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CHEMICAL AND BIOLOGICAL INVESTIGATIONS OF TWO RUMEX SPECIES GROWING IN YEMEN

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ABSTRACT

The phytochemical examination of the polar and non-polar fractions of two ethanol extracts of *Rumex* species viz; *R. obtusifolia* L. and *R. steudelii* Hochst. Ex A. Rich afforded seven anthraquinones and eight flavonoids. They were identified as chrysophanol, physcion, emodine, aloe-emodin, chrysophanin, emodin-8-O-glucoside, physcion-8-O-glucoside, kampferol, luteolin, catechin, luteolin-7-O-glucoside, isovetexin, isoorientin, vitexin and orientin. The identification was established through physical and spectral analysis as well as comparison with reference samples. The antibacterial and antifungal activities of the different fractions were assessed.

KEYWORDS: *Rumex obtusifolia*, Rumex steudelii antimicrobial. Yemen, phytochemical analysis.

INTRODUCTION

With more than 200 species, *Rumex* L. is the second biggest genus in the Polygonaceae family and is mostly found in the northern temperate zone.[1] Most of the phytochemical and pharmacological studies were performed on up to 50 species. Most of the investigated species under this genus are rich in anthraquinones, naphthalenes, flavonoids, stilbenoids, triterpenes, carotenoids, and phenolic acids.[2] The plant's leaves, roots, and aerial parts are used as vegetables and to treat a variety of illnesses, including inflammation, constipation, mild diabetes, infections, diarrhea, oedema, and jaundice.[2,3,4] The genus showed a broad array of

biological activities such as diuretic and analgesic, antitumor, anti-asthmatic, antitussive, insecticide, antioxidant, antimicrobial, antihyperglycemic, antihyperlipidemic, antiinflammatory, analgesic and antihelminthic activity.[5,6,7,2]

The genus *Rumex* is represented in Yemen by five species.[8] Harshaw et al reported the antibacterial activity of methanolic extract of *R. obustifolia* against against *B. cereus, B. subtilis, E. coli, S. aureus* and *Salmonella* tryphi.[9] While aqueous fractions of *R. obtusifolius* L. root extract had shown moderate inhibition against *S. epidermidi, S. aureus, and MRSA*, aqueous fractions of herb extract from the plant demonstrated mild inhibition against same bacterial strains.[10] Most Rumex species are generally used in Yemeni traditional medicine as laxatives, antinfective and against gastrointestinal disorders. *R. nervosus* Vahl, is the most widely used species in Yemeni traditional medicine compared to the other species.[11,12,13,14] This study reports for the first time the chemistry and antimicrobial activity of two *Rumex* species growing in Yemen, *R. obustifolia* and *R. steudelii*.

EXPERIMENTAL

Instruments: IR-spectrophotometer, Pye Unicam Sp. 1000; UV-spectrophotometer, Perkin Elmer 550 S; MS: (EI) VG-70 E. Authentic samples from previously isolated and identified compounds in Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt.

Plant materials

The aerial parts and roots of *R. Obtusifolia* and *R. steudelii*, Hochst. Ex A. Rich were collected at the flowering stage and identified in the Botany Department, Faculty of Pharmacy, Sana'a University. A voucher specimens are deposited in Pharmacognosy Department, Faculty of Pharmacy, Sana'a University. The folk medicinal uses, distribution and location of plant collection above the sea level[11-14] are shown in Table (1).

Extraction and isolation

The powdered leaves and roots (500 g each) of the three species were separately exhausted with boiling ethanol (3L). Each alcoholic extract was concentrated, diluted with water (200 ml), and extracted with petroleum ether, ether and ethyl acetate respectively. Each extract was examined chromatographically. Extracts of the same chromatographic pattern were combined together, fractionated over silica gel column (150 g, Merck) by gradient elution starting with petroleum ether (b.r. 60-80º) followed by increased ratio of ethyl acetate. The

obtained fractions were monitered by TLC (silica gel GF_{254}, Pet. ether -EtOAc 7:3, alcoholic KOH 5% w/v spray reagent for detection). Seven substances **1-4, 8-10** could be isolated. However further purification was performed by preparative TLC. The combined ethyl acetate extracts were fractionated by preparative TLC (silica gel GF_{254}, EtOAc-MeOH-H_2O, 100:15:10 and NH_3 /UV for detection) to afford eight substances **5-7** and **11-15**. Substances 12-15 were further purified by paper chromatography (Whatmann 3mm, 20 % acetic acid, and NH3/UV for detection). Each isolated band was eluted by methanol, concentrated and crystallized.

Identification of the isolated substances

1. **Chrysophanol**: Eluted with 2% EtoAc, 20 mg, yellow prisms (from acetone), m.p. 193-195º, lit; 195-197º.[15,16]

IR: ν(KBr) 3490 (OH), 1680 (C=O), 1635 (chelated C=O), 1610, 1570, 1480, 1450, 1390, 1210 and 900 cm^{-1}.

UV: λ(CH_3OH) 228, 245, 273 and 434 nm.

MS: EI m/z 254 (100) M^+ for $C_{15}H_{10}O_4$.

2. **Physcion:** Eluted with 4% EtOAc, 20 mg, yellow plates (from acetone), m.p. 204-206º, lit; 204-206º.[15,16]

IR: ν(KBr) 3500 (OH), 1680 (C=O), 1630 (chelated C=O), 1615, 1570, 1480, 1325, 1235, 11170 and 895 cm^{-1}.

UV: λ(CH_3OH) 224, 255, 260, 290 and 340 nm.

MS: EI m/z (rel. int) 284 (100) M^+, 255 (10), 241 (10), 227 (6), 213 (9), 198 (5), 185 (7), 128 (14), 115 (5) and 11 (8).

3. **Emodin:** Eluted with 10% EtOAc, 30 mg, yellow needles (from methanol) , m.p. 251-252º, lit; 250-252º.[15,16]

IR: ν(KBr) 3495 (OH), 1685 (C=O), 1630 (chelated C=O), 1600, 1575, 1480, 1345, 1300, 1230, 1170 and 910 cm^{-1}.

UV: λ(CH_3OH) 225, 255, 292 and 442 nm.

4. **Aloe-emodin:** Eluted with 12% EtoAc, brown crystals (from methanol), m.p. 218-220º, lit; 218-220º.[15]

IR: ν(KBr); 3400 (OH), 1680 (C=O), 1630 (chelated C=O), 1575, 1460, 1395, 1280, 1090, 1060, 1040 and 870 cm^{-1}.

UV: λ(CH_3OH) 214, 226, 256, 276 and 432 nm.

5. Chrysophanin: Yellow needles (from methanol) 25 mg, m.p. 240-242, Lit; 245-246°[15]

IR: ν(KBr) 3450 (OH), 2960 (CH), 1675 (C=O), 1625 (chelated C=O), 1455, 1270 and 1080 cm^{-1}.

UV: λ(CH_3OH) 228, 272, 280, ,408 and 430 nm.

6. Emodin 8-O-glucoside: Red prisms (from methanol) 30 mg, m.p. 190-193º, lit 194-196º.[15]

UV: λ(CH_3OH) 232, 275, and 418 nm.

7. Physcion 8-O-glucoside: Red prisms (from methanol) 30 mg, m.p. 235º, Lit; 236-240º.[15]

IR: ν(KBr) 3490 (OH), 3000(CH), 1680 (C=O), 1630 (chelated C=O), 1590, 1460, 1275 and 1090 cm^{-1}.

UV: λ(CH_3OH) 225, 270, 282, and 410 nm.

8. Kampferol: Yellow needles (from methanol) 25 mg, (UV in Table 3).

9. Luteolin: Yellow needles (from methanol) 25 mg, (UV in Table 3).

10. Catechin: Eluted by 50% EtOAc, 10 mg, yellowish-brown crystals (from methanol), m.p. 148-150º. It gave orange color with KOH spray reagent.

IR: ν(KBr) 3405 (OH), 2962 (CH), 1636, 1534, 1476, 1295, 1154, 1085 and 825 cm^{-1}.

UV: λ(CH_3OH) 217 and 277 nm.

11. Luteolin 7-O-glucoside: Yellow crystals (from methanol) 15 mg, m.p. 238-240º

Identified through comparison with authentic sample (m.p., chromatography, UV).

12. Isovitexin: Yellow needles (from methanol), 20 mg, m.p. 246-248º, (UV in Table 2).

13. Isoorientin: Yellow powder (from methanol), 25 mg, m.p. 235 - 237º, (UV in Table 2).

14. Vitexin: Yellow crystals (from methanol), 15 mg. Identified through comparison with authentic sample (m.p., chromatography, UV).

15. Orientin: Dark yellow powder (from methanol), m.p. 260-262º.

Substances 5-7 and 11-15 gave positive Molish`s test.

Acid hydrolysis of substances 5-7 and 11-15

10 mg of each substance was separately refluxed with 10 ml 5% HCl in ethanol for three hours. The resultant water insoluble hydrolysate was extracted by ether, crystallized from methanol and identified as chrysophanol, physcion, emodin and luteolin respectively (m.p., Co- chromatography and UV). The water -soluble hydrolysates were separately neutralized with Ag_2O and filtered. The filtrate of each was evaporated to dryness, the residue of each was dissolved in 0.5 ml pyridine and subjected to TLC (Cellulose precoated plates Merck), EtOAc-pyridine- water 2:1:2 developer and aniline hydrogen phthalate spray reagent). Glucose was identified as the suger moity of substances 5-7 and 11. Substances 12-15 failed to produce aglycones or sugars even after prolonged treatment with acid indicative to their non-hydrolysable C-glycosidic natures.

Ferric chloride oxidation of substances 12-15

About 7 mg of the flavonoids 12-15 were separately mixed with 0.2 g $FeCl_3$ in 0.8 ml water and refluxed for 6 hours. The resultant sugar moiety was identified by TLC on Cellulose precoated plates (Merck) using EtOAc-pyridine-n-butanol-acetic acid-water (25:20:20:50:10) solvent system (two runs) and aniline hydrogen phthalate spray reagent. The chromatograms were visualized by heating at 110° for 5 min. Glucose was detected in each case (Rf. 0.46) identifying C-glycosidic nature of substances 12-15.

Anti-microbial activity

All prepared extracts (Table 3) were investigated for their antibacterial and antifungal activities against representative Gram positive, Gram negative and some fungal strains. The media were sterilized by autoclaving at 120° for 15 min.

Microorganisms

The bacterial and fungal strains used in the investigation were *staphyllococcus aureus* (ATCC 29737), *staphyllococcus epidermidis* (ATCC 12228), *Bacillu subtilis* (ATCC 10031), Escherchia coli (ATCC 10536), *K. pneumonia* (ATCC 10031), *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans* and *Fusarium solani* (clinical isolates).

Antibacterial Assay

A modified disc diffusion method[17,18] was used to determine antibacterial activity. Sterile nutrient agar was inoculated with 0.1ml of diluted organism. Paper discs were impregnated with plant extracts (4mg/disc) using ethanol 80%v/v as negative control and ampicillin (10 µg/disc) as positive control. The discs are dried and placed on the seeded plate, which were kept for 2 hr in refrigerator to enable prediffusion of the substances into the agar and then incubated for 24 hr at 37°. The diameter of inhibition zones around each well or paper discs were measured and recorded at the end of the incubation period. The average zone of inhibition was calculated for three replicates (Table 3).

Anti-fungal assay

A modified agar-well diffusion method[19] was used for determination the antifungal activity. Nutrient agar (sabouraud dextrose agar, OXOID Ltd, Basingstoke, Hants, England) was used as culture medium. It was inoculated with the fungal strains, wells 4 mm in diameter were punched into the agar and injected with plant extracts (4mg /well). Solvent blanks and standard solutions of antibiotic standards (0.8 mg/well) (ketoconazol, clotrimazol and Nystatin). The plates were incubated at 37° and observed after 48 h. The diameter of inhibition zones around each well was measured and recorded at the end of the incubation period (Table 3).

RESULTS AND DISCUSSIONS

The alcoholic extracts of the different *Rumex* species afforded after extensive fractionation and chromatography fifteen compounds. Compounds 1-7 gave red color with KOH spray reagent indicating their anthraquinone natures. The UV spectra revealed the presence of absorption bands characteristic of α-hydroxyanthraquinone. This was confirmed by the IR which showed free C=O and chelated C=O groups. Furthermore, comparison with authentic samples (m.p., MS and Co-chromatography with authentics) confirmed the identity with Chrysophanol 1, physion 2, emodin 3, aloe-emodin 4, chrysophanin 5, emodin 8-O-glucoside 6 and physcion-8-O-glucoside 7 respectively. Compounds 8, 9 and 11-15 gave yellow color with KOH and $AlCl_3$ spray reagents indicating their flavonoidal nature. The UV spectra revealed absorption bands characteristic for flavonol 8 and flavone skeletons 9, 11-15. Compounds 11-15 gave positive Molish's test assigned to their glycosidic nature. Acid hydrolysis afforded glucose for compound 11 (co-chromatography) and failed to produce aglycones or sugar even after prolonged treatment (compounds 11-15) indicative to their

stability and non-hydrolysable C-glycosidic nature.[20,21] Application of $FeCl_3$ oxidation degradative procedure [Markham et al 1982, Mabry et al 1970]for flavonoids, 12-15 afforded glucose in each case indicating the presence of glucose in the form of C-glycosidic linkage.[29,21] Careful examination of UV spectra with different shift reagents for compounds 8-9 and 11-15 and comparing the data with those obtained from authentic flavonoids confirmed the identity with Kamferol 8, luteolin 9, luteolin -7-O-glucoside 11, isovetexin 12, isoorientin 13, vitexin 14 and orientin 15 respectively. Compound 10 gave brown color with KOH spray reagent. Direct comparison (TLC, m.p. and UV) with authentic flavonoids confirmed the identity with catechin, a common flavan in the polygonaceae.[22] The antibacterial and anti-fungal activities (Table 4) revealed that all different extracts of *Rumex* species have no antifungal activities against five fungal strains. Ether extract of *R. obustifolia* roots has the most potent antibacterial activity against *S. aureus* (Table 4). Ethyl acetate extract of *R. Obstufolia* roots have the most potent antibacterial activity against *B. subtilis* (inhibition zone = 10 mm). All *Rumex* species extracts have no antibacterial activities against *E. coli* and *K. pneumonia*. in contrast, Harshaw et al reported the antibacterial activity of methanolic extract of *R. obustifolia* against against *B. cereus, B. subtilis, E. coli, S. aureus* and *Salmonella tryphi*.

Table 1: Folk uses and traditional names of *Rumex* species in Yemen.

Plant species	Local name	Location	Traditional uses and pharmacological activities	Height above sea level
R. obtusifolia L.	Hammed	Kawkaban	Mild laxative, anti-inflammatory and antiviral[11, 12]	2400-3000 m [wood
R. steudelii Hochst. Ex A. Rich	Tibil	Dhamar	Anti-diarrheal, mild laxative[13,14]	1800-3200 m [wood]

Table 2: UV spectral data (nm) of the isolated flavonoids 8,9 and 12, 13, 15.

No.	Compound	MeOH	$NaOCH_3$	$AlCl_3$	$AlCl_3$/HCl	NaoAc	NaOAc/ boric acid
8	Kampferol	250, 266, 365	245, 266, 334sh, 420(dec)	260sh, 266, 301sh, 253, 420	245sh, 260sh, 266, 350, 420	252, 324, 385, 420sh	264sh, 225sh, 370
9	Luteolin	268, 293, 349	268, 402*	273, 332, 415	295, 356, 383, 275	275, 300, 368	269, 370
12	Isovitexin	270, 333	280, 325, 400*	269sh, 274, 300, 345, 385	269sh, 277, 300, 345, 384	275, 300, 381	267, 300sh, 344
13	Isoorientin	258, 269, 247	270sh, 277, 332sh, 406*	276, 301sh, 339, 422	269sh, 277, 296sh, 346, 382	274, 345sh, 393	265, 373, 428sh
15	Orientin	253, 267, 346	267, 275, 331sh, 401*	275, 301sh, 326, 423	266sh, 275, 295sh, 356, 382	269, 275, 329, 390	264, 302sh, 375, 434sh

sh: shoulder

dec: decomposition

*: increase in intensity

Table 3: Antibacterial and antifungal activities of different extracts of two Yemeni *Rumex* species.

Species	Extract	S. a	S. e	B. s	E. c	K. p	A. f	A. n	C. a	F. s
R. obtusifolia (leaves)	Ethanol.	5	nd	6	nd	nd	nd	nd	nd	nd
	Pet. ether	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Ether	nd	nd	nd	nd	nd	nd	nd	nd	nd
	EtoAc	nd	nd	nd	nd	nd	nd	nd	nd	nd
R. obtusifolia (roots)	Ethanol	7	6	7	nd	nd	nd	nd	nd	nd
	Pet. ether	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Ether	10	nd	9	nd	nd	nd	nd	nd	nd
	EtoAc	9	nd	10	nd	nd	nd	nd	nd	nd
R. steudelii (leaves)	Ethanol.	4	nd	5	nd	nd	nd	nd	nd	nd
	Pet. ether	4	nd	nd	nd	nd	nd	nd	nd	nd
	Ether	nd	nd	nd	nd	nd	nd	nd	nd	nd
	EtoAc	5	nd	7	nd	nd	nd	nd	nd	nd
R. steudelii (roots)	Ethanol	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Pet. ether	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Ether	3	nd	nd	nd	nd	nd	nd	nd	nd
	EtoAc	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ampicillin		30	nd	23	15	nd				
Gentamycin		12	11	nd	11	11				
Clotrimazole							30	26	30	32
Nystatin							32	29	30	30

Ethanol did not show any inhibitory activity. nd = non detectable

S. aureus (S. a): ATCC 29737; *S. epidermidis* (S. e): ATCC 12228; *B. subtilis* (B. s) ATCC 6633; *E. coli* (E.. c) ATTC 10536; K. pneumonia (K. p): ATTC 10031; A. flavus (A. f); A. niger (A. n); C. albicans (C. a) and F. solani (F. s).

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