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CHARACTERIZATION OF FLAVONOIDS FROM *CISSUS QUADRANGULARIS* LEAVES BY LCMSMS

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ABSTRACT
Cissus quadrangularis Linn. (Vitaceae) leaves that constitute only 5-8% of the aerial plant parts have not been explored for its chemical constituents, hence it was thought worthwhile to elaborate on the phytochemical characterization of *Cissus quadrangularis* leaves. Four flavonoids were isolated from *Cissus quadrangularis* leaves. Characterization was achieved by liquid chromatography coupled with electron spray ionization mass spectrometry (ESI-MS/MS) with negative ion detection.Vitexin and kaempferol 3-O-rhamnoside are reported for the first time in this species while quercetin and quercitrin have been reported for the first time from *Cissus quadrangularis* leaves.

KEYWORDS: Quercetin, Kaempferol 3-O-rhamnoside, Quercitrin, Vitexin, Electron spray ionization mass spectrometry.

INTRODUCTION
Cissus quadrangularis Linn. (Vitaceae) is distributed throughout the hotter parts of India and Sri Lanka and is known as Asthisamdhani in Sanskrit. The plant is reported to be useful in treatment of bone fracture, diarrhoea, skin disorders and scurvy.[1,2] Ketosteroids, triterpenoids (δ-amyrin and δ-amyrone),[3, 4] stillbene derivatives,[5] lipids[6] and Flavonoids (quercetin, quercitrin)[7] have been reported from aerial parts of the plant, specifically from the stems.

Leaves that constitute only 5-8% of the aerial plant parts[8] have not been explored for its chemical constituents, hence it was thought worthwhile to emphasize on the phytochemical characterization of *Cissus quadrangularis* leaves. In continuation of our phytochemical investigations on *Cissus quadrangularis* [9,10,15-18] we have now characterized flavonoids from *Cissus quadrangularis* leaves.

EXPERIMENTAL
Materials and method
General
TLC was carried out on precoated silica gel $60F_{254}$ plates (Merck) using ethyl acetate: acetic acid: formic acid: water (100:10:10:27) as solvent system. Compounds were detected by UV fluorescence before and after spraying with ammonia vapors. Column chromatography was carried out using silica gel G (60-120).

Plant material
The leaves of *C. quadrangularis* were collected locally and authenticated by Prof. H.M. Pandit, Botany Dept., Khalsa College, Mumbai, India, a voucher specimen has been deposited in Medicinal Natural Product Research Laboratory, ICT, Mumbai, India.

Instrumental details
LC-MS analysis was performed on Finnigan electron LC attached with Finnigan LCQ Advantage Max model (Thermo Electron Corporation, USA). The column consisted of Thermo Hypergold RP C18 (particle size 5 µm, diameter 4.6 mm, length 250 mm) and Finnigan Surveyor photo diode array (PDA) plus detector was used for recording chromatogram and UV spectra. The mobile phase comprises of 75:25 ratio of A and B at flow rate of 400 µL/min. The reagents were of analytical grade and purchased from Merck and J.T. Baker.

The MS and MS/MS analyses were performed using electron spray ionization mass spectrometry (ESI-MS/MS) in both positive and negative ion mode. The following parameters were optimized for present analysis: spray voltage, 5.5 kV; collision gas (N_2), 10 A.U.; sheath gas flow 38 A.U.; auxiliary gas flow rate 12 A.U.; curtain gas flow rate 12 A.U.; capillary voltage 36 V and capillary temperature 275 ºC. The collision energy was optimized for each constituent. Product ion mass spectra were recorded in the mass range of m/z 145-1000.

Extraction and isolation

Dried and powdered leaves (1.0 kg) were defatted by extracting with hexane. The defatted leaf powder was extracted with methanol using Soxhlet extractor for 48 h. The extract was concentrated and fractionated into hexane, chloroform, ethyl acetate and n-butanol soluble fractions. The ethyl acetate fraction was concentrated and chromatographed over silica gel, eluted initially with chloroform and sequentially with chloroform-methanol (2% increments of methanol). Fractions (250 ml) were collected and monitored by TLC. Initial fractions (12-16) eluted with 4% methanol gave compound **1** (25mg), fractions 40-45 eluted with 8% methanol resulted in compound **2** (40 mg)**,** 10% methanol eluate (fractions 50-58) gave compound **3** (50 mg) and compound **4** (15 mg) was obtained from fraction 60-62 eluted with 10% methanol. The purified compounds were analyzed by LC-MS/MS.

RESULTS AND DISCUSSION

Four flavonoids quercetin, kaempferol 3-O-rhamnoside, quercitrin (quercetin 3-O-rhamnoside), and vitexin (apigenin 8-C-glucoside) have been isolated from methanol extract of leaves of *C. quadrangularis* by solvent fractionation and column chromatography. The isolated compounds were characterized mainly by LC/MS/MS.

Compound 1 showed UV λ_{max} at 372 nm and 256 nm, a pattern typical of flavonol (11). LCMS showed peak at RT 21.86 min and corresponding m/z at 603 $(M+H)^+$ and 301 $(M-H)^-$ in positive ion and negative ion mode respectively. Since spectra obtained in negative ion mode are reported to be clean with intense ions due to the efficiency of deprotonation of flavonoids (12), hence for further fragmentation $(M-H)^-$ ions were taken as precursors in the ESI-MS/MS and yielded spectra corresponding to deprotonated molecule. Fragmentation of m/z 301 showed ions at m/z 300$(M-2H)^-$, m/z 283 $(M-H-H_2O)^-$, 271 $(M-H-CH_2O)^-$, 255 $(M-H-H_2O-CO)^-$,227 $(M-H-H_2O-2CO)^-$, 273 $(M-H-CO)^-$ and 245 $(M-H-2CO)^-$ due to the loss of neutral molecules and at m/z 179$(M-H-122)^-$ and 151(M-H-122-CO) due to further degradation of C-ring, confirming compound **1** as quercetin.

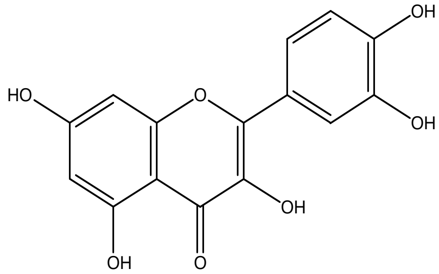

Fig 1: Structure of Compound 1 (Quercetin)

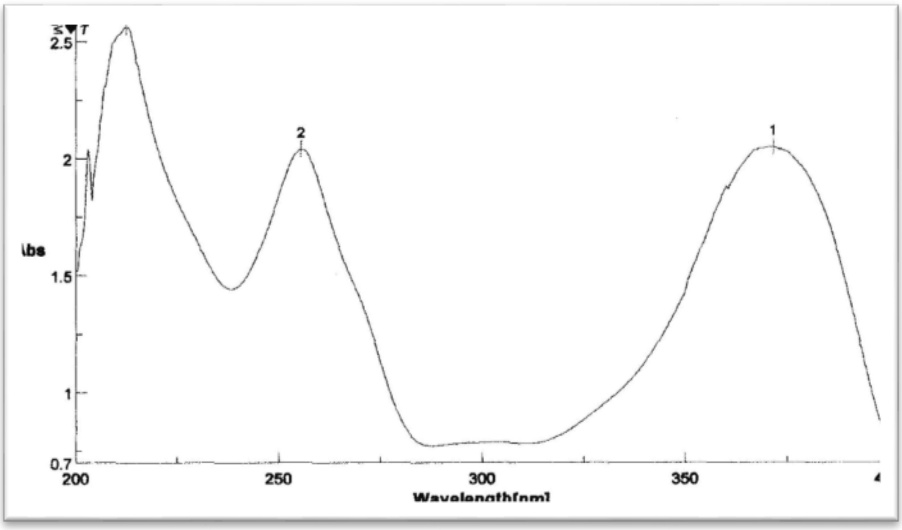

Fig. 1.1: UV spectra of compound 1 showing λ_{max} at 370 nm and 255 nm

Mobile phase: Water (0.1 % formic acid): acetonitrile [75:25] at 400 μl/min flow rate

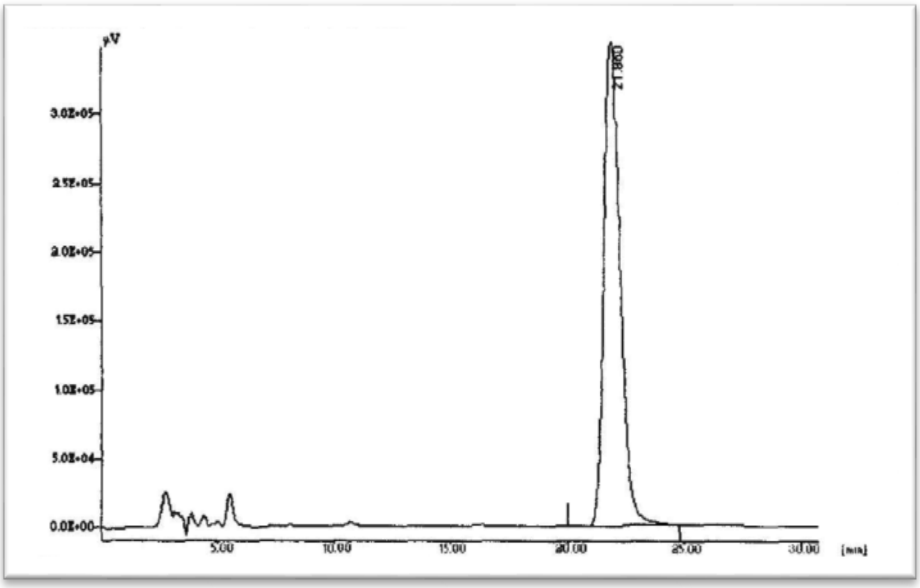

Fig. 1.2: Chromatogram of isolated compound 1

Mobile phase: Water (0.1 % formic acid): acetonitrile [75:25] at 400 μl/min flow rate

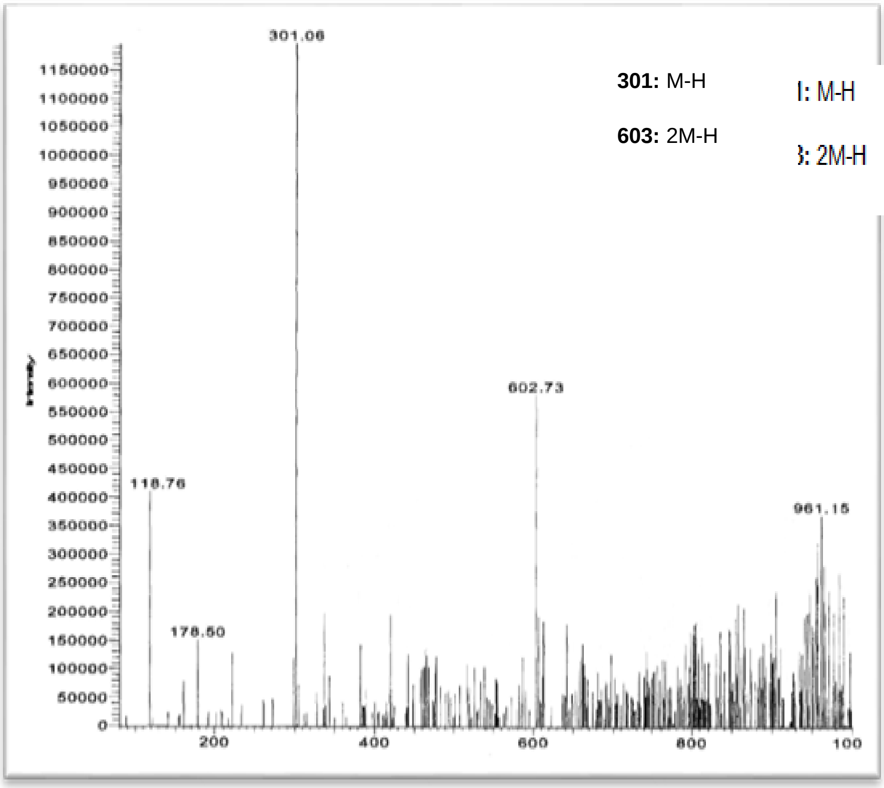

Fig. 1.3: Full MS scan of isolated compound 1 in LCMS (Negative ion mode)

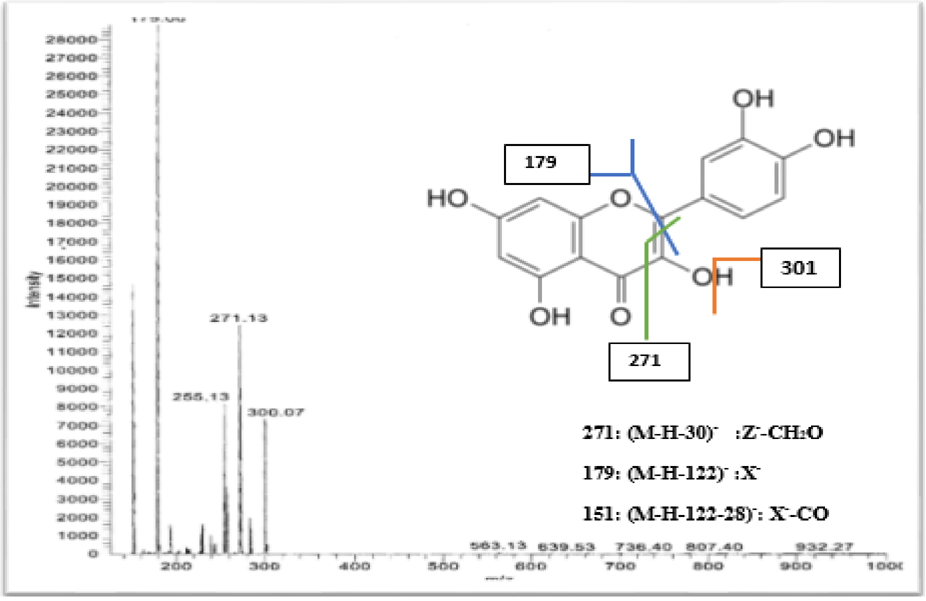

Fig. 1.4: MSMS spectra of peak at m/z 301 in LCMS/MS

Compound 2 showed UV λ_{max} at 343 nm and 264 nm, a pattern typical of tetrahydroxy flavonol with substitution at 3 position. LCMS showed peak at RT 18.7 min and corresponding m/z at 433 $(M+H)^+$ and 431 $(M-H)^-$ in positive ion and negative ion mode respectively. Fragmentation of m/z 431 gave ions at m/z 285 with a loss of 146 units corresponding to kaempferol and rhamnose. Further fragmentation of m/z 285 showed ions at 267 $(M-H-H_2O)^-$, 239 $(M-H-H_2O-CO)^-$, 211 $(M-H-H_2O-2CO)^-$, 257 $(M-H-CO)^-$ and 229 $(M-H-2CO)^-$ confirming compound 2 as kaempferol 3-O-rhamnoside.

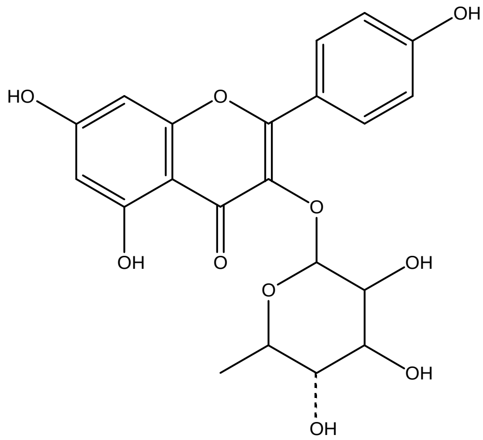

Fig 2: Structure of compound 2 (Kaempferol 3-O-rhamnoside)

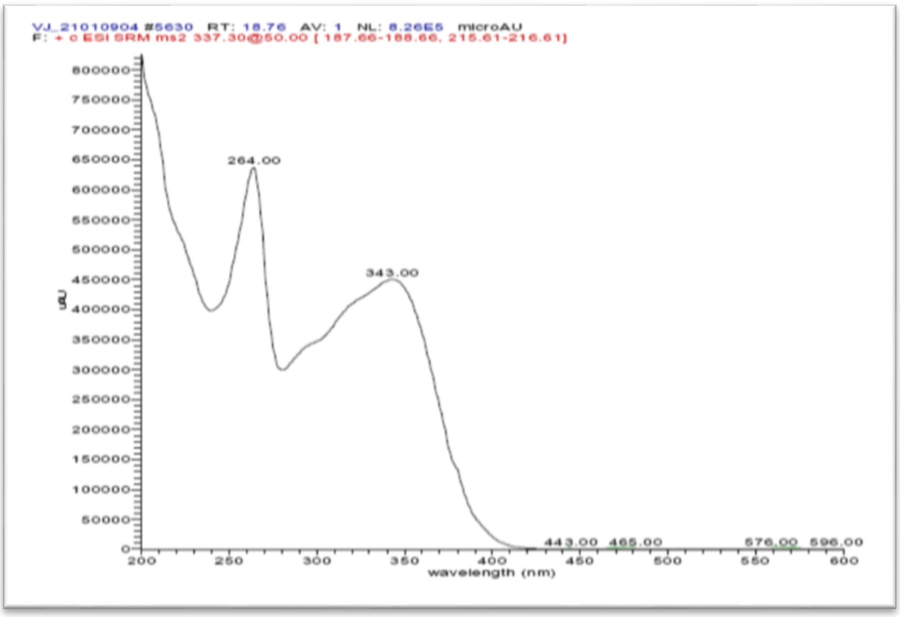

Fig. 2.1: UV spectra of compound 2 showing λ_{max} at 264nm and 343 nm

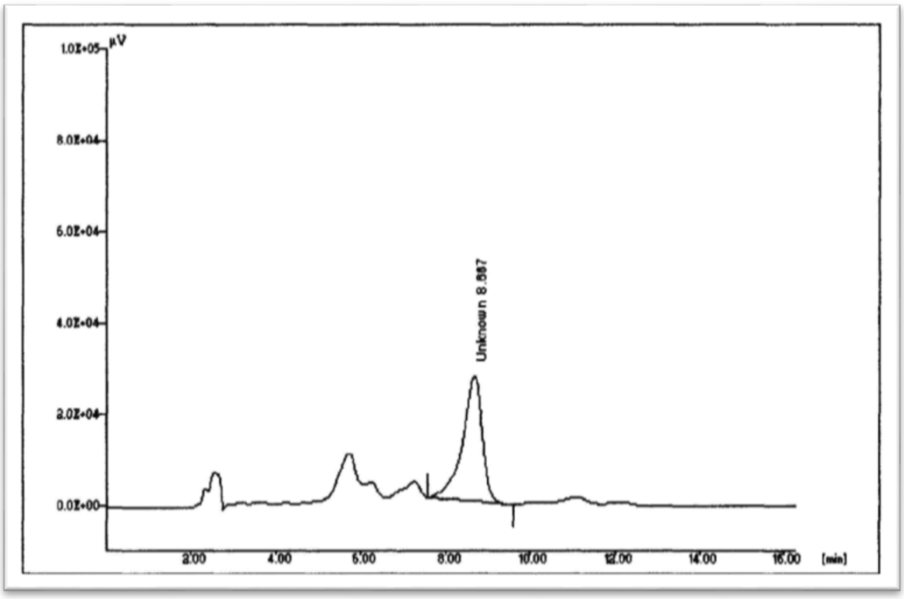

Fig. 2.2: Chromatogram of isolated compound 2 in HPLC

Mobile phase: Methanol: water (0.1% formic acid) [65:35] at a flow rate of 1 ml/min

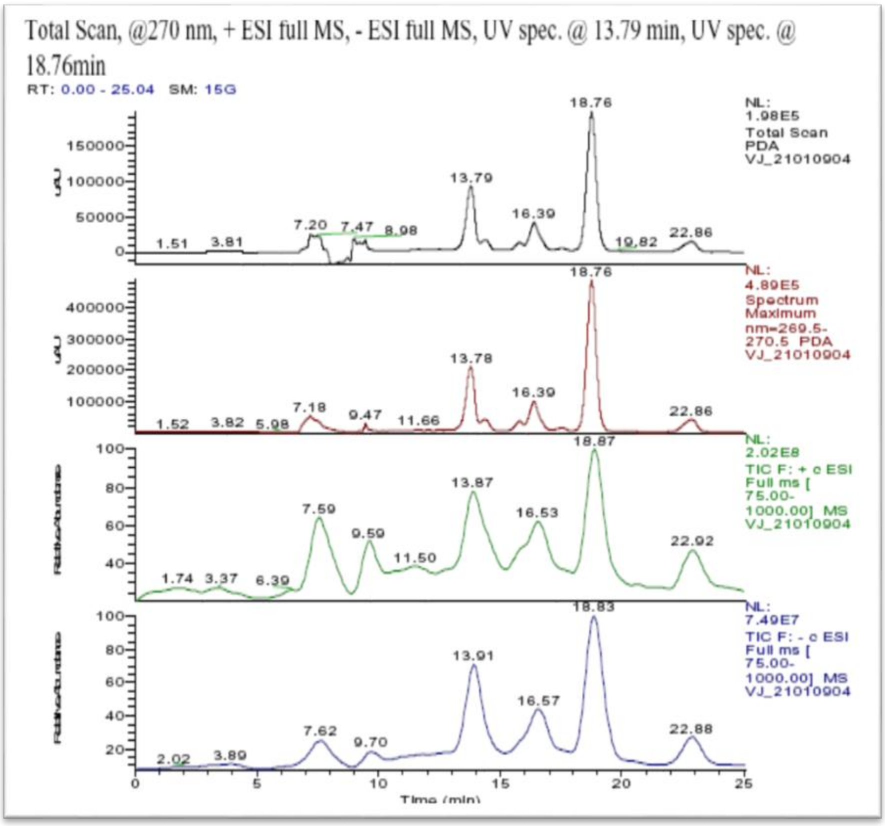

Fig. 2.3: Chromatogram of compound 2 in LCMS

Mobile phase: Water (0.1 % formic acid): acetonitrile [75:25] at 400 μl/min flow rate

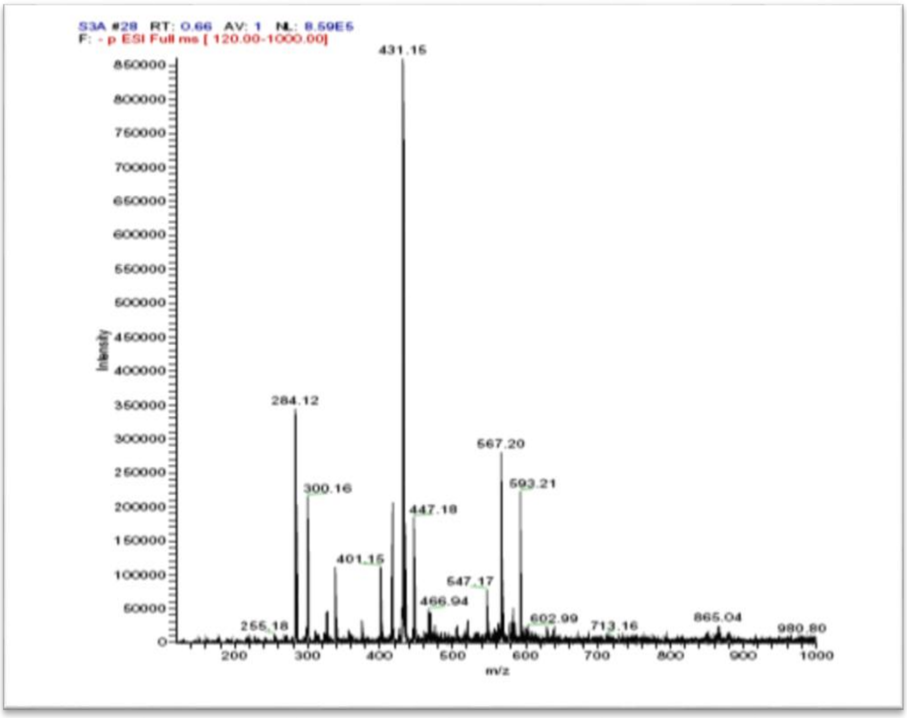

Fig. 2.4: Mass spectra of compound 2 in LCMS (Negative ion mode)

The mass spectra shows major ion peak at m/z 431 (M-H) along with other peaks

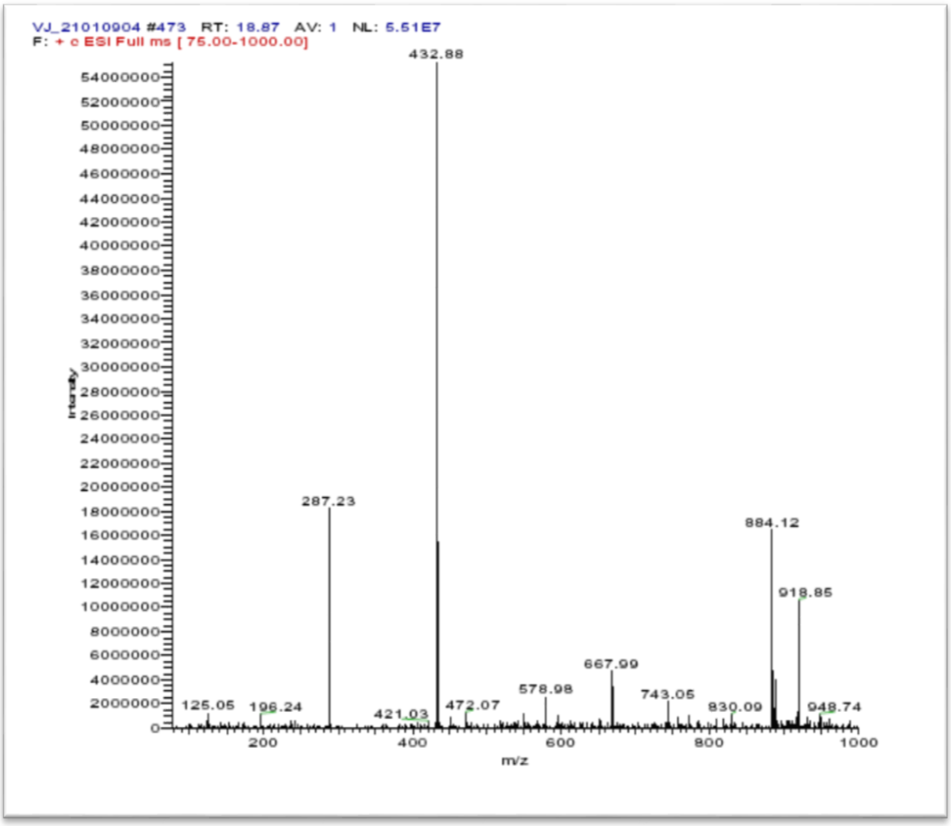

Fig. 2.5: Mass spectra of compound 2 in LCMS (Positive ion mode)

The mass spectra shows major ion peak at m/z 433 (M+H) and 287 (M+H-146)

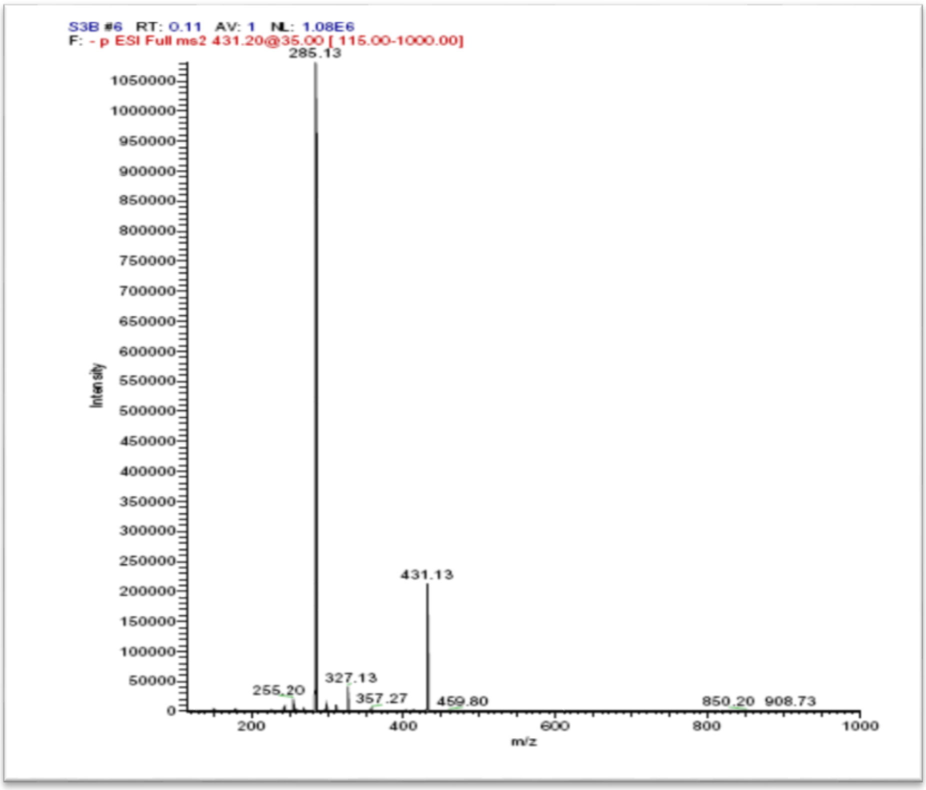

Fig. 2.6: MSMS spectra of peak at m/z 431 in LCMS (Negative ion mode)

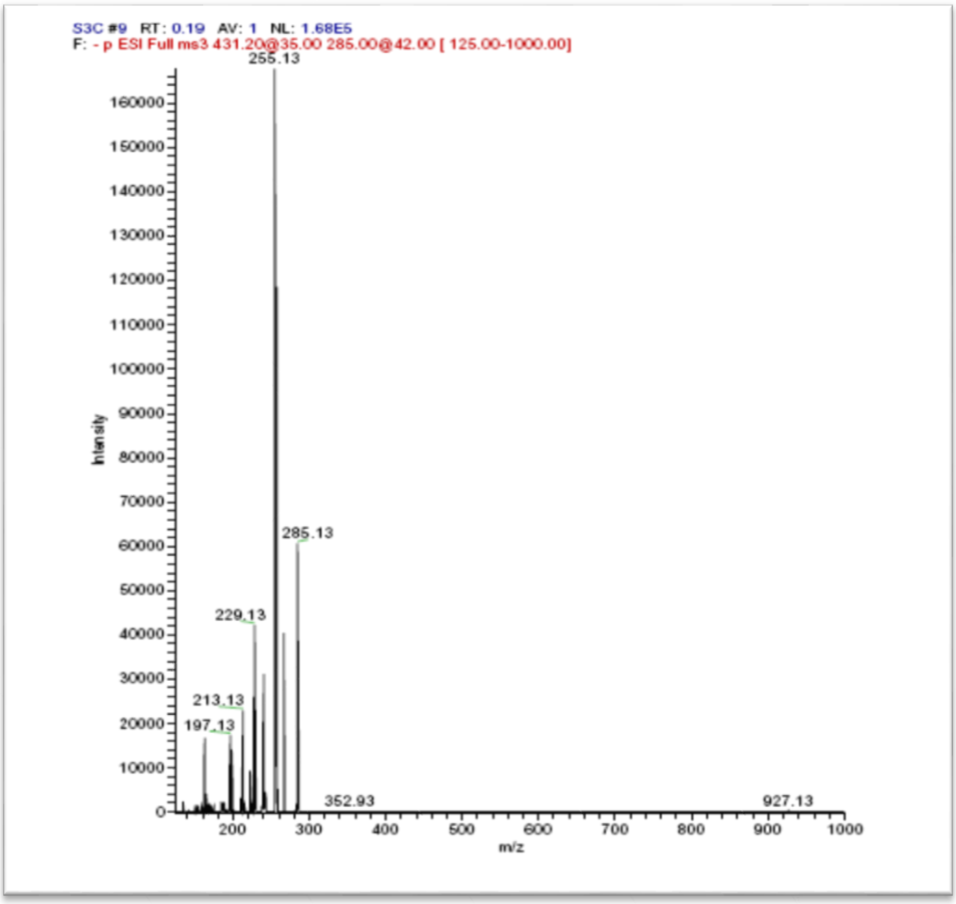

Fig. 2.7: MSMS spectra of peak at m/z 285 in LCMS/MS (Negative ion mode)

Compound 3 showed UV λ_{max} at 349 and 255 nm, indicates presence of flavonol with glycosylation at position 3 (substitution at 3 position gives a hypsochromic shift of 17-20 nm in Band A). LCMS showed peak at RT 14.4 min and corresponding m/z at 449 $(M+H)^+$ in positive ion and 447 $(M-H)^-$ and 895 $(2M-H)^-$ in negative ion mode. Fragmentation of m/z 447 gave ions at m/z 301, indicates the presence of quercetin aglycone and rhamnose as sugar. Further fragmentation of m/z 301 showed fragmentation pattern similar to that of compound 1. On the basis of above data compound **3** is characterized as quercetin 3-O-rhamnoside (quercitrin).

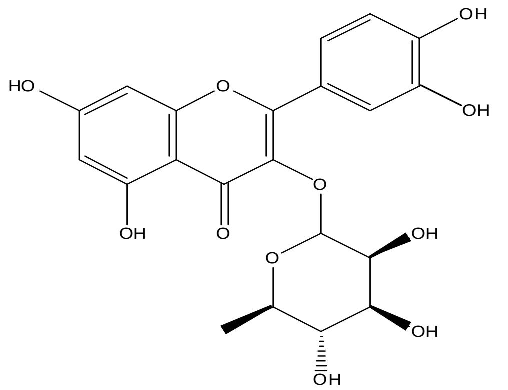

Fig. 3: Structure of compound 3 (Quercitrin)

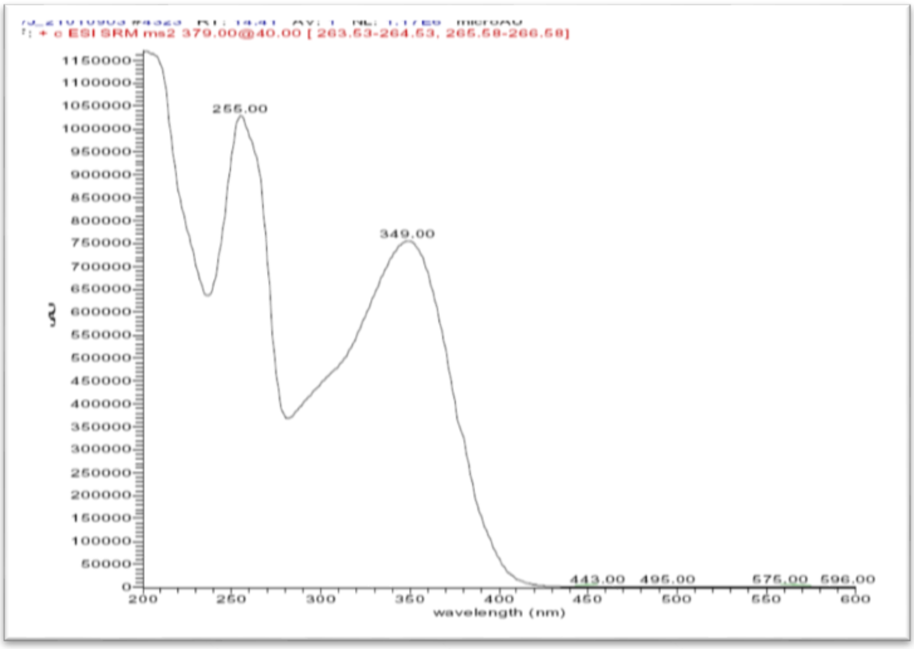

Fig. 3.1: UV spectra of isolated compound 3

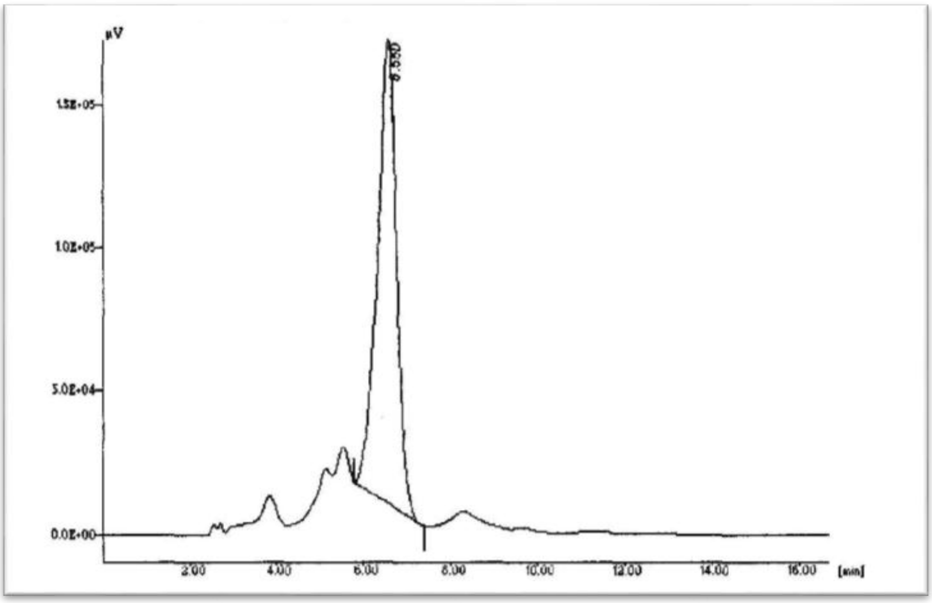

Fig. 3.2: Chromatogram of isolated compound 3

Mobile phase: Methanol: water (0.1 % formic acid) [65:35] at a flow rate of 1 ml/min

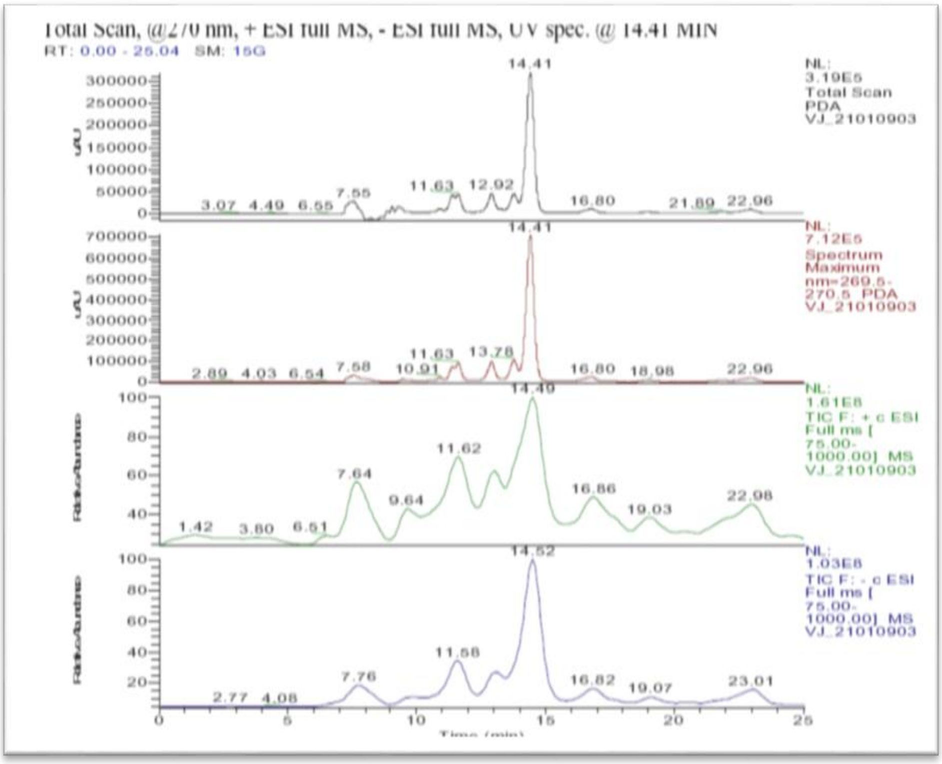

Fig. 3.3: Chromatogram of isolated compound 3 in LCMS

Mobile phase: Water (0.1 % formic acid): acetonitrile [75:25] at 400 μl/min flow rate

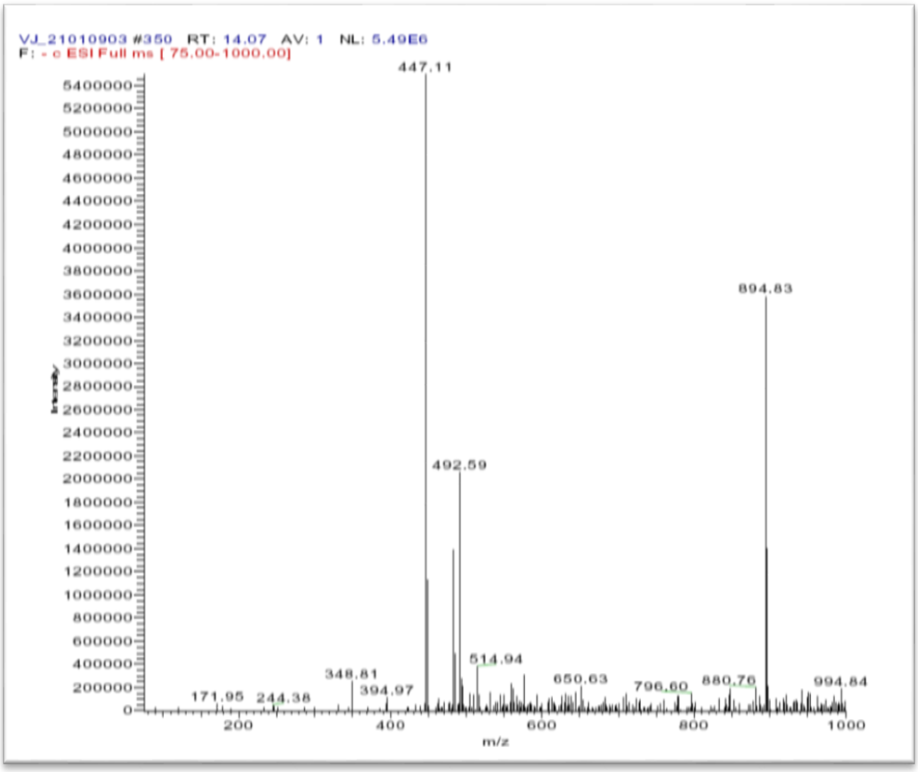

Fig. 3.4: Mass spectra of isolated compound 3 in LCMS

(Negative ion mode)

The mass spectra of compound IX shows peak at m/z 447 (M-H) and 895 (2M-H)

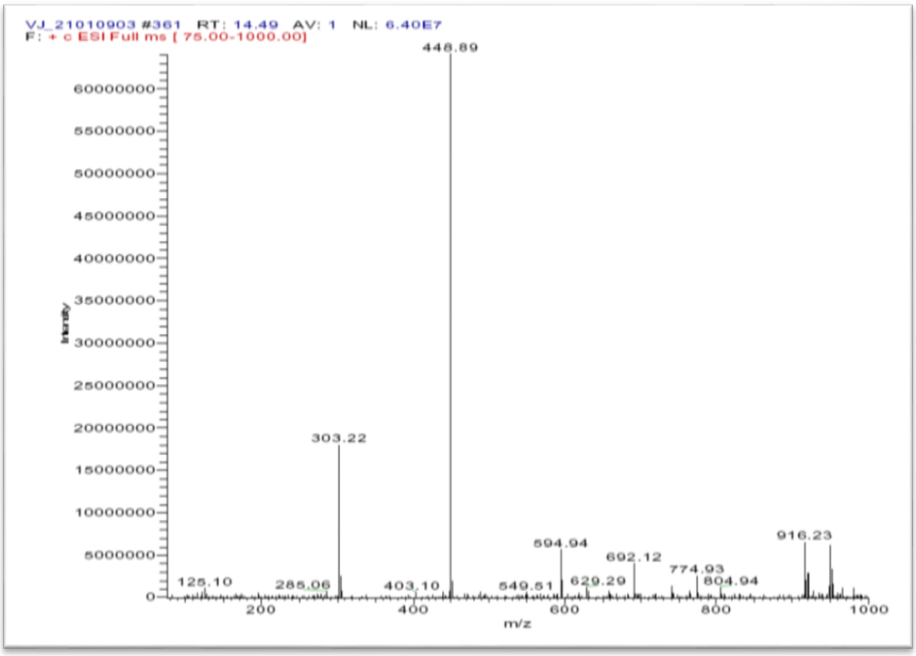

Fig. 3.5: Mass spectra of isolated compound 3 in LCMS

(Positive ion mode)

The mass spectra of compound IX shows peak at m/z 449 (M+H) and 303 (M+H-146)

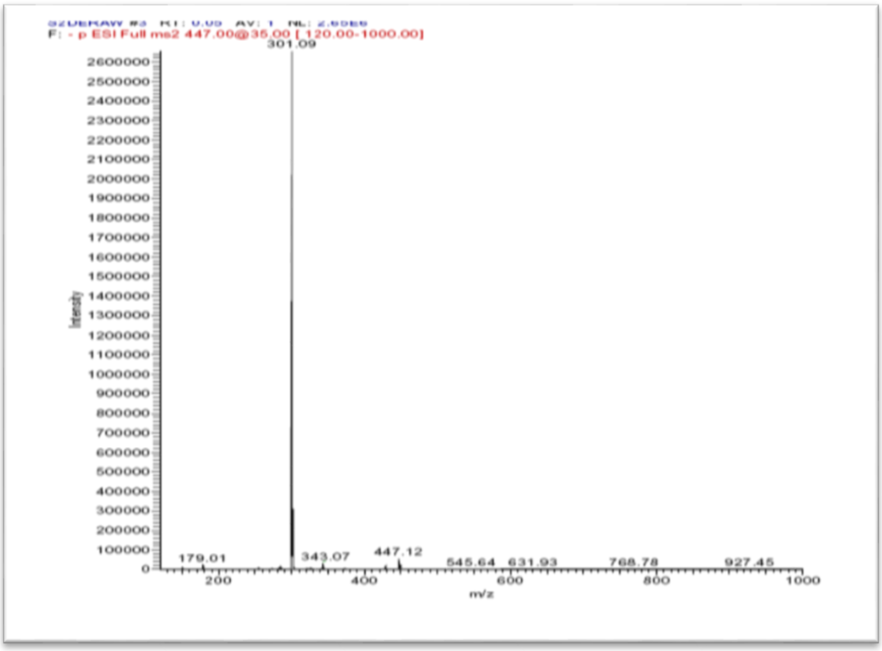

Fig. 3.6: MSMS spectra of peak at m/z 447 in LCMS/MS

(Negative ion mode)

The MSMS spectra of peak at m/z 447 shows fragment ions at m/z 301 (M-H-146)

Table 1: Flavonoid glycosides with molecular ion peak at m/z 447

FLAVONOIDS	m/z	MS/MS	Loss
Orientin	447	327	120
Kaempferol-3-O-glucoside	447	284	163
Luteolin 4-O-glucoside	447	285	162
Luteolin 7-O-glucoside	447	285	162
Quercetin 3-O-rhamnoside (Quercitrin)	447	301	146
Quercetin 7-O-rhamnoside	447	301	146

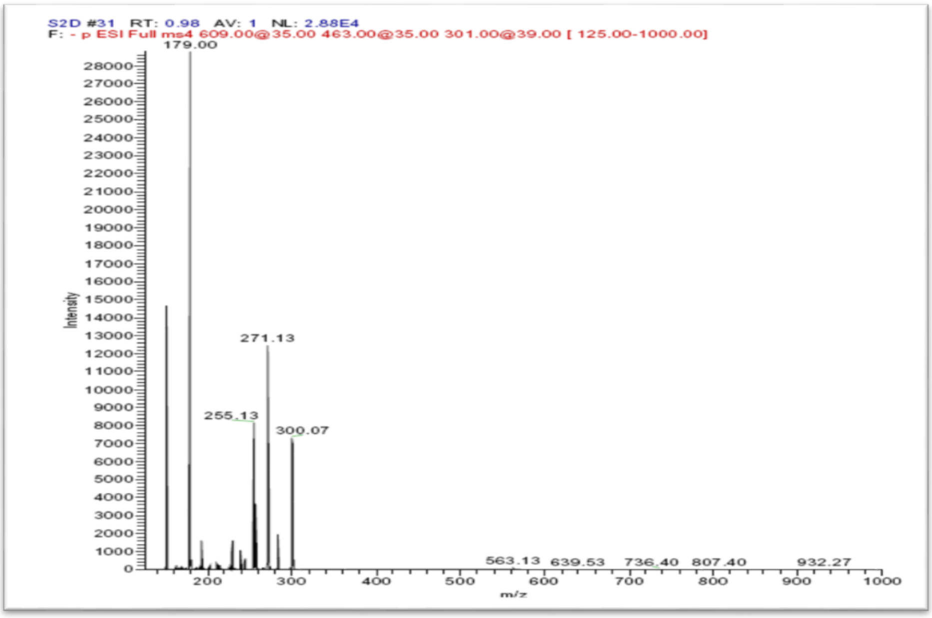

Fig. 3.7: MSMS spectra of peak at m/z 301 in LCMS/MS (Negative ion mode)

Compound 4 showed UV λ_{max} was observed at 337 nm and 269 nm, a pattern typical of flavones. In LCMS/MS the compound showed a peak at RT 10.8 and corresponding m/z at 863 $(2M\text{-}H)^-$ and 431 $(M\text{-}H)^-$ in negative ion mode and at 887 $(2M+Na)^+$ and 433 $(M+H)^+$ in positive ion mode indicating a molecular mass of 432. Fragmentation of m/z 431 showed ions at m/z 341$(M\text{-}H\text{-}90)^-$ and 311$(M\text{-}H\text{-}120)^-$, due to cross link cleavages in the sugar unit, characteristic feature of C-bonded glycosides (12, 13). Further fragmentation of m/z 311 gave ions at 283 and 269. Distinction of vitexin (apigenin-8-C-glucoside) from isovitexin (apigenin-6-C-glucoside) is based on the fact that fragmentation of m/z 311 in 6-C isomer gives daughter ions at m/z 175 and 147 due to retro-Diels-Alder fragmentation, which are absent in mass fragmentation of 8-C-isomer (14). On the basis of above spectral and chromatographic data, the compound is identified as vitexin.

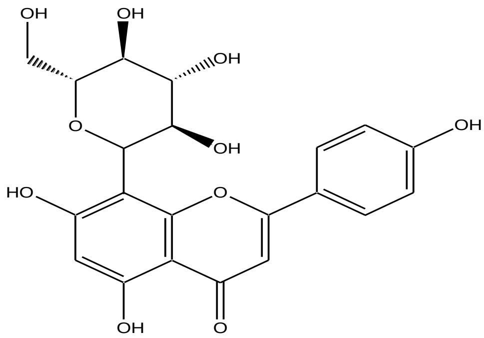

Fig 4: Structure of compound 4 (Vitexin)

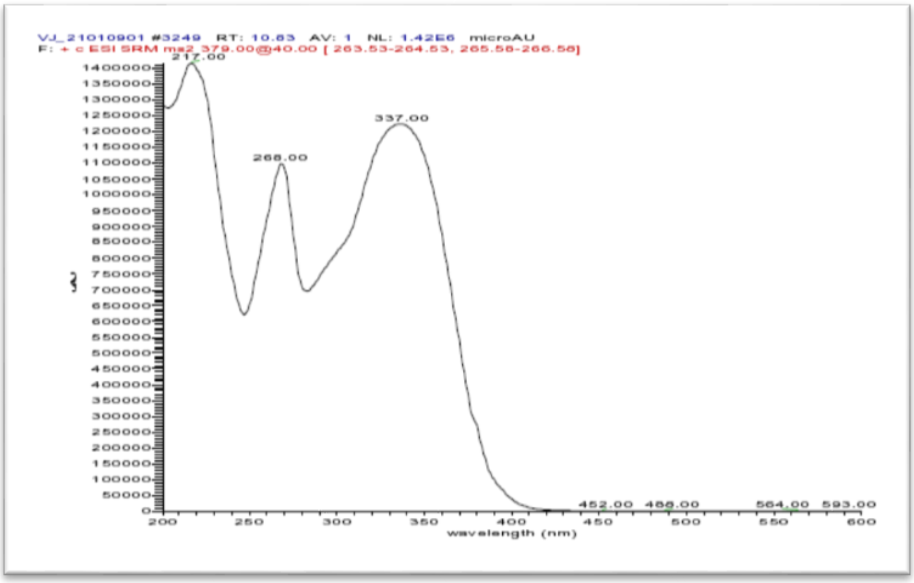

Fig. 4.1: UV spectra of isolated compound 4

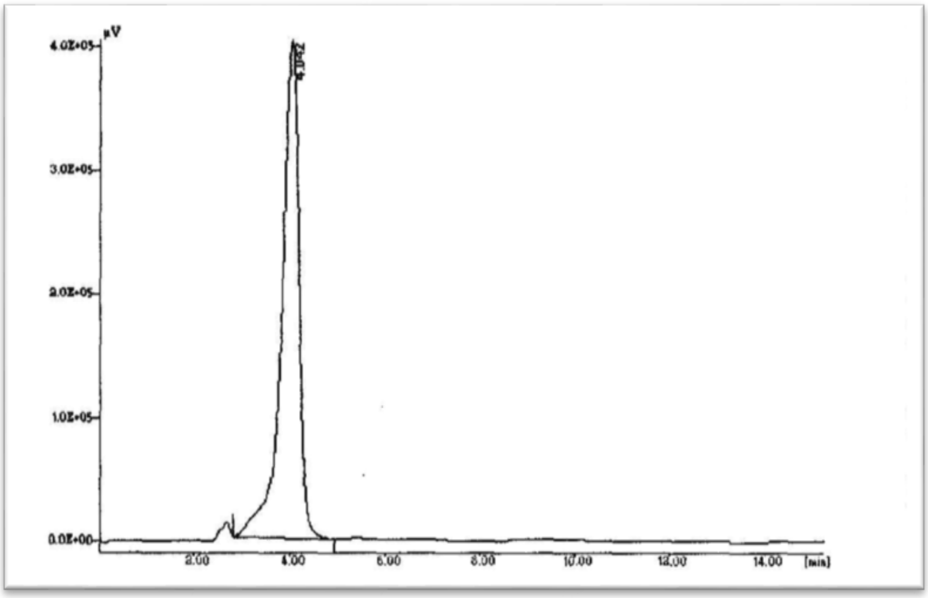

Fig. 4.2: Chromatogram of isolated compound 4 in HPLC

Mobile phase: Methanol: water (0.1% formic acid) [65:35] at a flow rate of 1 ml/min

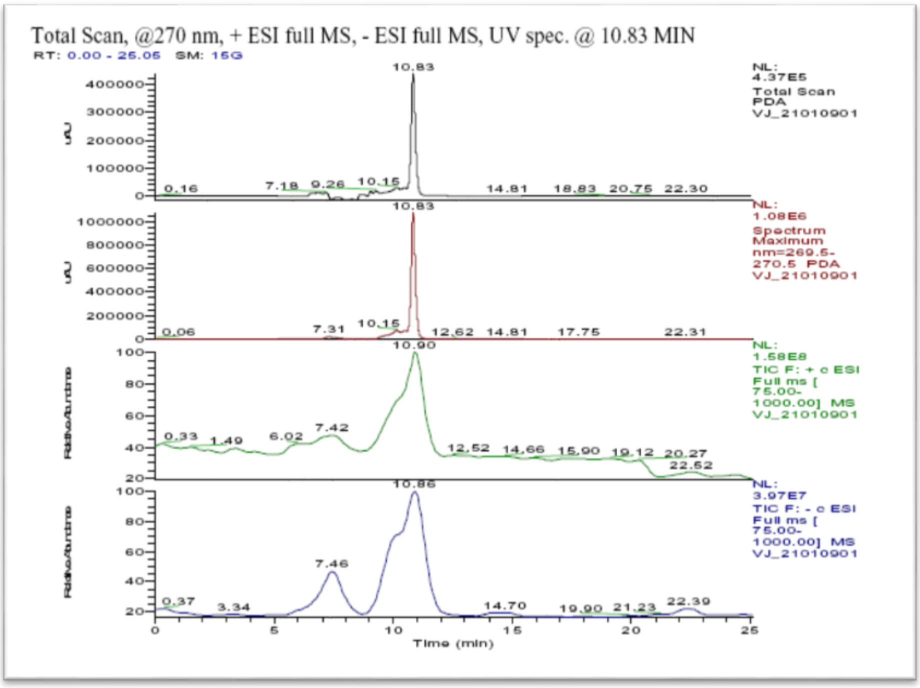

Fig. 4.3: Chromatogram of isolated compound 4 in LCMS

Mobile phase: Water (0.1% formic acid): acetonitrile [75:25] at 400 μl/ml flow rate

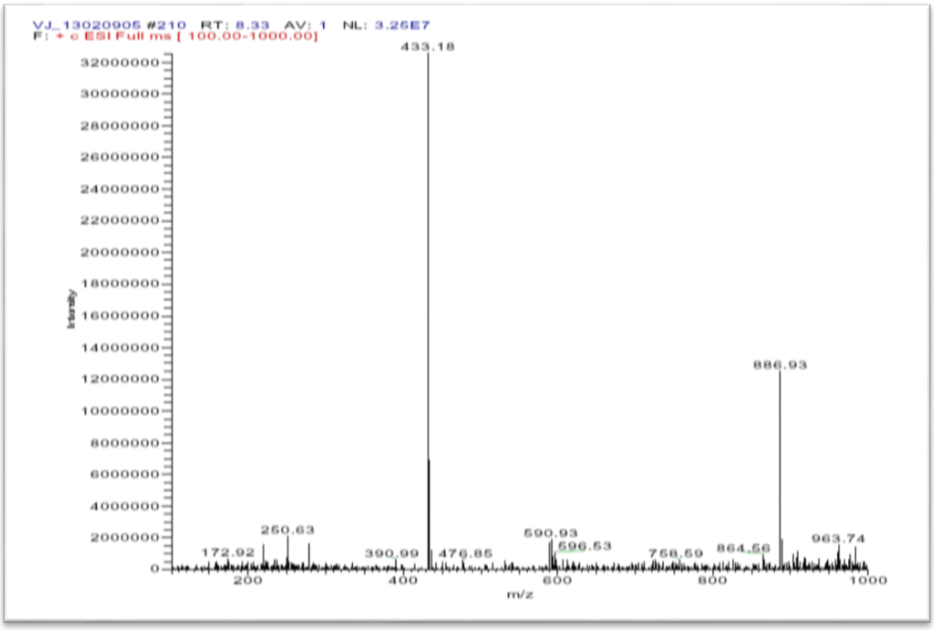

Fig.4.4: Mass spectra of isolated compound 4 in LCMS

(Positive ion mode)
The mass spectra of compound 4 shows peak at m/z 433 (M+H) and 887 (2M+Na)

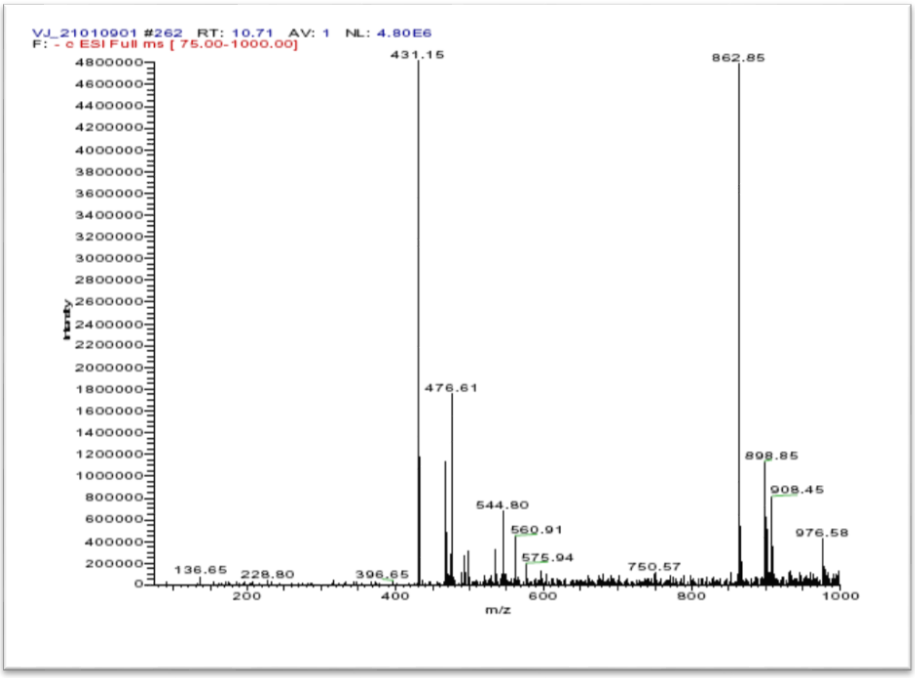

Fig. 4.5: Mass spectra of isolated compound 4 in LCMS

(Negative ion mode)
The mass spectra of compound 4 shows peak at m/z 431 (M-H) and 863 (2M-H)

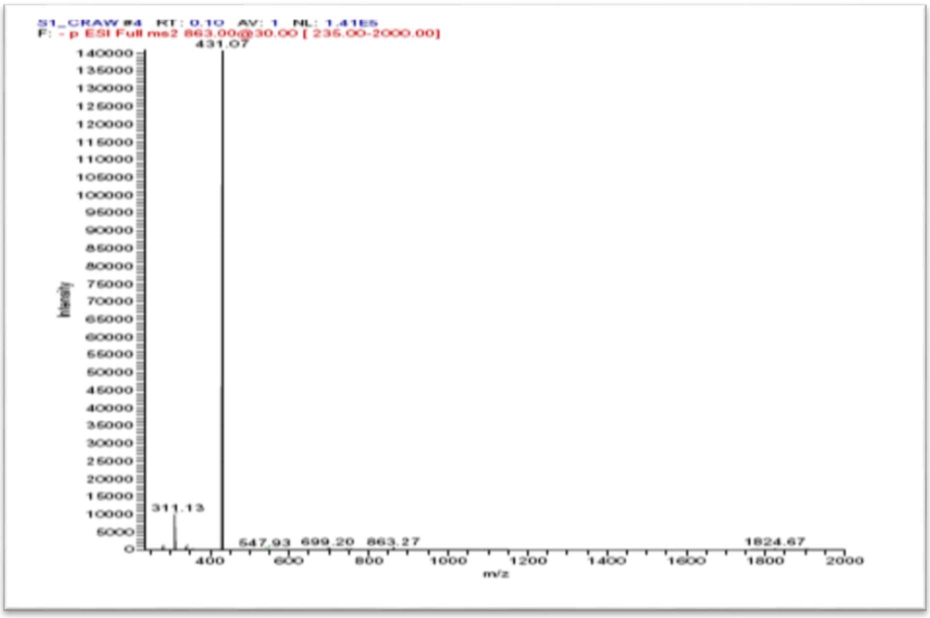

Fig. 4.6: MSMS spectra of peak at m/z 863 in LCMS/MS

(Negative ion mode)

The MSMS spectra of peak at m/z 863 shows fragment ions at m/z 431 (M-H) and 311 (2M-H-120)

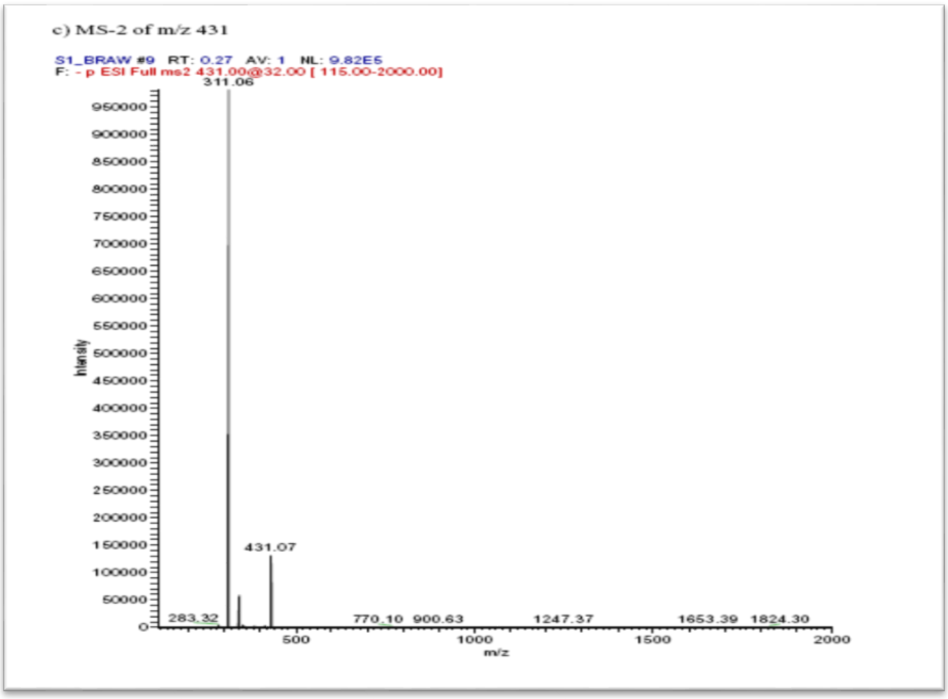

Fig. 4.6 (a): MSMS spectra of peak at m/z 431 in LCMS/MS

(Negative ion mode)

The MSMS spectra of peak at m/z 431 shows fragment ions at m/z 341 (M-H-90) and 311 (M-H-120)

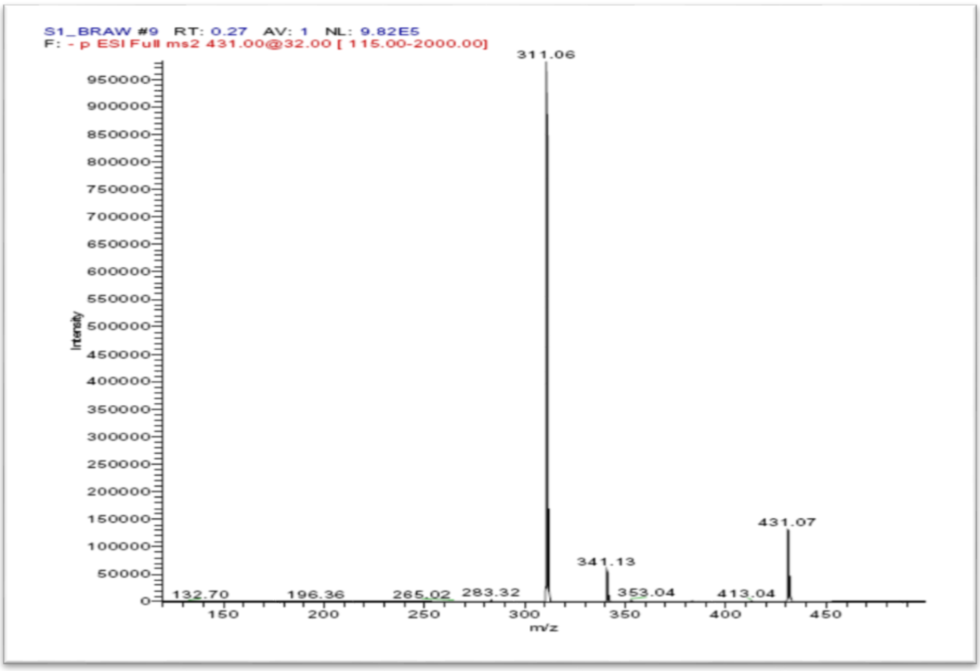

Fig. 4.7: Enlarge view of MSMS spectra of peak at m/z 431 in LCMS/MS

Table 2: Flavone glycosides with a molecular ion peak at m/z 431 (Negative ion mode)

Flavonoid	m/z	MS/MS	Loss
Apigenin-7-glucoside	431	269	162
Vitexin (apigenin 8-C-glucoside)	**431**	**311**	**120**
Isovitexin (apigenin 6-C-glucoside)	**431**	**311**	**120**

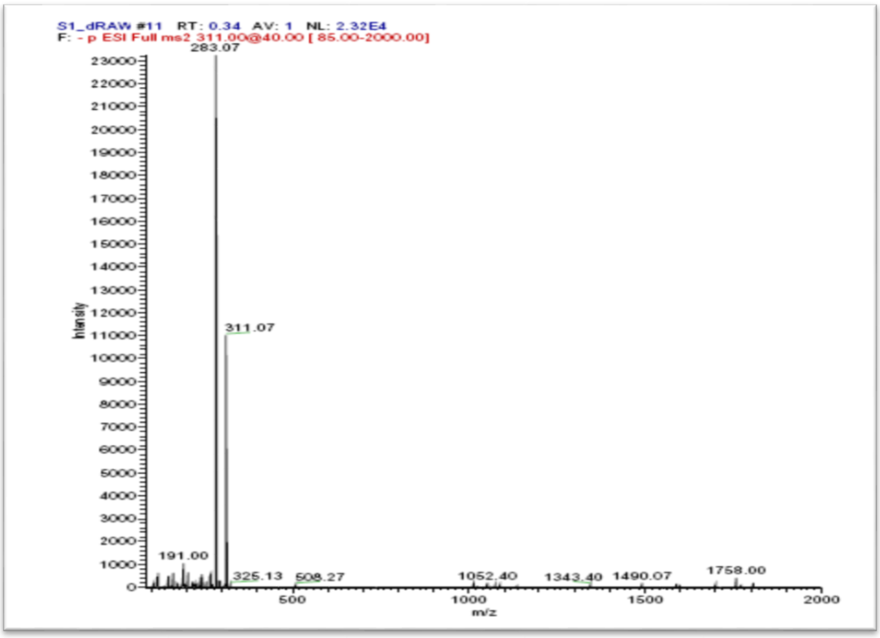

Fig. 4.8: MSMS spectra of peak at m/z 311 in LCMS/MS

(Negative ion mode)

The MSMS spectra peak at m/z 311 shows fragment ions at m/z 283 (M-H-120-18)

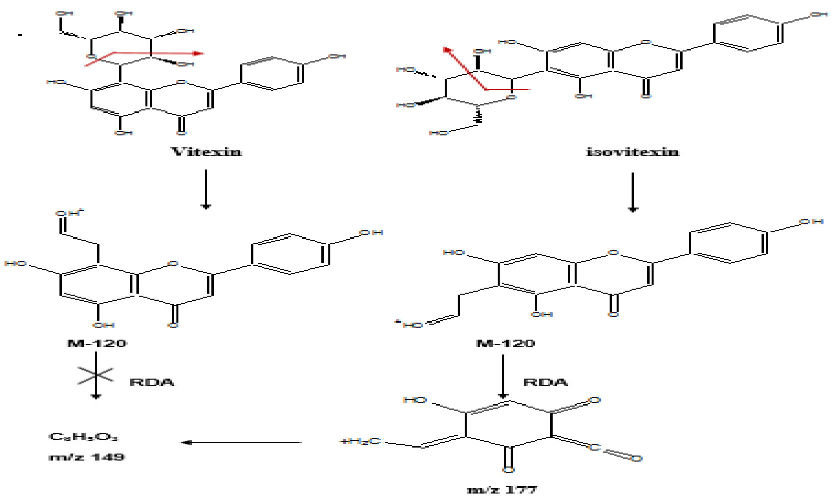

Fig. 4.9: Distinguishing fragmentation pattern of vitexin and isovitexin

CONCLUSION
In conclusion, four flavonoids quercetin, kaempferol 3-O-rhamnoside, quercetin 3-O-rhamnoside (quercitrin) and vitexin have been isolated and characterized by LC/MS/MS analysis, from the leaves of *C. quadrangularis*. The newly reported phytoconstituents can serve as important marker for the standardization of *C. quadrangularis* extracts and its formulations.

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Author(s)' Statement Competing Interests*:* The authors declares no conflict of interest.

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