Journal of Applied Pharmaceutical Science Vol. 5 (Suppl 3), pp. 022-026, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.510.S4
ISSN 2231-3354

Phytochemical screening and evaluation of biological activity of *Calligonum polygonoides* L. subsp. *comosum*

Hayam S. Ahmed[1*], Abeer S. Moawad[1], Asmaa I. Owis[1], Sameh F. AbouZid[1], Rehab F. Abdel-Rahman[2]

[1]Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt.
[2]Pharmacology Department, National Research Centre, Dokki, Giza, Egypt.

ARTICLE INFO

Article history:
Received on: 24/07/2015
Revised on: 16/08/2015
Accepted on: 16/09/2015
Available online: 12/11/2015

Key words:
Calligonum polygonoides, TLC screening, estrogenic, antimicrobial.

ABSTRACT

Calligonum polygonoides L. subsp. *comosum*; locally known as "arta", is tall woody shrub, perennial desert plant. TLC screening, estrogenic and antimicrobial activities of different fractions of hydroalcoholic extract of the leaves; *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH, were studied in order to find the correlation between the phytoconstituents and the biological activity. Estrogenic activity was studied in immature ovariectomized female Wistar rats by oral administration of 75 and 150 mg extract/kg body weight for seven days using 1 µg estradiol/rat/day as positive control. The antimicrobial activity against *Aspergillus fumigatus*, *Candida albicans*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* was carried out using agar diffusion method with determination of the minimum inhibitory concentration (MIC). The CH_2Cl_2 fraction showed significant estrogenic and antimicrobial activity by significantly increasing uterine weight and low MIC values for all tested microorganisms ranging from 0.03 and 3.9 mg/mL. The *n*-hexane fraction showed mainly the presence of sterols and/or triterpenoids. The EtOAc and *n*-BuOH fractions were the richest in flavonoids while that of methylene chloride was found to contain both classes of chemical compounds. In conclusion; flavonoids, sterols and/or triterpenes exhibit synergistic effect to the antimicrobial and estrogenic activity of *Calligonum polygonoides* leaves.

Abbreviations
CH_2Cl_2: methylene chloride, EtOAc: ethyl acetate, *n*-BuOH: normal butanol, MIC: minimum inhibitory concentration, TLC: thin layer chromatography, b. wt. body weight, p. o.: oral administration, HE: *n*-hexane extracts of leaves, ME: methylene chloride extract of leaves, EE: ethyl acetate extract of leaves, BE: *n*-butanol extract of leaves, RCMB: Regional Center for Mycology and Biotechnology, NA: No activity.

INTRODUCTION

The genus *Calligonum* (family Polygonaceae) comprising about 80 species; is widely distributed in North Africa, South Europe, and West and Central Asia. In the Saharan areas, *Calligonum* species are dominant perennial shrubs and trees in active sand dunes and in stabilized sand fields. They can tolerate extreme drought conditions by the loss of leaves and branches during the driest months (Gouja *et al.*, 2014). *C. polygonoides* L. subsp. *comosum*; locally known as "arach", "abal" or "arta", is a tall woody perennial desert shrub indigenous to Egypt (Centre for Mediterranean Cooperation, 2005). It exhibits diverse biological activities including antimicrobial activity (Alkhalifah, 2013), cytotoxicity and antioxidant activity (Badria *et al.*, 2007), anti-ulcer, anti-inflammatory activity (Liu *et al.*, 2001), hypoglycemic activity (El-Hawary and Kholief, 1990). Previous phytochemical investigation led to the isolation of some compounds that were mostly phenolic from the herb (El–Sayyad and Wagner, 1978, Badria *et al.*, 2007). Members of the family Polygonaceae in Chinese medicine are used traditionally to treat menopausal symptoms; *Polygonum cuspidatum; Rheum palmatum*, *Polygonum multiflorum* (Zhang *et al.*, 2005). Previous investigation of antimicrobial activity showed that the aqueous ethanol (90%) extract of the leaves significantly inhibited growth of some Gram positive and Gram negative bacteria (Alkhalifah, 2013), however, nothing could be traced concerning the fraction responsible for this activity.

* *Corresponding Author*
Hayam Ahmed, Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt
mail id:hymsalah@yahoo.com

Therefore, it was interesting to screen different extractives of *C. polygonoides* leaves growing in Egypt for its estrogenic and antimicrobial activities.

MATERIALS AND METHODS

Plant material

The plant (*C. polygonoides*) was collected from western desert, Giza governorate, Egypt, on April 2012 during flowering stage. The authenticity of the collected plant was confirmed by Dr. Abdelhalim Mohamed (Plant Taxonomy Department, Agricultural Research Institute, Egypt). Voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University under the registration number BUPD-40.

Preparation of the extract

Two kilograms of the fresh leaves were air-dried in shade at room temperature, powdered, and exhaustively extracted by cold maceration with aqueous ethanol (70%). The extract was evaporated under reduced pressure at 40^{o}C to yield 400 g residue. The residue was suspended in distilled water and partitioned successively with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH saturated with H_2O. Each extract was evaporated under reduced pressure to yield 2, 5, 10 and 45 g residues, respectively.

Phytochemical and TLC screening

Preliminary phytochemical screening was performed on total alcohol extract to test for the presence of volatile oil, carbohydrates and/or glycosides, sterols and/or triterpenes (Raaman, 2006), tannins, saponins, alkaloids and/or nitrogenous bases, anthraquinones (Evans and Evans, 2002), flavonoids (Geissman, 1962), and cardiac glycosides (Kar, 2003). Different fractions were subjected to TLC screening using standard qualitative methods (Wagner and Bladt, 1996).

Reagents and solvents

The following reagents were prepared according to the Egyptian Pharmacopoeia monographs (Ministry of Health and Population, 2005); 1% $FeCl_3$, 1% Aluminum chloride, Mayer's, Wagner's and Dragenorff's reagents, alcoholic α-naphthol, Fehling's solution, picric acid and ferric chloride (T.S.). *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH, MeOH, and EtOH were of analytical grade.

Animals and ethics

Mature albino mice (20–25 g b.wt. each, of both sexes) that used in the acute toxicity study were fasted for 12 h prior to the experiment. Immature female Wistar rats (50-60 g and 25 days old) that used in the assessment of the estrogenic activity were allowed to adapt to the laboratory environment for one week before experiment. All animal procedures were performed following the rules of the Ethical Committee of The National Research Centre, Egypt and in accordance with the recommendations of the proper care and use of laboratory animals.

Acute toxicity study

Mice were divided into six groups (n = 10) for each extract. Mice were given *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH extracts orally in graded doses from 500 to 2500 mg/kg body weight then closely observed for 48 h for changes in behavior, symptoms of toxicity and death. Control mice received the vehicle and kept under the same conditions without any treatment. The oral LD_{50} of the tested extract was calculated mathematically (Gad and Weil, 1982).

Estrogenic activity

Doses of 75 and 150 mg/kg b.wt. of the *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH fractions were used. The tested fractions were freshly prepared by dissolving 0.5 g residue of each extract in 25 mL of dimethyl sulfoxide (DMSO). The final solution concentration was 2%.

Experimental design

Seventy seven rats were divided into eleven groups each of seven rats. All rats; except the normal control (1st group), were bilaterally ovariectomized by dorso-lateral approach under light, ether anesthesia and semi-sterile conditions. Afterward, all rats were housed under standard conditions of natural 12 h light and dark cycle with free access to food and water. The 2nd group was kept as ovariectomized control group. The 3rd group was subcutaneously injected with 1 µg estradiol/rat/day, as a reference drug, for 7 successive days. The other eight groups were orally administered extracts at doses of 75 and 150 mg/kg, for 7 successive days. On the 8th day of the experiment, vaginal smears from all rats were microscopically examined and cornification of vaginal epithelial cells was observed according to (Sharaf, 1951). Animals were then sacrificed by cervical decapitation and the uteri were dissected out and separated from the adherent tissues and weighed up using an electronic balance. The relative uterine weight of each rat was calculated according to (Chavalittumrong *et al.*, 2004), using the following formula: Relative organ weight (kg) = [organ weight (g)/body weight (g)] × 1000

Statistical analysis

Data are expressed as mean ± standard error (SE) of mean. Statistical analysis was performed, using a one-way analysis of variance (ANOVA). When the F-value was found statistically significant ($P < 0.05$), further comparisons among groups were made using Dunnett's multiple comparisons test. All statistical analyses were performed using SPSS software 17.0 (Released Aug. 23, 2008), Chicago, USA.

Antimicrobial activity

Fractions under investigation were individually tested against a panel of Gram-positive and Gram-negative bacterial pathogens, yeast and fungi using the agar well-diffusion method (Smania *et al.*, 1999). Nutrient agar (NA) medium for bacteria and Sabouraud dextrose agar (SDA) for fungi were prepared then wells (6 mm in diameter) were made containing 100 µl of the tested

extracts solutions in DMSO. Microbial inoculum was uniformly spread using sterile cotton swab on a sterile Petri dish. The inoculated plates were then incubated for 24 h at 37°C for bacteria and yeast, 48 h at 28 °C for fungi. Negative controls were prepared using DMSO. Ampicillin (100 µg/mL), Gentamicin (100 µg/mL) and Amphotericin B (100 µg/mL) were used as standards for Gram positive bacteria, Gram negative bacteria, and fungi, respectively. After incubation, antimicrobial activity was evaluated by measuring the zone of inhibition against tested microorganisms. Antimicrobial activity was expressed as inhibition diameter zones in millimeters (mm). The experiment was carried out in triplicate and the average zones of inhibition ± S.D were calculated. Minimum inhibitory concentration (MIC) was determined by the broth micro dilution method using 96-well micro-plates (Saini *et al.*, 2005, Bhuiyan *et al.*, 2011). The microbial strains inoculate was prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Each sample (1.0 mg) was dissolved in DMSO (1 mL) to obtain 1000 µg/mL stock solution. A number of wells were reserved in each plate for positive and negative controls. Sterile broth (100 µl) was added to the well from row B to H. The stock solutions of samples (100 µl) were added to the wells in rows A and B. Then, the mixture of samples and sterile broth (100 µl) in row B was transferred to each well in order to obtain a two-fold serial dilution of the stock samples (concentrations of 500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.9, 1.95, 0.98 and 0.49 µg/mL). The inoculums (100 µl) were added to each well and a final volume 200 µl was obtained in each well. Plates were incubated at 37°C for 24 h in case of antibacterial activity and 48 h at 25°C for antifungal activity. Microbial growth was indicated by the presence of turbidity and a pellet at the bottom of the well.

RESULTS AND DISCUSSION

Phytochemical and TLC screening of the different extracts of *C. polygonoides* leaves was performed and the biological study on the fractions of the extract was carried out to evaluate their antimicrobial and estrogenic activities. Phytochemical screening of the plant leaves extract showed that carbohydrates and/or glycosides, tannins, free and combined flavonoids, as well as sterols and/or triterpenes are present in the leaves while steam volatile substances, saponin, alkaloids and/or nitrogenous bases, cardiac glycosides, free and combined anthraquinones were absent. TLC screening of the different fractions (Tables 1 and 2) revealed that the *n*-hexane fraction is rich in sterols and/or triterpenoids. The EtOAc and *n*-BuOH fractions were the richest in flavonoids, while CH_2Cl_2 fraction contains both of these classes of compounds.

Acute oral toxicity

No signs of toxicity or mortality were recorded in mice following single administration of all of the tested fractions of *C. polygonoides* leaves at oral doses up to 2.5 g/kg. The safe therapeutic doses used for the evaluation of its possible estrogenic activity were $1/20^{th}$ (75 mg/kg) and $1/10^{th}$ (150 mg/kg) of the oral LD_{50} dose.

Table 1: Results of TLC investigation of the *n*-hexane and methylene chloride fractions of the leaves of *Calligonum polygonoides* L.

Fraction	Solvent system	Spot No	R_f	Color		
				Day light	UV (365 nm)	*p*-anisaldehyde
***n*-Hexane**	**S_1**	**1**	0.88	Colorless	Colorless	Violet
		2	0.15	Colorless	Colorless	Violet
		3	0.07	Colorless	Colorless	Violet
Methylene chloride	**S_2**	**1**	0.62	Pale Yellow	Yellowish green	Yellow
		2	0.48	Yellow	Yellow	Intense Yellow
		3	0.46	Colorless	Colorless	Violet

S_1, *n*-Hexane/ EtOAc (9:1 v/v) S_2, CH_2Cl_2/ MeOH (9:1 v/v)

Table 2: Results of TLC investigation of the ethyl acetate, and *n*-butnol fraction of the leaves of *Calligonum polygonoides* L.

Fraction	Solvent system	Spot no	R_f	Color in		Color with NH_3		Color with $AlCl_3$		Color with *p*-anisaldehyde reagent
				Vis. light	UV	Vis. light	UV	Vis. light	UV	
Ethyl acetate	**S_3**	**1**	0.86	-	-	-	-	Faint yellow	Yellowish green	Faint yellow
		2	0.79	Yellow	Purple	Intense yellow	Orange yellow	Yellow	yellow	Yellow
		3	0.57	-	Purple	-	Yellow	Faint Yellow	Yellow	Faint yellow
		4	0.43	Yellow	Purple	Yellow	Orange yellow	Yellow	Yellow	Yellow
		5	0.29	Yellow	Yellow	Yellow	Yellow	Yellow	Intense yellow	Yellow
		6	0.14	Yellow	Yellow	Yellow	Yellow	Yellow	Yellowish green	Faint yellow
***n*-Butanol**	**S_4**	**1**	0.86	Yellow	Purple	Yellow	Yellow	Intense yellow	Yellowish green	Yellow
		2	0.79	Yellow	Purple	Intense yellow	Yellow	Intense yellow	Yellow	Yellow
		3	0.57	-	-	-	Faint Yellow	Faint Yellow	Faint yellow	Faint yellow
		4	0.43	-	Faint yellow	-	Faint yellow	-	Yellowish green	Yellow

S_3, CH_2Cl_2/MeOH (8.5:1.5 v/v) S_4, Ethyl acetate/Formic acid/Acetic acid/Water (100:11:11:27 v/v)
Vis: visible UV: at 365 nm

Estrogenic activity

Estrogen synthesis in the ovary controls estrus cycle, normally with duration of 4-5 days. During a normal rat estrus cycle, there are three cell types. Presence or absence of these types of cells and their relative proportions determine the stage of estrus cycles phase (El-Alfy *et al.,* 2012). The effects of the fractions obtained from the hydroalcoholic extract of *C. polygonoides* leaves were tested on two doses 75 and 150 mg/kg/d. In the ovariectomized immature female Wistar rats, oral administration of the different fractions for 7 days at dose 75 mg/kg b.wt was found to increase the weight (g) of the uterus. The *n*-butanol fraction appears to be the least potent, while *n*-hexane, methylene chloride and ethyl acetate fractions appear to be nearly with the same activity when compared with ovariectomized control rats (1.5 ± 0.09). On increasing dose to 150 mg/kg, it was clear that methylene chloride fraction resulted in significant increase in the uterine weight ($P < 0.05$) when compared with ovariectomized control rats with different cell type phases. The uterine weights did not significantly increase on increasing the dose of administration for other tested fractions. Data of estrogenic activity is shown in Table 3. Examination of vaginal smears obtained from rats administered *n*-hexane, methylene chloride, ethyl acetate, *n*-butanol fractions or estriol; showed different phases of estrus cycle following administration of 150 mg/kg/d more than 75 mg/kg/d. Untreated ovariectomized control rats (positive control) revealed anoestrus cells (no cornified). Both *n*-hexane and ethyl acetate fractions showed only one rat out of seven was in metoestrus phase and one in dioestrus phase. The ethyl acetate fraction showed one rat in estrus phase. The methylene chloride fraction showed one rat in metoestrus phase and three rats in estrus phase. The *n*-butanol fraction showed three rats in metoestrus phase and two rats in estrus phase while the group receiving the standard estriol, showed three rats in metoestrus and three in estrus phase (Table 3). These results indicate that all fractions have estrogenic activity to some extent at both doses; 150 mg/kg/day being more significant; *n*-butanol and methylene chloride fractions are more potent. The activity of *n*-hexane fraction may be related to presence of hydrocarbons, fatty acids; sterol and/or triterpenes other than β-sitosterol since β-sitosterol previously isolated from this plant was reported to have no binding ability to human estrogen receptors (ER) and did not exert estrogenic effect in female rats (Dixon, 2004, Nishihara *et al.,* 2000). Activity of CH_2Cl_2 fraction may be related to presence of flavonoids aglycons; kaempferol and quercetin previously reported from *C. comosum* herb (El–Sayyad and Wagner, 1978). Kaempferol was reported with high significant estrogenic activity nearly the same results produced by 17β-estradiol while it was found to be 10 fold more active than quercetin (Miksicek, 1995). TLC screening of EtOAc and *n*-butanol fractions showed that these fractions are rich in flavonoids. Activity of these fractions may be related to their flavonoids content as flavonoids were reported to support an estrogen response when they are present in sufficient quantities (Miksicek, 1993).

Antimicrobial activity

Antimicrobial activity of different fractions obtained from the hydroalcoholic extract of *C. polygonoides* was tested against a panel of microorganisms. Data of antimicrobial activity is shown in Table 4. Antifungal activity was tested against *Aspergillus fumigatus* and *Candida albicans*. Amphotericin B was used as a positive control. All of the tested fractions were active against *Aspergillus fumigatus*, while only methylene chloride fraction showed activity against *Candida albicans*. The MIC of methylene chloride fraction against *Aspergillus fumigatus* was 0.24 µg/mL.

All of the tested fractions showed antibacterial activity against Gram positive bacteria. The methylene chloride fraction was the most active with MIC values of 0.12 and 0.03 µg/mL against *Staphylococcus aureus* and *Bacillus subtilis*, respectively. The same fraction showed moderate activity against *Pseudomonas aeruginosa* and high activity against *Klebsiella pneumoniae* of the tested Gram negative bacteria. The other fractions were less active against *Klebsiella pneumoniae* and did not show any activity against *Pseudomonas aeruginosa*.

Table 3: Effect of *n*-hexane, methylene chloride, ethyl acetate, and *n*-butanol fractions on relative uterine weight and estrus cycle stage in rats.

Groups	Dose/Route of administration	Relative Uterine Weight (kg)	Estrus cycle stage
Control	---	2.4 ± 0.21*	Proestrus (3/7), Metoestrus (1/7), Dioestrus (1/7), Estrus (3/7).
Ovariectomized control	---	1.5 ± 0.09	Anoestrus (7/7).
n-Hexane fraction	75 mg/kg/day, p.o	2.1 ± 0.10	Anoestrus (2/7), Proestrus (2/7), Metoestrus (2/7), Dioestrus (1/7).
	150 mg/kg/day, p.o.	2.2 ± 0.09	Anoestrus (2/7), Proestrus (3/7), Metoestrus (1/7), Dioestrus (1/7).
Methylene chloride fraction	75 mg/kg/day, p.o	2.0 ± 0.19	Anoestrus (1/7), Proestrus (1/7), Metoestrus (2/7), Dioestrus (2/7), Estrus (1/7).
	150 mg/kg/day, p.o.	2.5±0.15*	Proestrus (3/7), Metoestrus (1/7), Estrus (3/7).
Ethyl acetate fraction	75 mg/kg/day, p.o.	1.9 ± 0.10	Anoestrus (7/7).
	150 mg/kg/day, p.o.	2.2 ± 0.20	Anoestrus (3/7), Proestrus (1/7), Metoestrus (1/7), Dioestrus (1/7), Estrus (1/7).
n-Butanol fraction	75 mg/kg/day, p.o	1.7 ± 0.11	Anoestrus (2/7), Proestrus (2/7), Metoestrus (2/7), Dioestrus (1/7).
	150 mg/kg/day, p.o.	2.2 ± 0.23	Proestrus (2/7), Metoestrus (3/7), Estrus (2/7).
Estradiol	1 µg/rat/day, s.c.	3.2 ± 0.29*	Proestrus (1/7), Metoestrus (3/7), Estrus (3/7).

Values represent the mean ± S.E. of seven rats for each group.

*P< 0.05: Statistically significant from ovariectomized control (Dunnett's test).

Table 4: Antimicrobial screening and minimum inhibitory concentration of *n*-hexane, methylene chloride, ethyl acetate and *n*-butanol fractions of the leaves of *Calligonum polygonoides* L.

Sample	Diameter of the zone of inhibition (mm)					Minimum inhibitor concentration (µg/mL)				
Tested Microorganism	**HE**	**ME**	**EE**	**BE**	**Standard**	**HE**	**ME**	**EE**	**BE**	**Standard**
Fungi					Amphotericin B					Amphotericin B
Aspergillus fumigatus (RCMB 02564)	18.3 ± 0.44	22.6 ± 0.44	12.3 ± 0.37	16.2 ± 0.44	23.7 ± 0.10	7.81	0.24	125	31.25	0.12
Candida albicans (RCMB 05035)	NA	20.9 ± 0.25	NA	NA	21.9 ± 0.12	NA	1.95	NA	NA	0.49
Gram +ve Bacteria					Ampicillin					Ampicillin
Staphylococcus aureus (RCMB 010027)	20.1 ± 0.28	24.2 ± 0.37	13.6 ± 0.63	19.6 ± 0.58	22.7 ± 0.14	3.9	0.12	62.5	1.95	0.24
Bacillus subtilis (RCMB 0000101(5)	23.1 ± 0.37	26.3 ± 0.25	13.9 ± 0.25	20.9 ± 0.25	27.9 ± 0.37	0.24	0.03	62.5	1.95	0.007
Gram -ve Bacteria					Gentamicin					Gentamicin
Pseudomonas aeruginosa (RCMB 000102(3)	NA	20.3 ± 0.37	NA	NA	19.7 ± .44	NA	3.9	NA	NA	1.95
Klebsiella pneumoniae (RCMB 0010093(12)	19.8 ± 0.44	25.9 ± 0.44	13.6 ± 0.25	17.1 ± 0.25	27.3 ± 0.44	3.9	0.03	62.5	15.63	0.03

CONCLUSION

The results obtained from the phytochemical screening and the antimicrobial evaluation of fractions of the hydroalcoholic extract of *C. polygonoides* leaves indicate that methylene chloride fraction could be potent candidates as phytotheraputic agent against the tested microorganisms. This may be related to its content of flavonoid aglycones, steroids and triterpenes. *C. polygonoides* leaves could be useful as a safe natural estrogenic food supplement for postmenopausal women. Phyto-constituents of the methylene chloride fraction of the extract could be isolated and identified to be incorporated in food supplements and adjunct therapy with antibiotics**.**

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How to cite this article:

Hayam S. Ahmed, Abeer S. Moawad, Asmaa I. Owis, Sameh F. AbouZid, Rehab F. Abdel-Rahman. Phytochemical screening and evaluation of biological activity of *Calligonum polygonoides* L. subsp. *comosum*. J App Pharm Sci, 2015; 5 (Suppl 3): 022-026.