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Antibacterial activity, cytotoxicity, antioxidant capacity and phytochemicals of *Rheum australe* rhizomes of Nepal

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ABSTRACT

Rhizome of *Rheum australe* D. Don of Nepalese origin was evaluated for its antibacterial, cytotoxic and antioxidant efficacies. The plant extracts significantly inhibited the growth of Gram-positive and Gram-negative bacteria tested. Cytotoxicity of the methanolic and aqueous extracts was tested on the brine-shrimp nauplii and the LC_{50} value was determined to be 194.98 and 18.28 µg/ml, respectively. The aqueous extract contained a higher amount of total phenolics than the methanolic extract and the result was in correlation with their Fe (III) reducing power ability as well. However, the methanolic extract contained more potent DPPH free radical scavengers than the aqueous extract. Two anthraquinones namely chrysophanol and emodin were isolated from the hexane extract.

Keywords: Brine-shrimp, Chrysophanol, Emodin, Ferric reducing antioxidant power, Free radical scavenging activity, Total phenolics.

1. Introduction

Rhubarb constitutes different species of *Rheum*, which considered to be mainly distributed in mountainous regions of the Qinghai-Tibetan Plateau area and Asian interior over 7 million years ago [1]. About 60 extant species of *Rheum* are reported, among which seven species *viz. R. australe* (synonym *R. emodi*), *R. nobile*, *R. acuminatum*, *R. moorcroftianum*, *R. delavayi* (synonym *R. nepalense*), *R. speciforme* and *R. webbianum* are naturally grown in Nepal [2, 3]. The rhizomes of rhubarb mainly constitute anthraquinones that can act as a stimulant and laxative, while the tannins present in rhubarb may act as astringent [4]. Traditionally in Nepal, the rhizomes of several rhubarbs are used in gastritis, stomachache, skin disease, sore throat and body ache; as laxative, antihelmintic, and to treat infection, gastric ulcer, liver disease, gout, dislocated bones and pregnancy-induced hypertension etc. [5]. The rootstocks are also used for colouring wools and to make pickle.

Rheum australe D. Don (family Polygonaceae) was first described by Don in Prodromus Florae Nepalensis in 1825 and the synonym *Rheum emodi* Wall. Ex Meisn. was given by Meissner in 1832 as a misnomer [2]. *Rheum australe* (local name "Padamchal") is a perennial, robust herb with stout rhizomes, endemic to the alpine and sub-alpine zones of the Himalayas distributed at 3200-4200 m [6].

Despite a significant researches has been done in Rhubarb (data searching with Scifinder using phrase *Rheum* hits 8686 research articles, while 716 papers have described species *R. emodi* alone, 11 July 2012), *R. australe* of Nepalese origin is rarely explored. Krenn *et al.* have reported isolation of anthraquinones (chrysophanol, physcion, emodin etc.) and phenolics (carpusin, maesopsin and epicatechin) from the rhizome of the plant material collected from Gorkha, Nepal [7]. Rokaya *et al.* have determined concentration of anthraquinones (chrysophanol, emodin and physcion) and stilbenes (piceatannol and resveratrol) using High Performance Liquid Chromatography (HPLC) in the rhizome collected from Gosaikunda, Nepal [8]. More recently, Kumar and Spandana also reported quantitative determination of anthraquinones by HPLC in the plant material obtained from Gyan herbal products Nepal [9]. In a recent review, Rokaya *et al.* have detailed the ethnobotany, phytochemistry and pharmacology perspectives of *R. australe* [6].

2. Materials and Methods

2.1 Materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu's Phenol reagents were purchased from Sigma-Aldrich. 2,4,6-Tripyridyl-*s*-triazine was purchased from Fluka Chemie. Brine-shrimp (*Artemia salina*) egg was procured from San Francisco Bay Brand Inc., USA. Analytical thin layer chromatography (TLC) was performed on 0.2 mm pre-coated plate Kieselgel 60 F_{254}. Spectrophotometry was carried out using 6715 UV/Vis Spectrophotometer JENWAY. EI-MS was recorded on JEOL-MS. ^{1}H NMR was recorded on Avance AV-500.

The rhizomes of *Rheum australe* D. Don were collected at 3200-3800 m above sea level from the Manaslu Conservation Area, Nepal in August 2011. The plant material was authenticated at the National Herbarium Laboratory, Godawari, Lalitpur, Nepal with Voucher Specimen No. 20116.

2.2 Extraction of the plant material

The rhizomes were chopped, air dried in the shade at room temperature and coarsely ground. The plant material (150 g) was successively extracted with hexane (1000 ml, 7 h), ethyl acetate (1000 ml, 14 h), methanol (1000 ml, 22 h) and finally with distilled water (1000 ml, 26 h) using a Soxhlet extractor. These plant extracts were concentrated using a rotary evaporator under reduced pressure to obtain residues.

2.3 Antibacterial susceptibility assay

Bacillus subtilis, *Staphylococcus aureus* (ATCC 25923), *Enterobacter cloacae*, *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATTCC 27853), *Salmonella typhimurium* (ATCC 14038) and *Shiegella flexneri* were used as bacterial strains for the Agar well diffusion assay [10]. The extracts displaying antibacterial activity were further used to evaluate the Minimum Bactericidal Concentration (MBC) by using the Two-fold micro dilution broth methodology [11].

2.4 Brine-shrimp bioassay

The methanolic and aqueous extracts were used to evaluate cytotoxicity using the Brine-shrimp bioassay [12]. Briefly, sample solutions were prepared by dissolving 200 mg of each plant extract in methanol up to the mark in a 10 ml volumetric flasks. To calculated volume of the sample solution for 10, 100 and 1,000 µg/ml dose levels in five replicates was introduced freshly hatched ten brine-shrimp nauplii in artificial sea water (total volume 5 ml). After 24 h, the percentage death was determined and the LC_{50} (Lethal concentration for 50% mortality) value with 95% CI (Confidence Interval) were computed.

2.5 Antioxidant assays

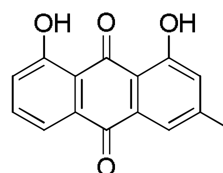
The methanolic and aqueous extracts were further used to calculate the total phenolic content by Folin-Ciocalteu (FC) assay. The evaluation of antioxidant capacity of these extracts was carried out by using the ferric reducing antioxidant power (FRAP) and free radical scavenging DPPH assays. We have detailed the experimental procedures before [13].

2.6 Isolation of anthraquinones from the hexane extract

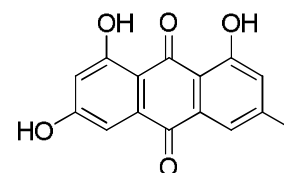
A remaining portion of the hexane extract residue was subjected to repeated column chromatography (silica gel 120-160 mesh) using the mixtures of hexane and ethyl acetate with increasing polarity. Two anthraquinones namely chrysophanol **(1)** and emodin **(2)** were isolated. These compounds were authenticated by comparing the analytical data.

Chrysophanol (1): Yellow solid; m. p. 198 ^{0}C; TLC R_f = 0.88 (silica gel, hexane: EtOAc, 9:1); UV-Vis λ_{max} (MeOH) nm: 228, 257, 279, 286, 434; EI-MS *m/z* (%): 254.0 (M^+, 100), 237.0 (11.3), 226.1 (36.7), 197.1 (22.8), 152.0 (16.5), 127.0 (6) (calcd. for $C_{15}H_{10}O_4$ 254.0579); ^{1}H NMR ($CDCl_3$, 500 MHz) δ ppm: 2.45 (s, 3H), 7.09 (d, *J* = 0.5 Hz, 1H), 7.28 (dd, *J* = 1.0, 8.5 Hz, 1H), 7.67-7.64 (m, 2H), 7.81 (dd, *J* = 1.0, 7.5 Hz, 1H), 12.00 (s, 1H), 12.11 (s, 1H) [14].

Emodin (2): Orange solid; m. p. 254 ^{0}C; TLC R_f = 0.80 (silica gel, hexane: EtOAc, 9:1); UV-Vis λ_{max} (MeOH) nm: 230, 251, 291, 438; EI-MS *m/z* (%): 269.9 (M^+, 100), 253.0 (9), 242.0 (27), 213.0 (19), 185.0 (9), 139.0 (11) (calcd. for $C_{15}H_{10}O_5$ 270.0528); ^{1}H NMR ($CDCl_3$, 500 MHz) δ ppm: 2.44 (s, 3H), 6.65 (d, *J* = 2.5 Hz, 1H), 7.08 (d, *J* = 1.9 Hz, 1H), 7.27 (d, *J* = 2.5 Hz, 1H), 7.61 (d, *J* = 1.9 Hz, 1H), 12.07 (s, 1H), 12.26 (s, 1H) [14].



Chrysophanol (**1**)



Emodin (**2**)

3. Results and Discussion

Upon a successive Soxhlet extraction of the rhizomes of *R. australe* (150 g) yielded the hexane extract (2.27 g, 1.51%, pale yellow), ethyl acetate extract (4.04 g, 2.69%, yellowish brown), methanolic extract (18.96 g, 12.64%, reddish brown) and aqueous extract (40.92 g, 27.28%, dark brown).

The result of antibacterial susceptibility assay is depicted in Table 1. After 24 h incubation, the methanolic extract of the plant material effectively inhibited the growth of both Gram positive and Gram negative bacteria with Zone of inhibition (ZOI) range of 19-23 mm. Ethyl acetate extract was also found effective against all bacteria tested except *E. cloacae.* Aqueous extract displayed antibacterial activity too, except for *E. coli* and *S. typhimurium* whereas the hexane extract was found effective only against *E. cloacae* and *E. coli*. Next, MBC values were evaluated and the results are tabulated in Table 2. The methanolic extract was found to possess highly antibiotic effect with MBC values of 0.78-1.56 mg/ml.

To evaluate cytotoxicity thereby the anticarcinogenic property of the plant material, the Brine-shrimp bioassay of the methanolic and aqueous extracts was carried out at concentrations of 10, 100 and 1,000 µg/ml. Table 3 shows the results of the assay after 24 h exposure of the samples to freshly hatched brine-shrimp nauplii. It can clearly be seen from the table that both the methanolic and aqueous extracts are cytotoxic (LC_{50} value <1,000 is considered as cytotoxic), therefore bear anticarcinogenic property. Comparably, the aqueous extract displayed 100% mortality at a concentration of 1,000 µg/ml with LC_{50} value 18.28 µg/ml, hence was found to be highly potentiality for cancer chemoprevention, when consumed.

We have determined the total phenolic content in the methanolic and aqueous extracts using FC assay. Figure 1 shows a plot of the observed absorbance values of the reference gallic acid and the extracts at 760 nm at different concentrations. The slope calculated for the reference gallic acid, methanolic and aqueous extracts are 0.0097, 0.0049 and 0.0116, respectively. Higher the slope, higher is the antioxidant property. Therefore, aqueous extract displayed potential antioxidant property. The total phenolic content in methanolic and aqueous extracts was calculated to be 6.85 and

14.51 g gallic acid equivalent/100 g of dried plant material. The result indicated that the aqueous extract is highly enriched with polyphenolic constituents.

The reducing capacity of the plant extract in the FRAP assay can be expressed as the slope value of a linear curve in which the concentration of extract added results in a corresponding increase in absorbance. The slope values 0.0193 and 0.0475 were obtained from the methanolic and aqueous extracts, respectively. Taking the ferrous sulphate as the reference of the calibration curve, the FRAP values calculated for the methanolic and aqueous extracts were 217.16 and 1709.01 mM/l Fe(II)/100 g of dried plant material. The results showed good correlation between the phenolic content and FRAP value in accordance with the structure-antioxidant relationship (SAR) principle, which express that a higher phenolic content increases the reducing capacity. In contrary to our results, Rajkumar *et al.* have reported that the methanolic extract of *R. emodi* rhizome, collected from Uttaranchal, India, showed higher ferric reducing power in comparison to the aqueous extract [15]. In their case, they have first extracted the plant material with methanol followed by water, but in our case, we began the extraction with less polar solvents.

In contrast to the FC and FRAP assays, the methanolic extract (IC_{50} = 16.63 µg/ml) was found to possess a high free radical scavenging activity in comparison to the aqueous extract (IC_{50} = 28.99 µg/ml) using the DPPH assay. A similar result was recently reported by Singh *et al.* from the plant extracts of *R. emodi* rhizomes of Indian origin [16]. We attributed that the effect was not only due to the phenolic content, but due to the presence of potent free radical scavengers in the methanolic extract.

Finally, we have isolated two known anthraquinones (chrysophanol and emodin) from the hexane extract.

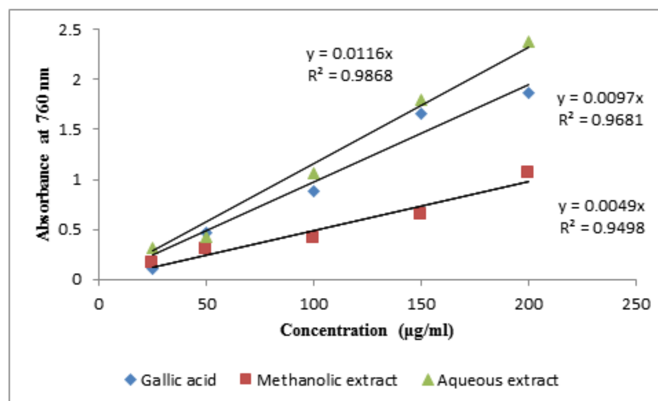


Fig 1: Concentration-response curve in the FC assay showing linear variance

Table 1: Antibacterial susceptibility assay of *R. australe* extracts

S. N.	Pathogenic bacteria used	ZOI shown by different extracts[a] (in mm diameter)				
		HE	EE	ME	AE	Gentamycin
1	*B. subtilis*	–	14	19	10	33
2	*S. aureus*	–	20	23	11	30
3	*E. cloacae*	13	–	21	10	23
4	*E. coli*	11	16	21	–	23
5	*K. pneumoniae*	–	20	21	11	30
6	*P. aeruginosa*	–	16	23	11	32
7	*S. typhimurium*	–	15	20	–	30
8	*S. flexneri*	–	14	21	15	32

[a]Values of the ZOI (in mm) include the diameter of well (6 mm) after 24 h incubation against different bacterial species in the Agar well diffusion assay. (–)-Sign indicates no significant zone of inhibition was observed. HE = Hexane extract, EE = ethyl acetate extract, ME = methanolic extract, and AE = Aqueous extract.

Table 2: MBC values of *R. australe* extracts

S. N.	Pathogenic bacteria used	MBC values for different extracts (mg/ml)			
		HE	EE	ME	AE
1	*B. subtilis*	ND	6.25	1.5625	6.25
2	*S. aureus*	ND	1.56	0.78	6.25
3	*E. cloacae*	6.25	ND	0.78	6.25
4	*E. coli*	6.25	3.125	0.78	ND
5	*K. pneumoniae*	ND	1.56	1.56	6.25
6	*P. aeruginosa*	ND	3.125	1.56	6.25
7	*S. typhimurium*	ND	6.25	1.56	ND
8	*S. flexneri*	ND	6.25	1.56	6.25

HE = Hexane extract, EE = ethyl acetate extract, ME = methanolic extract, and AE = Aqueous extract. ND = Not determined.

Table 3: Brine-shrimp bioassay of *R. australe* extracts

Plant extracts	Percentage death at 24 h/dose			LC_{50} (µg/ml)	95% CI (µg/ml)
	10 (µg/ml)	100 (µg/ml)	1,000 (µg/ml)		
Methanolic extract	12	28	80	194.98	194.98 ± 2.26
Aqueous extract	44	68	100	18.28	18.28 ± 2.26

4. Conclusion

In conclusion, the rhizomes of *R. australe* displayed antimicrobial as well as anticarcinogenic properties. The methanolic and aqueous extracts of the plant material were found cytotoxic against brine-shrimp nauplii hence are potential for cancer treatment. The total phenolic content was found higher in the aqueous extract. Comparably, the aqueous extract displayed a high reducing power to reduce Fe(III) to Fe(II) ions, while the methanolic extract bore potent free radical scavenging constituents. Chrysophanol and emodin were isolated.

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