, 17).Infectious diseases are among the most common diseases in Yemen (7, 8, 22). Antibiotics and other antimicrobial agents are effective in the prevention and treatment of these infections, but the wide and indiscriminate use of common anti-infective drugs has contributed substantially to the persistence of infections, as a major cause of morbidity and mortality. Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action (2,31). Herbal remedies, used in Yemeni traditional medicine for the treatment of infectious diseases may provide an excellent source to be investigated for the development of new therapeutic agents without the disadvantages of growing resistance and toxicity of the currently available commercial antibiotics. Therefore, the objectives of this work are to screen different extracts obtained from *Cissus subaphylla*(Balf .f.) Planch., locally known as (Atirheh) (23) and *Euphorbia spiralis* Balf.f., locally named as Qisho, Qash'ho, Zo'hor(23) that are used as traditional endemic plant remedies for the treatment of infectious and skin diseases in the island of Socotra. These medicinal plants were screened for their antibacterial properties and bioactive constituents in order to search for new antibacterial active agents as well as to provide a scientific basis for their traditional use in Socotra Island for the treatment of infectious diseases.

Materials and Methods

Plant materials:

Plants used in this study were stems of *Cissus subaphylla* (Balf.f.) Planch. (Vitaceae), and stems of *Euphorbia spiralis* Balf.f. (Euphorbiaceae). They were collected in Socotra Island in April 2014. The authentication was made under the supervision of Abdul Naser Al-Gifri, Department of Biology, Collage of Education, University of Aden, Yemen. Voucher specimens were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Aden-Yemen. After collection, the plant materials were subsequently shade dried at ambient temperature and then ground in a grinder.

Microorganisms:

Two Gram-positive bacteria: *Staphylococcus aureus* (305-864-669)and *Staphylococcus epidermidis* (505-864-689), and two Gram-negative bacteria: *Pseudomonas aeruginosa* (909-825-1793) and *Escherichia coli* (909-825-1793) were used as test microorganisms.

Standard antibiotics:

Susceptibility test discs of standard antibiotics, Amoxicillin 10µg, Erythromycin 15µg, and Gentamicin 10µg used in the antibacterial assay as positive controls, were purchased from Himedia Laboratories Pvt. Ltd., (India).

Preparation of extracts:

The air-dried and powdered plant materials (25 g of each) were extracted successively under shaking with chloroform for three times at room temperature and then with 70% ethanol for three times. The obtained extracts were filtered and then concentrated to dryness and weighed. All extracts were stored in refrigerator until used in the test for the antibacterial activity and phytochemical screening.

Phytochemical screening

The extracts were subjected to phytochemical screening for plant secondary metabolites (sterols,triterpenoids, cardiac glycosides, saponins, alkaloids, anthraquinones, flavonoids, tannins, carbohydrates**)** using standard procedures described by Farnsworth(15), Harborne (19),Stahl and Schild (35) and Trease and Evans (36). As confirmatory evidence of the presence and/or absence of alkaloids, sterols, triterpenoids, saponins, anthraquinons, flavonoids and tannins, thin layer chromatographic tests were performed on silica gel 60 F_{254} coated sheets as listed (35).

Antibacterial assay:

Antibacterial activity of the extracts was evaluated by using agar well diffusion assay (25, 27).The nutrient agar plates, prepared according to the manufacture instruction, were swabbed with 24 hour old prepared inoculum of respective bacteria (*Staphylococcus aureus* (305-864-669), *Staphylococcus epidermidis* (505-864-689), *Pseudomonas aeruginosa* (909-825-1793) and *Escherichia coli* (909-825-1793)) and the inoculum was allowed to dry for 5mins. 4 wells (6 mm diameter holes 25mm apart from one another) were made in each of the nutrient agar plates by using sterile cork borer. The different amounts of the extracts dissolved in dimethyl sulphoxide (40µl, and 80µl equivalent to 5 mg, and 10 mg of the dried extract) were added to each of the 4 wells. Reference commercial discs of Amoxicillin 10µg, Erythromycin 15µg and Gentamicin10µg were placed on the agar surface, served as positive controls. 40 and 80 µl of the solvent dimethyl sulphoxide added to each of the 2 wells served as negative controls. The plates were then incubated for 24h.at 37°C. The inhibition of bacterial growth was determined by measuring the diameter of a clear inhibition zone (in mm) around each well and compared with established inhibition zone size around the disc of each individual reference antimicrobial agent. An average zone of inhibition was calculated for three replicates.

Determination of the activity index (6, 18)

The activity index of the crude plant extract was calculated as follows:

$$\text{Activity index (AI)} = \frac{\text{Zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

Statistics

Data are presented as mean ± standard deviation from three independent experiments.

Results

The yield of each plant extract (w/w), presented in Table 1, was estimated as dry extract weight/ dry starting material × 100.

Table 1: The percentage of extract yield of the tested plants

Test sample	Yield in %	
	Chloroform extract	**70% Ethanol extract**
Cissus subaphylla	4%	8%
Euphorbia spiralis	8%	16.4%

The results of the phytochemical screening of chloroform and 70% ethanol extract of *Cissus subaphylla* and *Euphorbia spiralis* are presented in the Table2.

Table 2: Phytochemical constituents of the tested plant extracts

Species/ Family	Alka-loids	Sterols/ triterpe-noids	Cardiac glycol-sides	Sapo-nins	Anthra-quinons	Flavo-noids	Tann-ins	Carbo-hydrates
Cissus subaphylla	-	+	+	-	+	+	+	+
Euphorbia spiralis	-	+	+	-	+	+	+	+

"+" indicates presence and "–" indicates absence.

Thin layer chromatography (TLC)

The results of TLC (R_f values of the positive colored bands, displayed by the 70% ethanol and chloroform extracts), presented in Table 3,revealed the presence of sterols and triterpenoids, cardiac glycosides, anthraquinones , flavonoids, and tannins(15,35).

Table 3: Results of TLCmanifested by the R_f values and colors of bands displayed bythe 70% ethanol and chloroform extracts of the *C. subaphylla* and *E. spiralis* for the presence of different phytochemical constituents

Tested plants	70% ethanol extract		Chloroform extract	
	Sterols and triterpenoids			
	Color	**R_f values**	**Color**	**R_f values**
Cissus subaphylla	2 Violet	0.05,0.25	Violet	0.06
			3 Yellow	0.46, 0.64, 0.95
Euphorbia spiralis	3Violet	0.09, 0.32, 0.75	2Violet	0.08, 0.33
			Yellow	0.65
			Violet	0.75
			2Yellow	0.89, 0.92
	Cardiac glycosides			
Cissus subaphylla	Brown	0.09	Bluish-gray	0.73
	2 Yellow	0.39, 0.53	Yellowish-brown	0.79
			Green	0.89
	Bluish-gray	0.78		
Euphorbia spiralis	Brown	0.09	11 Brown spots	0.06-0.84
	Anthraquinones			
Cissus subaphylla	3 yellowish-brown	0.24, 0.34, 0.77	3 Green	0.47, 0.52, 0.63
Euphorbia spiralis	Light yellow	0.76	2 Light yellowish-brown	0.31, 0.49
	Flavonoids			
Cissus subaphylla	Light-brown	0.06	2 Light-yellow	0.15, 0.34
	2 Yellow	0.19, 0.25	Green	0.85
	2 Light-yellow	0.40, 0.82		
Euphorbia spiralis	Yellowish-brown	0.15	Yellowish-brown	0.18
	Light yellow	0.83	Light yellow	0.84
	Tannins			
Cissus subaphylla	2 Blue	0.48, 0.77	Green	0.86
	Light green	0.87		
Euphorbia spiralis	Light brown	0.74	Light brown	0.82

The chromatograms of the 70% ethanol and chloroform extracts of the tested plants showed no bands for the presence of saponins and alkaloids.

Antibacterial activity

The results of the tests for the antibacterial activity of the different extracts of the tested plant materials are given in Table 4.

Table 4: Results of the antibacterial activity of plant extracts

Test sample	Microorganism			
***Cissus subaphylla* stems**	***S. aureus***	***S. epidermidis***	***E. coli***	***P. aeruginos*a**
Chloroform extract-5mg				
DIZ[a] (in mm)	10.3±0.58	12.3±1.16	14±1.73	12.7±0.58
AI_1	0.41	0.54	0.61	0.55
AI_2	0.34	0.35	0.52	0.40
AI_3	0.32	0.36	0.47	0.47
Chloroform extract-10mg				
DIZ (in mm)	12.3±1.53	14.7± 0.58	16.7±1.53	15.3±0.58
AI_1	0.49	0.64	0.73	0.67
AI_2	0.41	0.42	0.62	0.48
AI_3	0.39	0.43	0.56	0.57
70% Ethanol extract 5mg				
DIZ (in mm)	12.7±0.58	11.7±1.53	14±1	12.3±0.58
AI_1	0.51	0.51	0.61	0.54
AI_2	0.42	0,33	0.52	0.38
AI_3	0.40	0.34	0.47	0.46
70% Ethanol extract 10mg				
DIZ (in mm)	13.7±0.58	13.7±0.58	17.7±1.52	13± 0
AI_1	0,59	0.60	0,77	0.57
AI_2	0.46	0.39	0,66	0.41
AI_3	0.43	0.40	0.59	0.48
***Euphorbia spiralis* succulent stems**				
Chloroform extract 5mg				
DIZ (in mm)	13±1	11.3±0.58	15±1	11.3±1.53
AI_1	0.52	0.49	0.65	0.49
AI_2	0.43	0.32	0.56	0.35
AI_3	0.41	0.33	0.50	0.42
Chloroform extract 10mg				
DIZ (in mm)	15±1	15.3±1.53	17.7±0.58	12.7±1.15
AI_1	0.60	0.67	0.77	0.55
AI_2	0.50	0.44	0.66	0.40
AI_3	0.47	0.45	0.59	0.47
70% Ethanol extract 5mg				
DIZ (in mm)	11.7±1.15	12.7±0.58	18.7±1.53	12.3±1.16

AI_1	0.47	0.55	0.81	0.54
AI_2	0.39	0.36	0.69	0.39
AI_3	0.37	0.37	0.62	0.46
70% Ethanol extract 10mg				
DIZ (in mm)	15±1	14.3±1.53	21.7±1.53	12.7±0.58
AI_1	0.60	0.62	0.94	0.55
AI_2	0.50	0,41	0.80	0.40
AI_3	0.47	0.42	0.72	0.47
Amoxicillin(10µg /disc)	25	23	23	23
Erythromycin(15µg /disc)	30	35	27	32
Gentamicin(10µg /disc)	32	34	30	27

[a] = DIZ= Diameter of inhibition zone (in mm); AI= activity index; AI_1= activity index comparing to amoxicillin; AI_2= activity index comparing to erythromycin;AI_3= activity index comparing to gentamicin. Negative control (DMSO) did not show any activity. Values are mean of triplicate readings (mean ± SD)

Discussion

Traditional medicine, especially the use of herbal medicine is widespread throughout the world and has been practiced for centuries. Despite the development and spread of modern scientific medicine, herbal medicine is still dominant today, for example, in China, traditional herbal preparations account for 30%-50% of the total medicine consumption, while is in Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicine at home (40). Herbal medicine is widely appreciated and used by a large number of populations in different areas in Yemen for the treatment of a number of diseases including infections **(**4, 10, 16, 17).Antibiotics are undeniably one of the most important therapeutic discoveries of the 20th century that had effectiveness against serious bacterial infections. However, only one third of the infectious diseases known have been treated from these synthetic products(32). This is because of the emergence of resistant pathogens that is beyond doubt the consequence of years of widespread indiscriminate use, incessant and misuse of antibiotics (14,39). One of the methods to reduce the resistance to antibiotics is by using antibiotic resistance inhibitors from plants (3, 21).It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant pathogens (1).Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. Hence, researchers have recently paid attention to safer phytomedicines and biologically active compounds isolated from plant species used in herbal medicines with acceptable therapeutic index for the development of novel drugs (29, 38).Socotra is distinguished by strong biodiversity and endemism. 307 plant types are endemic of Socotra, which constitutes about 37% of the total flora of Socotra (9,23, 37). Plants used in herbal medicine in Socotra represent a valuable source to be explored for new biologically active compounds. Thus, the purpose of this study is to screen for the phytochemical constituents and antibacterial activity of two endemic plants used in herbal medicine by people in Socotra for the treatment of infectious diseases and other diseases. Ethnobotanical studies of the selected plants were performed by using all available literature sources such as scientific journals, books and internet as well as by consultation with the native people in Socotra. To the best of our knowledge, this is the first time, the two selected plants *C. subaphylla* and *E. spiralis* were screened for their phytochemical constituents. The phytochemical screening, confirmed by thin layer chromatography, of the 70% ethanol extracts and chloroform extracts of *C. subaphylla* and *E. spiralis*, revealed the presence of sterols, triterpenoids, cardiac glycosides, anthraquinones, flavonoids, tannins and carbohydrates.

However, all the tested extracts showed the absence of alkaloids and saponins. A search in the literature indicated that the chemical structures of antibacterial agents, found in higher plants, belong to most commonly encountered classes of higher plant secondary metabolites such as alkaloids, coumarins, chromans, flavonoids, anthraquinones, saponins, terpenoids and tannins (11-13, 29).Consequently, the results of our work on phytochemical screening of different extracts of *C. subaphylla* and *E. spiralis* suggest that a number of plant constituents, such as terpenoids, flavonoids, anthraquinones, and tannins, may be involved in the antibacterial activity of these plant materials.

Testing the 5 and 10mgof the 70% ethanol and chloroform extracts of *C. subaphylla* and *E. spiralis* for antibacterial activity against two gram positive bacteria (*S. aureus* and *S. epidermidis*) and two gram negative bacteria (*E. coli* and *P. aeruginosa*) revealed that all extracts showed different grades of antibacterial activity against all tested microorganisms (Table 4). The activity index of the test substance, above 0.5 ,is considered as significant activity (20). From the Table 4, it is obvious that:

- Significant antibacterial activity was demonstrated by all tested extracts against test microorganisms except the chloroform extract of *C. subaphylla*, which was found inactive against *S. aureus.*
- The70% ethanol extract (5mg and 10mg)of *E. spiralis* demonstrated higher antibacterial activity (AI_1=0.81 and 0.94 respectively) against *E. coli* ,compared to all other extracts. This outstanding antibacterial activity is similar or approaching those produced by positive control (amoxicillin). To the best of our knowledge this is the first time to reveal the significant antibacterial activity of *E. spiralis* especially against *E. coli*. *E. coli,* among other enteric bacteria that causes food-borne illnesses and gastrointestinal problems in the developing countries and human beings around the world(5). Hence, *E. spiralis* could be of special interest as a potential source to be further investigated for new antibacterial agent, especially for the treatment of infections caused by *E. coli*. However, a number of Euphorbia species have been reported to contain skin irritant, toxic and cancer promoting substances (26, 28, 32, 34, 42). Hence, it is important to subject *E. spiralis* to pharmacological investigations to test their toxicity, irritancy and carcinogenic activity.
- The chloroform extracts (5and 10mg) of *C. subaphylla* possess significant activity (AI=0.52-0.73) against *S. epidermidis*, *E. coli* and *P. aeruginosa*, while the 70% ethanol extracts (5 and 10mg) of the same plant showed a significant activity (AI=0.51-0.77) against all tested bacteria.
- The chloroform extract (5mg) of *E. spiralis* demonstrated a significant activity (AI=0.52-0.65) against *S. aureus,* and *E. coli*, whereas the chloroform extract (10mg) was significantly active (AI=0.55-0.77) against all tested bacteria.
- The 70% ethanol extract (5mg) of *E. spiralis* showed a significant activity (AI=0.54-0.81) against *S. epidermidis*, *E. coli* and *P. aeruginosa*, while the70% ethanol extract (10mg) displayed a significant activity against all tested bacteria(AI=0.55-0.94).

The microorganisms showed different sensitivity to the plant extracts. *E. coli* was the most sensitive, followed by *S. epidermidis* and *P. aeruginosa*. *S. aureus* was the least sensitive. A direct linear relationship between the concentrations of the tested extracts and their antibacterial activity was observed , except for the 70% ethanol extracts (5 and 10mg) of *E. spiralis* against *P. aeruginosa*, where both extracts showed almost similar inhibitory effect. In a previous study (24), a small concentration (4mg) of chloroform extract of *C. subaphylla* from Socotra was reported to be inactive against *S. aureus*, *E. coli* and *P. aeruginosa*. This could confirm our result on the concentration-activity relationship. Consequently, extracts displayed activity indices above 0.5 and, especially, those with higher activity indices justify the traditional uses of the tested plants in Socotra for the treatment of infectious diseases. These plants are deserving further phytochemical and pharmacological investigations to reveal their active constituents and testing them for antimicrobial activity against a vast array of microorganisms as well as for other pharmacological activities.

Conclusion

In conclusion, data obtained in this study, revealed the main phytochemical constituents (sterols, triterpenoids, cardiac glycosides, anthraquinones, flavonoids and tannins) presented in the two endemic Socotrean plants *Cissus subaphylla* and *Euphorbia spiralis* , illustrated their significant antibacterial activity, especially the antibacterial activity of *E. spiralis* against *E. coli*. These data provide scientific justification for the use of *Cissus subaphylla* and *Euphorbia spiralis* in Socotra for the treatment of infectious diseases. However, further phytochemical and pharmacological investigations are required to establish their efficacy and safety against a wide range of microorganisms, as well as to search for further biological activities.

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الفحص الكيميائي والكشف عن النشاط المضاد للبكتيريا لنبات (*Cissus subaphylla*) ونبات (*Euphorbia spiralis*) المتوطنة في جزيرة سقطرى

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الملخص

تم اختيار نباتين متوطنين في جزيرة سقطرى وهما سيقان نبات عطرها(*Cissus subaphylla*) والسيقان العصارية لنبات القشر (*Euphorbia spiralis*) لإجراء الفحص الكيميائي عن المواد الفعالة فيها وكذلك إجراء تجارب لاختبار فعاليتهما ضد الميكروبات. الفحص الكيميائي لمستخلصات الكلوروفورم و 70 % إيثانول للنباتين دل على احتوائهما على المواد الأتية: sterols, triterpenoids, cardiac glycosides, anthraquinons, flavonoids, tannins and carbohydrates . تم فحص فعالية مستخلصات 70 % إيثانول و الكلوروفورم (5 و 10 ملجم) لكلا النباتين ضد:

Staphylococcus aureus (305-864-669), *Staphylococcus epidermidis* (505-864-689), *Pseudomonas aeruginosa* (909-825-1793), *Escherichia coli* (909-825-1793) ،

باستخدام agar well diffusion assay هذا وقد تم التوصل إلى أنَ كل المستخلصات لكلا النباتين، بيُنت فعاليتها الأكيدة ضد البكتيريا، حيث وجد أن مؤشر فعاليتها(AI) كان فوق0.5 ضد البكتيريا الخاضعة للاختبار ماعدا مستخلص الكلوروفورم لنبات عطرها *C. subaphylla*والذي كان غير فعال ضد بكتيريا*S. aureus*. مستخلص 70 % إيثانول تركيز(10ملجم) لنبات القشر*E. spiralis* أظهر فعالية عالية (AI=0.80-0.9) ضد بكتيريا *E. coli*.

الكلمات المفتاحية:*Cissus subaphylla* ، *Euphorbia spiralis*، الفحص الكيميائي، النشاط المضاد للبكتيريا، جزيرة سقطرى.