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Phytochemical Analysis of *Danae Racemosa* L. Moench Leaves

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ABSTRACT

Background: *Danae racemosa* (L.) Moench (*Ruscus racemosa* L., Asparagaceae) is an erect, muchbranched evergreen shrub, native in the mountains from Syria to Iran, commonly used for its decorative green foliage in fresh flower arrangements. Despite its importance, far too little attention has been paid to gather detailed documentations of chemical constituent present in extract of *D. racemosa*. ***Methods:*** In this study, leaves of *D. racemosa* were collected from north of Iran, next, extracted via maceration followed by partitioning through solvents of different polarity. The qualitative–quantitative chemical composition of the extract was determined using high-performance liquid chromatography. ***Results:*** Two major flavonoids, quercetin and kaempferol, were separated whilst their identities were confirmed by UV-visible, shift reagents and ^{1}H-NMR spectroscopy.

Introduction

In a time of increased considerations to natural products as potential safe remedies, it is as important as ever to gather detailed documentations of the latter in different aspects. Accordingly, natural resources, particularly higher plant species, continue to be important sources of natural medicines which represent a challenge to science due to various aspects, including their chemical constituents.

Danae racemosa (L.) Moench (Syn.; *Ruscus racemosus* L.) ("Hamishak" in Persian and "Alexandrian Laurel" in English) belongs to the family Asparagaceae.[1] The family Asparagaceae is in the major group Angiosperms (Flowering plants) that is placed in the order Asparagales of the monocots. The most complete revision of the family was Baker's classification (1875), who divided the natural order Liliaceae into three suborders; Liliaceae proper, Colchicaceae and Asparagaceae, based on the fruit and stamen characteristics.[2] The Asparagaceae was characterized by baccate fruit and introsed stamens and then subdivided globally into 143 genera and over 3500 species.[3] *D. racemosa* member of family Asparagaceae is native in the mountains from Syria to Iran and Transcaucasia.[4] The plant naturally grows in higher lands mainly in the north parts of Iran including Guilan, Mazandaran and Golestan provinces that are located in the south of the Caspian Sea and north of the Elburz mountains on latitude of N35°50', 38°30' and longitude E48°30', 56°10' and ranges of 700-1600 mm precipitation.[5] *D. racemosa,* an erect, much branched evergreen shrub growing under the shade of forest trees, with green shoots and glossy leaves along with thick unarmed alternate cladophylla and terminal racemese of white-yellow flowers followed by red berries is frequently used for its decorative green foliage in fresh flower arrangements.[4] Although there have been a great number of studies referring the beneficial of plants containing biologically active chemical constituents, far too little attention has been paid to gather detailed documentations of chemical constituents present in extract of *D. racemosa*. Hence, the present study was conducted to detect some biologically active chemical constituents, naturally occurring in extract of the plant. This survey is an ongoing point in our study on the plants of Iranian flora. Regarding our previously published papers on the elicited systemic dose-related antinociceptive properties of *D. racemosa* extracts on both chemical and thermal nociceptive rat models[6] and spermatogenesis in rats,[7] herein we report on the extraction, isolation and structural elucidation of two flavonoids from the leaves of *D. racemosa*.

Materials and Methods

General experimental procedures

All solvents in the present work were purchased from Merck (Germany). HPLC separation was performed in a Shimadzu prep-HPLC System coupled with UV detector. A Shim-Pak CLC – RP18 preparative high performance column chromatography (HPLC) column (10 µm, i.d.:

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250 mm × 20 mm; mobile phase: 0-80 min, acetonitrile - distilled water (40-60) as the eluent; flow-rate: 6 ml/min, injection volume: 0.5 ml and detection at 254 nm was used in the chromatography. UV-visible spectrum of each compound was determined using a Shimadzu UV-2100A spectrometer in methanol and after addition of different shift reagents such as NaOMe, $AlCl_3$, $ALCl_3$/HCl, NaOAc and NaOAc/H_3BO_4 at 190-500 nm. H-NMR spectra were recorded in MeOH-d4 on a Bruker DRX 200 MHz NMR spectrometer.

Plant material

The leaves of *Danae racemosa* (L.) Moench were collected during June–July from Shirgah forests in Mazandaran province in Iran. A voucher specimen of the plant (Tbz-Fph-751) representing this collection has been retained in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Extraction, isolation and structure elucidation of flavonoids

The dried aerial parts (200g) were grounded and defatted by treatment with n-hexan (1L). The defatted powdered aerial parts were extracted with 70% methanol in water (2L) by maceration at room temperature. The methanol of resultant hydroalcoholic extract was evaporated at 40 ◦C under reduced pressure, affording the aqueous residue. Subsequently, the aqueous residue was fractionated with EtOAc and n-butanol respectively. According to the TLC analysis of the fractions on analytical silica gel GF_{254} plate using a mixture of n-hexane-EtOAc-MeOH (6:3:1) as eluent and 5% $AlCl_3$ reagent for detection, the EtOAc fraction was determined to be rich in flavonoids. As a consequence, the EtOAc fraction was subjected to preparative HPLC providing several fractions. On separation, two fractions with retention times of 36.46 and 52.11 minutes each containing one major flavonoid (Figure 1), were dried and dissolved in small volume of methanol and kept in refrigerator at +2°C. The formed crystals were separated by filteration and dried to provide compound 1 and compound 2. The structures of compound 1 (45mg) and 2 (32mg) were elucidated by interpretation and comparison of their spectral data (UV and ^{1}H-NMR) with those published references.[8,10]

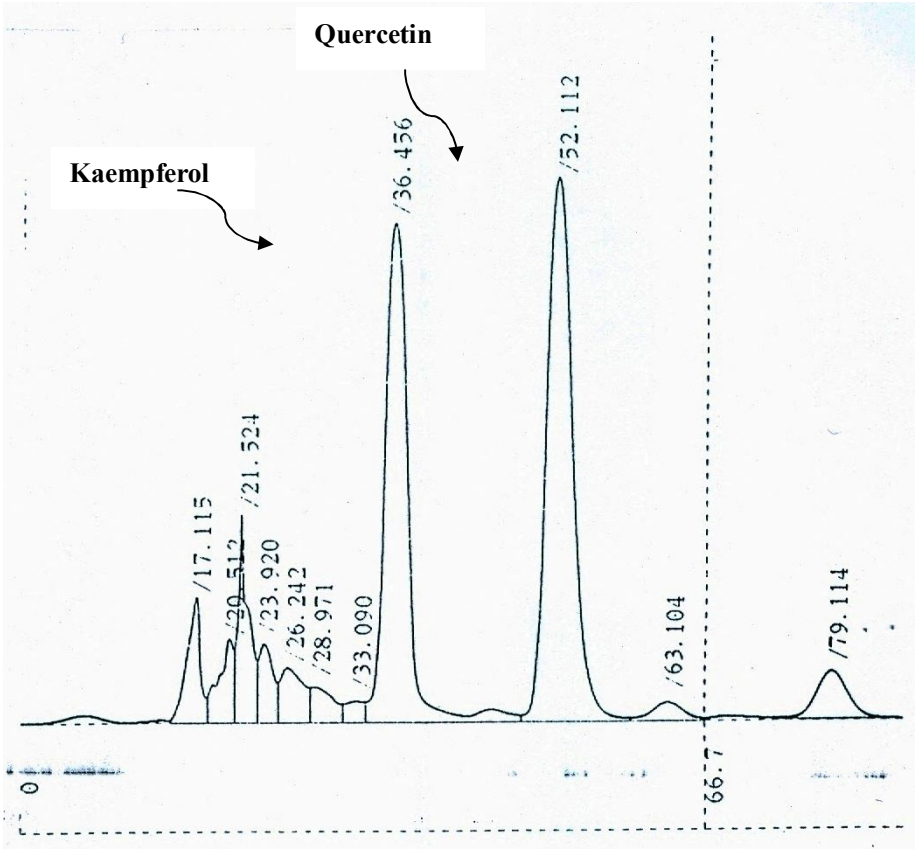


Figure 1. HPLC chromatogram of the flavonoids in EtOAc fraction of *D. racemosa*

Results and Discussion

Two major flavonoids were detected in EtOAc fraction of the leaves of *D. racemosa,* where the structures were deduced by UV-visible and ^{1}H-NMR spectral data analyses. The spectroscopic data of the separated flavonoids are outlined in tables 1 and 2. The UV spectrum of methanolic solution of compound 1 which was separated as yellow crystals exhibited two major absorption bands at 371 nm (band-I, cinnamoyl system) and 255 nm (band-II, benzoyl system) (Table 1), which confirmed the flavonol structure.

Table 1. UV-visible absorption peaks of the compounds 1 and 2 in methanol and with various shift reagents.

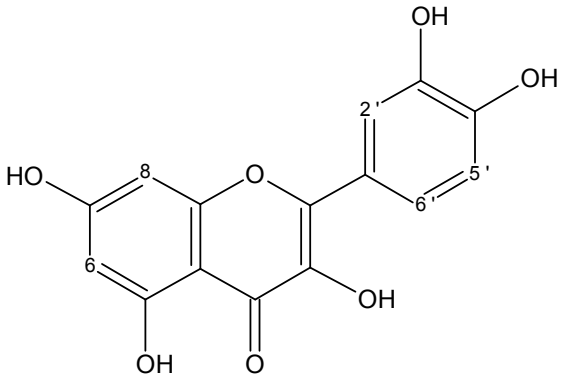
Compound	MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3
1	370 298 (sh) 254	419 (sh) 328	454 364 (sh) 313 (sh) 271	424 358 (sh) 300 (sh) 264	393 325 (sh) 273	386 259
2	363 322 (sh) 295 (sh) 264	426 317 (sh) 280	422 349 (sh) 300 (sh) 267	422 349 (sh) 300 (sh) 267	390 274	370 264

Table 2. ^{1}H-NMR (300 MHz) data of the compounds 1 and 2 in CD_3OD.

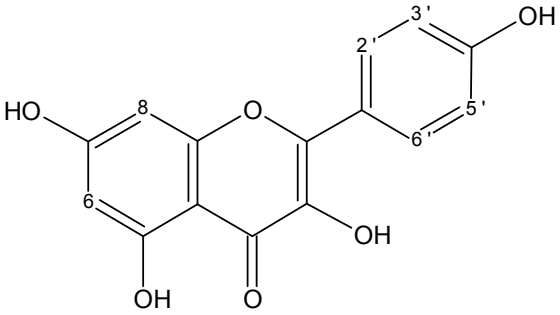
Position	Chemical shift (δ_H) in ppm 1 (Quercetin)	Chemical shift (δ_H) in ppm 2 (Kaempferol)
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	6.16 (d, J = 2.1 Hz)	6.17 (d, J = 2.1 Hz)
7	-	-
8	6.37 (d, J = 2.1 Hz)	6.38 (d, J = 2.1 Hz)
1'	-	-
2'	8.07 (d, J = 2.1 Hz)	8.01 (d, J = 9.0 Hz)
3'	-	6.90 (d, J = 9.0 Hz)
4'	-	-
5'	6.90 (d, J = 8.4 Hz)	6.90 (d, J = 9.0 Hz)
6'	7.62, 7.60 (dd, J = 8.4 , 2.1 Hz)	8.01 (d, J = 9.0 Hz)

The structure of each flavonol compound was verified according to UV absorbance spectra and known shift reagents. In the case of $AlCl_3$ and $AlCl_3$/HCl reagents, aluminum chloride forms a complex with the hydroxyl groups at C3- or (and) C-5 and the ketone group at C-4 of the flavonol structure and also forms an acid-labile complex with the catechol functional group in a compound like quercetin. The flavonol-aluminum complex presents a bathochromic shift corresponding to form a number of complexes. On addition of acid, the acid-labile catechol complex will dissociate bringing about a hypsochromic shift compared to the aluminum chloride spectrum. Furthuremore, NaOAc ionizes only the flavonols with a free 7-hydroxyl exhibiting a Band II shift (5–20 nm).

Besides, decomposition would occur only if alkali-sensitive hydroxyl patterns are present (e.g., 3-, 3′-, 4′-). Thus, degeneration of the band I in the presence of NaOMe, indicates free hydroxyl groups at C-3 and C-4'.[11] Herein, hypsochromic shifts with $AlCl_3$/HCl and bathochromic shift in bond-I (16 nm) with NaOAc/H_3BO_3 supported the presence of 3, 3′, 4′ trihydroxy system in the compound. Bathochromic shifts with NaOAc were related to 7-hydroxyl and the bathochromic shift with $AlCl_3$/HCl to 5-hydroxyl. Regarding the ^{1}H-NMR spectrum (Table 2), the data indicated a 5,7-dihydroxylated pattern for ring A (two meta-coupled doublets at δ_H 6.16 and 6.37 ppm, (J = 2.1 Hz) and a 3′,4′-dihydroxylation pattern for ring B: ABX system signals at δ_H 6.90 ppm (1H, d) with J = 8.4 Hz attributed to hydrogen atom H-5' and δ_H 7.62 and 7.60 ppm, (1H, dd) with J = 8.4, 2.1 Hz for H-6' followed by 8.08 ppm, (1H, d) with J = 2.1 Hz for H-2', allowing the compound to be recognized as quercetin which is in consistence with the previously published data for this compound (Figure 2) .[12-16]



Quercetin



Kaempferol

Figure 2. Chemical structures of the flavonoids in EtOAc fraction of *D. racemosa*.

In the case of compound 2**,** UV absorption spectrum in methanol exhibited two absorption maximums at 363 nm (band-I, cinnamoyl system) and and 264 nm (band-II, benzoyl system) indicating a flavonol compound. Furthermore, shifts obtained with diagnostic reagents: NaOMe, 280, 317 (sh), 426; $AlCl_3$: 267, 300 (sh), 349 (sh), 422; $AlCl_3$/HCl: 267, 300 (sh), 349 (sh), 422; NaOAc: 274, 390; and NaOAc/H_3BO_3: 264, 370 indicated the presence of some free hydroxyls. Considering the UV spectrum in methanol and its changes after the addition of the customary shift reagents in comparison with the reported data, as it has been stated above, it was suggested that the compound 2 might be a flavonol with free hydroxyl groups at positions C-5, 7 and C-3 and 4′. Additionally, ^{1}H-NMR spectrum of compound 2 displayed characteristic signals of the kaempferol nucleus: two doublets at δ_H 6.17 and 6.38 ppm (J = 2.1 Hz), assigned to the H-6 and H-8 protons, respectively and a pair of A2B2 aromatic system protons at δ_H 6.90 and 8.01 ppm (J = 9.0 Hz), assigned to H-3', 5' and H-2', 6', respectively. Consequently, based on the ^{1}H NMR and UV-visible data and also comparison of the data given in the literature, the structure of the compound 2 was identified as kaempferol (Figure 2).[17-20]

The results of quantitative determination by HPLC of quercetin and kaempferol in *D. racemosa* indicated that the quercetin and Kaempferol content of *D. racemosa* leaves were 0.6% and 0.8% (based on dry weight of the leaves), respectively. However, it can be used as an economical source of quercetin and kaempferol in pharmaceutical industry. Formerly in our published paper on assessment of the *D. racemosa* hydroalcoholic extract effect on its antinociceptive properties we determined marked dose-related antinociception in a number of chemical nociception models: formalin-induced hindpaw licking, acetic acid-induced abdominal constriction, hot-plate and rota-rod tests in rat models.[6] We also found that *D. racemosa* hydroalcoholic extract could increase sperm count, viability and motility along with increasing in serum TAC (total antioxidant capacity) and decreasing in serum MDA (malondialdehyde) in rats. These findings confirm the antinociceptive and inducing spermatogenesis activities of *D. racemosa* extract in vitro via antioxidant properties of the isolated flavonoids.[21-23] Likewise, it has been widely accepted that flavonoids encompass quite a lot of health benefits and diets rich in flavonoids alleviates and prevents many serious diseases.[23-30]

In consequence, *D. racemosa* rich in natural potent flavonoids, quercetin and kaempferol, might be an appealing candidate as a valuable source of natural antioxidants to replace with the synthetic antioxidants and neutralizing stress induced free radicals.[31,32]

Acknowledgement

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