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Original Article

Isolation and characterization of antimicrobial compound from the stem-bark of the traditionally used medicinal plant *Adenium obesum*

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ABSTRACT

Background: Medicinal plants constitute a natural reservoir for medicines worldwide. They serve mainstream therapeutics and are central in folklore medicine. In case of *Adenium obesum* (Lav, Apocynaceae), indigenous people of Oman use it for the treatment of venereal diseases, wounds, skin diseases, headaches, muscle pain as well as joint pain; yet, the active ingredients have not been classified. To screened the antioxidant and antimicrobial activities of an identified compound that we isolated from the highest active chloroform extract.

Methods: The antioxidant and antimicrobial activities of the extracts and the isolated compound were determined by diphenyl-1-picrylhydrazyl (DPPH) and disc diffusion methods. To characterize the compound, we used TLC, column, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, IR and MS.

Results: The highest antioxidant activity was found in chloroform extract with EC_{50} value of 28.32 μg/ml followed by water, methanol, butanol, ethyl acetate and hexane extracts, their IC_{50} being 29.95, 39.17, 42.40, 43.20 and 57.00 μg/ml respectively. All crude extracts and pure compound displayed moderate antimicrobial activity against one Gram positive *Staphylococcus aureus* and three Gram negative *Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris* within growth inhibition range of 0 −13 mm. The active metabolite was identified as 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester which is a common plant ingredient known as rosmarinic acid.

Conclusion: The results indicate that walnut chloroform fraction may contain effective compounds with a broad spectrum of curative applications. This is the first report on isolation and characterization of a compound from chloroform crude extract of *A. obesum*.

1. Background

Adenium obesum (Lav, Apocynaceae) is a wild plant found in selected areas of Oman.[1] Plants in this family grow well in rocky and sandy soil.[2] Plants belonging to the genus *Adenium* occur mainly in dry bush land or woodland and wooded grassland up to 2100 m altitude. Now-a-day some species belonging to this family are commercially cultivated in Saharan Africa, Sudan, Kenya, Senegal and Swaziland due to their biological and medicinal importance.[2] Some rare species, including *A. obesum*, are available in the Arabian Peninsula. *A. obesum* is designated long-lived plant by virtue of its growth regulator being very slow. The plant is considered a small tree, as it grows up to four meters in height.[1] Some species of this plant have a fleshy taproot, and a stem swollen at its base reaching up to one meter in diameter. The bark is pale greyish-green, grey, brown, smooth, with sticky, clear or white latex, the branchlets glabrescent, pubescent at the apex. The leaves are arranged spirally, clustered at the end of branchlets.[3,4] The plant shows diversity of flower characteristics depending on environmental conditions such as rainfall, temperature, etc.[2] The shape, size and colour of flowers are completely different from each other where the plant grows.[1] A few species of this family are available in Oman being used by the local ethnic communities as a medicine for the treatment of different diseases.[5] Most of the species belonging to this family, including *A. obesum*, show medicinal values and they exude a milky sap.

A. obesum contains different chemical compounds such as alkaloids, steroids, saponins, glycosides, anthraquinones, tannins and

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flavonoids.[3,6,7] As a medicine, the whole plant is used by different ethnic communities for the treatment of a variety of ailments including venereal diseases. The crude extracts from root and bark are used to prepare a lotion for the treatment of different skin diseases and to eliminate lice.[4] The latex is claimed to be a very good medicine for the treatment of decaying teeth and septic wounds[4] and in Somalia, it is traditionally used as nasal drops.[6] In Kenya, the stems and barks powder is used for eliminating skin parasites in camels and cattle[5] and in India, the bark of *A. obesum* is used as an abortifacient.[5,6] In Nigeria, the whole plant is used as antiplasmodial, anti-trypanosomal and anti-leishmanial drug.[1,2] However, Omani ethnic community use this plant for the treatment of venereal diseases, wounds, skin diseases, headaches, muscle pain and joint pain.[5] Limited information is available regarding the biological activity of this species since there is lack of extensive work on analysis of Omani species of *A. obesum*.

Therefore, in this paper, we describe the isolation and structure elucidation of a pure compound characterized as 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (**1**, rosmarinic acid) from stem-bark of *A. obesum* grown in Oman using different spectral techniques, showing antimicrobial activities using selected microbes. To our best knowledge, this is the first study to isolate and characterize this compound from the chloroform crude extract of stem-bark of *A. obesum*.

2. Methods

2.1. General

Chloroform, ethyl acetate, ethanol, methanol, butanol, DPPH (diphenyl-1-picrylhydrazyl), silica gel GF_{254}, were obtained from Sigma Chemical Company, UK. Silica gel (60–120 mesh), dimethyl sulphoxide (DMSO), potassium bromide (IR grade), deuterated chloroform and amoxicillin were collected from E. Merck, Germany. Evaporation was performed under reduced pressure on a rotary evaporator (Yamato Rotary Evaporator, Model RE 801, Japan). Melting point was determined on an electrochemical micro-melting point apparatus (Gallenkamp). ^{1}H-NMR spectra were recorded on a Bruker (600 MHz) instrument in $CDCl_3$ with TMS as an internal standard (chemical shifts δ, ppm). UV spectra were recorded on HATACHI, U-2000 spectrophotometer Ultrospeck in methanol (λ_{max} in nm). IR spectra were recorded (KBr discs) on a FT-IR spectrometer, validation (υ_{max} in cm^{-1}). Mass spectra (MS) were recorded on Waters Quattro Premier XE Tandem Quadrupole system (Waters Inc. USA) with ESI^+ technique.

2.2. Microorganism

The microorganisms used in this study include *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris* which were collected from Nizwa Hospital, Nizwa, Sultanate of Oman on March 14, 2014.

2.3. Plant materials

The stem-bark samples were collected from Al Mughsayl, Salalah, Sultanate of Oman during the month of November 29, 2013. The plant was identified by Ismail Al-Rashdi, Horticulture Senior Specialist, Ministry of Agriculture, Sultanate of Oman and voucher specimens No. 175 was deposited at Herbarium of this Ministry. The plant species was photographed for documentation and further taxonomic identification at Natural Product Laboratory, School of Pharmacy, University of Nizwa, Sultanate of Oman.

2.4. Processing of samples

The collected samples were washed and dried under sunlight for seven days and further dried under sunlight for seven days more after slicing to achieve complete dryness. The samples were then ground into a coarse powder by a ball mill. The powdered samples were preserved in clean polyethylene bags and kept away from light, heat and moisture until analyzed.

2.5. Extraction

Powdered dry stem-bark samples of *A. obesum* (70 g) were extracted with methanol by using Soxhlet extractor for period of 36 h. The extract was filtered through Buchner funnel with Whatman filter paper No. 1. After complete filtration, the methanol solvent was evaporated under reduced pressure at 24 °C using a rotary evaporator and the extract (9.17 g) was then suspended in water (150 ml). The whole mixture was transferred into a separatory funnel and extracted successively with differently polar solvents to give hexane (2.3 g), chloroform (2.68 g), ethyl acetate (1.51 g), butanol (1.32 g) and water (0.93 g).[5]

2.6. Antioxidant activity

Antioxidant activity in different polarities of stem-bark crude extracts of *A. obesum* was measured by 1,1-diphenyl-2-picrylhydrazil (DPPH) with slightly modified methods of Hossain et al.[8] Approximately (2 mg) of each polarity crude extract of *A. obesum* was placed in a test tube and dissolved in methanol (10 ml). Different concentrations (12.5, 25, 50, 100 and 200 mg/L, respectively) were prepared by the addition of methanol. 0.004% of DPPH was prepared by addition of methanol. 300 μl of each concentration crude extract was taken in a separate test tube to which 3 ml of DPPH solution was added and shaken by hand. All test tubes were incubated in a dark place for one and half hour. The absorbance of all incubated concentrations was measured by UV-visible spectroscopy at wavelength 517 nm. The antioxidant activity of each concentration of crude extracts of *A. obesum* was calculated by using a standard formula.

$$\% \text{ Inhibition} = \text{A control} - \text{A extract/A control}^*100$$

2.7. Antimicrobial activity

Antibacterial activity of different polarities crude extracts and the isolated compound of stem-bark of *A. obesum* were measured against one Gram (+) bacteria *S. aureus* and three Gram (−) bacteria *E. coli, P. aeruginosa* and *P. vulgaris* on nutrient agar plates using disc diffusion method with modification.[5,9] Different concentrations (2, 1, 0.5, and 0.25 mg/ml) of each stem-bark crude extracts of *A. obesum* were prepared by the addition of DMSO solvent and for the pure compound two concentrations (0.5 and 1 μg/ml) were prepared by same solvent. Positive control was also prepared by the addition of DMSO solvent. Whatman filter paper was used as a disc of 6 mm diameter. The discs were impregnated with the prepared concentration of each polarity stem-bark crude extracts and the pure compound of *A. obesum* and then placed on the inoculated agar plates. The ready discs were incubated at 37 °C for 24 h. The diameter of the zones of inhibition against the tested bacteria was measured and compared with broad spectrum antibiotics amoxicillin. Each method in this experiment was replicated three times.

2.8. Isolation of antimicrobial compound from chloroform extract

The chloroform crude extract (2.50 g) was subjected to column chromatography on silica gel eluted with ethyl acetate-hexane

(95:5) solvent system and this was repeated chromatography to give several fractions. Each fraction contained 3 ml and it was examined by TLC. Similar R_f values fractions were combined to give fractions T_1, T_2, T_3, T_4, T_5 and T_6. All collected fractions (T_1–T_6) were kept in a fume hood to dry. Yellowish amorphous solids were obtained from the fraction T_3. The solids were washed with petroleum ether followed by dichloromethane, dissolved in chloroform and checked for purity and the developed TLC plate was then viewed in an iodine chamber. Two different spots were detected. The amorphous solids were further purified by column chromatography over silica gel GF_{254} using ethyl acetate-hexane (99.5:0.5) solvent system. The solvent was evaporated to dryness and kept for 24 h. A whitish crystal was precipitated out at the bottom of the test tube. It was recrystallized from petroleum ether as white needles (1. 62 mg, 0.060% of dried chloroform extract); m.p. 176–177 °C (Kelley et al.[10] 1976; mp 177 °C). ESI-MS (100% methanol); (M^+, 360.89). IR (KBr): 3165, 1707, 1617, 1515, 1348, 1285, 1260, 1231, 1200, 1154, 1113, 1075, 972, 851, 818, 781 cm^{-1}. The ^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC and ^{1}H-^{1}H COSY are presented in Table 3.

2.9. Statistical analysis

The methanol extract and it fractions were assayed for their antioxidant and antimicrobial activities. Each experiment was run in triplicate, and mean values were calculated. A *t*-test was computed for the statistical significance of the results.

3. Results

This is the first report of isolation of the caffeoyl ester from *A. obesum*. The present study was conducted to prepare different polarities crude extracts from the stem-bark of *A. obesum* and to select the highest activity crude extract among them. The highest activity chloroform crude extract was subjected to column chromatography to give one major compound. On the basis of ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, IR and MS the isolated compound was identified as 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester also known as rosmarinic acid.[10,11]

3.1. Antioxidant activity of different polarities stem-bark crude extracts of Adenium obesum

The detection of antioxidant activity of crude extracts of *A. obesum* was determined by using DPPH reported by Hossain et al.[8] Phenols and flavonoids from plants are highly effective free radical scavengers and antioxidants. The highest antioxidant activity was obtained from chloroform crude extract and the lowest was in hexane crude extract among the six tested crude extracts. The order of activity was chloroform > water > methanol > butanol > ethyl acetate > hexane extract.

3.2. Antimicrobial activity

The antimicrobial activity of different polarities crude extracts and isolated pure compounds was determined by disc diffusion method reported by Hossain et al.[5,8,9] modification, and the results were presented in Tables 1 and 2. Most of the crude extracts and isolated pure compound at different concentrations showed moderate antimicrobial activity against all employed bacterial strains were in the range of 0–13 mm.

3.3. Structural elucidation of the antimicrobial compound

The spectral data and physical properties of the isolated compound matched those of 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (**1**), which is a common plant metabolite referred as rosmarinic acid in the literature.[10–12]

Table 1
Antimicrobial activity of different polarities stem-bark crude extracts of *A. obesum*.

Extracts	Concentration (mg/ml)	*E. coli* (mm)	*P. aeruginosa* (mm)	*S. aureus* (mm)	*P. vulgaris* (mm)
Hexane	2	nd	6 ± 0.13	nd	6 ± 0.29
	1	8 ± 0.05	nd	nd	7 ± 0.15
	0.5	7 ± 0.19	nd	nd	7 ± 0.21
	0.25	8 ± 0.10	7 ± 0.18	nd	7 ± 0.11
	Control	30 ± 0.45	nd	21 ± 0.44	nd
Chloroform	2	7 ± 0.09	8 ± 0.11	nd	8 ± 0.30
	1	nd	7 ± 0.23	nd	nd
	0.5	7 ± 0.22	nd	7 ± 0.09	nd
	0.25	nd	7 ± 0.47	nd	7 ± 0.32
	Control	47 ± 0.17	nd	23 ± 0.65	nd
Ethyl acetate	2	7 ± 0.13	nd	nd	nd
	1	nd	nd	nd	nd
	0.5	nd	nd	nd	nd
	0.25	7 ± 0.46	nd	nd	nd
	Control	45 ± 0.12	nd	19 ± 0.23	nd
Butanol	2	7 ± 0.09	7 ± 0.16	nd	nd
	1	7 ± 0.65	7 ± 0.22	nd	nd
	0.5	nd	7 ± 0.29	7 ± 0.24	nd
	0.25	7 ± 0.55	7 ± 0.70	nd	nd
	Control	40 ± 0.12	nd	22 ± 0.17	nd
Methanol	2	8 ± 0.09	nd	nd	nd
	1	nd	nd	nd	nd
	0.5	7 ± 0.12	7 ± 0.09	nd	nd
	0.25	nd	nd	nd	nd
	Control	45 ± 0.22	nd	20 ± 0.72	nd
Water	2	7 ± 0.16	nd	nd	nd
	1	7 ± 0.55	nd	nd	nd
	0.5	7 ± 0.12	nd	nd	nd
	0.25	7 ± 0.41	nd	nd	nd
	Control	42 ± 0.15	nd	22 ± 0.09	nd

nd = not detected.

4. Discussion

Omanis use this plant traditionally for treating venereal diseases, wounds, skin diseases, headaches, muscle pain and joint pain.[5] Here we note that the plant has antioxidant and antimicrobial properties.[6,8] The isolated compound is a well-known natural product extracted from *Perilla frutescens*, *Mentha dumetorum* and other members of Boraginaceae and Lamiaceae families.[13,14] However, there is no evidence in the literature designating compounds with medicinal properties from the chloroform extract of this plant. Therefore, we describe the extraction, isolation and characterization of an active compound from chloroform extract of *A. obesum* selected from Oman, which we screened for their antioxidant and antimicrobial activities. The plant phenols and flavonoids are highly effective free radical scavengers which can protect against damage incurred by free radicals on humans. DPPH was commonly used for the evaluation of free radical-scavenging activity of natural antioxidants.[15–19] It contains a free radical with purple colour. DPPH changes into a stable compound with a yellow colour by reacting with an antioxidant. In such a case, the antioxidant donates an electron to DPPH and change in colour is measured spectrophotometrically at 517 nm. The antioxidant activity of crude extracts was determined by using DPPH method.[8] DPPH scavenging activity of the six crude extracts of *A. obesum*, all polarities crude extracts exhibiting a significant antioxidant activity against DPPH; however, the highest antioxidant activity was found in chloroform extract with EC_{50} value of 28.32 μg/ml followed by water, methanol, butanol, ethyl acetate and hexane extracts, their IC_{50} being 29.95, 39.17, 42.40, 43.20 and 57.00 μg/ml respectively.

Table 2
Antimicrobial activity of isolated pure compound **1** of *A. obesum*.

Pure compound	Concentration (μg/ml)	*E. coli* (mm)	*P. aeruginosa* (mm)	*S. aureus* (mm)	*Valgurais* (mm)
3,4-dihydroxycinnamic acid	0.5	8 ± 0.05	11 ± 0.13	10 ± 0.05	9 ± 0.29
(*R*)-1-carboxy-2-3,4-dihydroxyphenyl)ethyl ester	1	10 ± 0.21	13 ± 0.31	12 ± 0.17	11 ± 0.15
	Control	28 ± 0.45	38 ± 0.66	21 ± 0.44	27 ± 0.50

Disc diffusion method was used for the determination of antimicrobial activity of crude extracts and isolated pure compounds[5] and the results were presented in Tables 2 and 3. The zones of inhibition for all crude extracts and isolated pure compound showed activity within the range of 0–13 mm. All crude extracts at most of the concentrations showed moderate antimicrobial activity against *E. coli*. On the other hand, butanol and hexane crude extract showed similar activity at all employed concentration against *P. aeruginosa* and *P. vulgaris*. However, methanol, water, butanol and ethyl acetate crude extracts did not show any activity against *P. aeruginosa, S. aureus* and *P. vulgaris* at any concentration. This might reflect the diversity of secondary metabolites and the compromise of their bioactivity by the polarity of extraction solvents.[20–22] In addition, it was evident that among the employed bacteria, Gram negative bacterial strains showed the highest sensitivity to all crude extracts at all concentrations. Our results are fully in line with earlier results presented.[20–22] Rosmarinic acid has previously been shown to possess significant antimicrobial activity against different strains of Gram (+ and −) bacterial.[11,12]

The chloroform crude extract was fractionated by different chromatographic techniques using petroleum ether (40–60 °C) followed by a mixture of petroleum ether with increasing amount of ethyl acetate. White crystals were precipitated at the bottom of the conical flask. These crystals were further purified by preparative TLC over silica gel GF_{254} using ethyl acetate-hexane (99.5:0.5) as a developing solvent. TLC examination showed a bright orange single spot upon exposure to iodine chamber. It was obtained as white needles with melting point 176–177 °C (Kelley et al.[10] 1976; mp 177 °C). High resolution mass spectrum of compound **1** exhibited molecular ion at m/z 360.83, which is consistent with the molecular formula $C_{19}H_{18}O_7$ as confirmed through 1D and 2D NMR analysis (Fig. 1). The IR spectrum showed absorption bands at 3165, 1707 and 1617 cm^{-1} indicating the presence of hydroxyl groups and

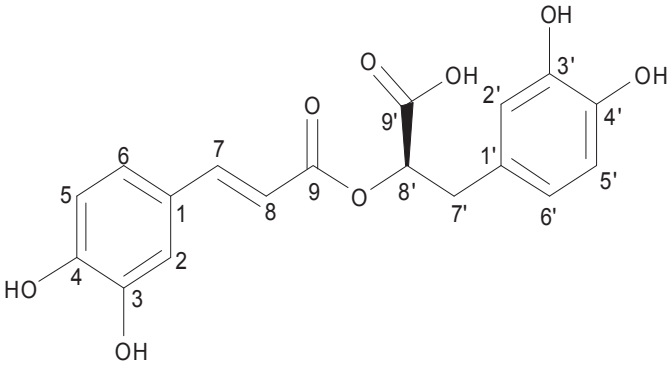

Fig. 1. Compound **1**.

carboxylic acid function. Its spectral data were in agreement with those previously reported in the literature for the same compound.[23–26] Different spectroscopic data like HMBC, HMQC, and ^1H-^1H COSY of the isolated compound is presented in Table 3.

The ^1H NMR spectrum showed a total of eleven signals. Two protons with doublet of doublet of aliphatic region with chemical shift at δ 2.99 and δ 3.01 (J = 10.1 and 10 Hz) indicating the presence of two methylene group of the 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid structural unit (Table 3). Four doublets at δ 7.02, δ 6.75, δ 6.73 and δ 6.67 indicating the presence of four protons at H-2, H-5, H-2′ and H-5′ in the aromatic rings (Table 3). Two doublets of doublets at the chemical shift δ 6.92 and δ 6.59 indication the presence of two aromatic ring protons at the position of H-6 and H-6′.

The ^{13}C NMR spectrum showed 18 signals for 18 carbons in agreement with the structure of 3,4-dihydroxycinnamic acid (*R*)-1-

Table 3
HMBC, HMQC, and ^1H-^1H COSY data of compound **1** in $CDCl_3$.

C	HMQC		HMBC	^1H-^1H COSY
	δ_H	δ_C		
1		127.71	H-5, H-7, H-8	
2	7.02 (d, 1H, J = 5.4 Hz, H-2)	115.18	H-6, H-7	H-6
3		145.21	H-5	
4		147.53	H-2, H-5, H-6	
5	6.75 (d, 1H, J = 8.12 Hz, H-5)	116.26	H-6	
6	6.92 (dd, 1H, J = 8.12 and 1.2 Hz, H-6)	123.11	H-2, H-7	H-2, H-5
7	7.51 (d, 1H, J = 15.8 Hz H-7)	146.81	H-2, H-6	H-8
8	6.24 (d, 1H, J = 15.8 Hz, H-8)	114.62		H-7
9		149.69	H-7, H-8, H-8′	
1′		129.57	H-5′, H-7′a, 7′b, H-8′	
2′	6.73 (d, 1H, J = 3.5 Hz, H-2′)	116.48	H-6′	H-6′
3′		146.14	H-5′	
4′		145.21	H-2, H-6′	
5′	6.59 (dd, 1H, J = 7.92 Hz, H-5′)	121.77		H-6′
6′	6.67 (d, 1H, J = 7.92 HZ, H-6′)	117.55	H-2′, H-7′a, 7′b	H-2′, H-5′
7′	2.99 (dd, 1H, J = 8.58 and 14.5 Hz, H-7b')	38.07	H-2′, H-6′, H-8′	H-7′b, H-8′
	3.01 (dd, 1H, J = 10.1 and 4.2 Hz, H-7a ′)		H-7′a, H-8′	
8′	5.14 (dd, 1H, J = 10 Hz, J = 2.8 Hz, H-8′)	75.10	H-7′a	H-7′a, H-7′
9′		168.55	H-8′	

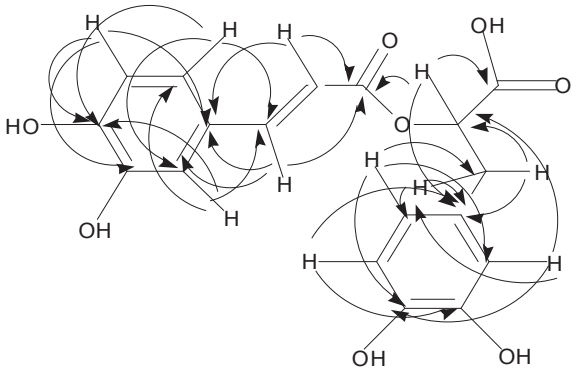

Fig. 2. HMBC correlations and full assignments of compound **1**.

carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (**1**, Fig. 1). The peaks between δ 114.62 ppm and δ 147.53 ppm are located in the two aromatic rings. The two signals at δ 168.55 ppm and δ 149.69 ppm is for carbonyl region are defined through the both carbonyl groups of the acid (COOH) and ester functions in the compound **1** (Table 3). The signal of carbon atom appeared at δ 38.07 ppm indicating the presence of $-CH_2-$ and was confirmed through DEPT and COSY analysis.

The HMBC correlations and assignments of the isolated compound are presented in Fig. 2. Thus, the MS, ^{1}H-NMR and ^{13}C-NMR spectral analysis along with physical properties established the identity of the compound as 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (**1**, rosmarinic acid). Based on the MS, 1D- and 2D-NMR analysis, the chemical-shift values of the protons and carbons were in agreement with those of the 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (**1**, rosmarinic acid), which was comparable to what is in the literature.[10,11]

5. Conclusions

The result of the present investigated compound **1** occurs in plant kingdom. MS, HMBC, HMQC, ^{1}H-^{1}H COSY and IR spectral analysis along with physical properties established the identity of compound **1** as 3,4-dihydroxycinnamic acid (*R*)-1-carboxy-2-(3,4-dihydroxy phenyl) ethyl ester (rosmarinic acid) from the active chloroform crude extract of stem-bark of *A. obesum*. The isolated compound **1** also showed significant antimicrobial activity against different Gram positive and negative bacterial strains. The title compound was isolated from this plant for the first time.

Authors' contributions

Mohammad Amzad Hossain and Sadri Abdullah Said designed the study and analyzed and interpreted data. Mohammed Sohail Akhtar, Mohammad Amzad Hossain, Sadri Abdullah Said collected the data. All authors read and approved the final manuscript.

Conflict of interest

The authors report no declarations of interest.

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