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Isolation of Compounds from the Aqueous Methanol Extract of *Cissus javana* DC Leaves and Determination of its Trace Element Content Through Wet Digestion

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A new alcohol was isolated from the aqueous methanol extract of the leaves of *Cissus javana* DC which is widely used in Manipur, India for dissolution and expulsion of kidney stones. In addition, known compounds Stigmasterol, Stigmasterol glucoside, Onocer-7-ene 3α,21β-diol, β-amyrin-[olean12(13)-en-3-one] were also isolated from the same methanol extract. Analysis of trace element contents of the plant leaf reflects its role in the ethno medicinal property of the plant. It was found that the plant leaf have exceptionally high calcium (2960 mg/100 g), magnesium (465 mg/100 g) and iron (520 mg/100 g) content.

Keywords: *Cissus*, Methanol extract, Kidney stone, Dissolution, Trace element.

INTRODUCTION

All ethnic groups have been used plants as a source of medicines. Traditional medicines are still an incredible part of primary healthcare in the developing countries. In the developed countries too, people are turning towards herbal treatment because of its low side effect as compare to synthetic drugs. Manipur, a north-eastern hilly state of India, had a great diverged flora and fauna because of its region in the biodiversity hot spots of the world and of its microclimatic conditions. Though the people of Manipur had a rich knowledge of traditional medicines from the plant sources and practiced them for their primary healthcare, but still many plants having ethno medicinal values remain unexplored. The present work reports the active chemical constituents from aqueous methanol extract of the leaves of *Cissus javana* DC. The leaves of *Cissus javana* DC have a sour taste and are a kind vegetable menu in Manipur. The plant itself is a weak perennial climber with a woody base[1,2]. Stems are prominently red and hairless and tendrils are forked. The leaves are ovate lance shaped, with a heart shaped base and pointed tip. The leaves are usually spotted with aluminum white above and purple on the underside. The aqueous boiled extract of the leaves are generally used in the traditional treatment for the dissolution and expulsion of stones in the kidney and its tract. The leaf decoction of the plant is also used in joint pains and healing of fracture of bones.

The kidney stone case is a serious clinical condition which may leads to major causes for acute and chronic renal failure. It is very common in man than woman but rare in children[3]. The main constituents of stones from the kidney and its tract are calcium oxalate, calcium phosphate, struvite stones which is caused by urinary tract infection, cystine stones which is caused by defect in protein metabolism, Uric acid stone[4]. In 1-5 % of the world population, the root cause of formation of stone is multifactorial and some factors that can induced stone formation may be dehydration, urinary tract infection by micro-organisms, alkaline pH and abnormality in calcium phosphate metabolism[5]. A low level of natural inhibitors of calculogenesis in urine is also another factor that tends to urolithiasis.

The kidney stone formation is influenced by the dietary habits. A diet low in calcium is more influencing in stone formation than a diet high in calcium[6]. Also ingestion of diet containing high amount of oxalate enhances stone formation than a diet containing more calcium. That is, oxalate is a very strong promoter of calcium oxalate precipitation, about 15 times stronger, than calcium. Low magnesium intake has been linked to an increase in development of kidney stone by increasing the solubility of calcium oxalate stone[7]. Therefore, a clear knowledge of elemental content of medicinal plants is highly essential to supplement their ethno medicinal value. The elemental contents particularly calcium and magnesium for those medicinal plants having antilithiatic property are very helpful in the evaluation of their action. Generally, the elemental content is determined by atomic absorption and atomic emission spectroscopy of the extract of leaves of the plant obtained by wet digestion method[8].

In the present investigation, the chemical components present in the aqueous methanol extract were isolated through different chromatographic techniques and purified. The spectra of the purified compounds were then used to construct the structures of the compounds which were then compared with the authentic data. Finally, the spectral data and structures of the compounds as well as the trace element contents were reported.

EXPERIMENTAL

Melting points of the isolated compounds were determined in capillary tube using digital auto melting point apparatus (Laptronics). Infrared (IR) spectra were recorded on an ATI Mattson Genesis FTIR spectrophotometer. All samples were run as thin film produced by mixing with KBr. Absorption maxima were recorded in wave number (cm^{-1}). Proton nuclear magnetic resonance (^1H NMR) were recorded on Varian unity 500 (500 MHz), Bruker AC 300 and varian XL (300 MHz) spectrometers. ^{13}C NMR spectra were recorded on Bruker AC 300 Varian XL(75) MHz spectrometer. Residual non-deuterated solvent was used as an internal reference and all chemical shifts (δ_H and δ_C) are quoted in parts per million downfield from tetramethylsilane (TMS). All samples were run in deuterochloroform ($CDCl_3$) and methanol (CD_3OD) as solvent unless otherwise stated. Mass spectra were recorded on a Kratas concept-IS mass spectrometer coupled to a Mach 3 data system or on a Jeol-D 300 mass spectrometer. The atomic absorption spectra were observed in Perkin Elmer, Analyst 200.

Collection of plant material was done during July-September, 2010. The healthy leaves were washed with plain water, dried in air and powdered. Fresh clean leaves were used to determine the moisture content.

Determination of trace elements through wet digestion method: 100 mg of the dried powdered leaves were taken in a 100 mL beaker. After adding 20 mL of concentrated HNO_3, it was kept for overnight. It was boiled gently to oxidize all the oxidizable materials on a hot plate until the production of red NO_2 fumes has ceased. The solution was cooled and 10 mL of 70 % $HClO_4$ was added slowly and boiled again until the solution was colorless and reduced to a small volume. It was cooled; doubled distilled water was added, filtered and diluted up to 100 mL with the same water[9-12].

The concentration of the element in per 100 mL of the sample was calculated by using the relationship:

$$\text{Concentration of the element } \frac{C.V}{10\ W}$$

where C is the spectrophotometric reading given by the spectrophotometer, V is the final volume of dilution and W, the original sample weight taken.

Extraction and isolation of active chemical compounds: The dried powdered leaves of *Cissus javana* DC (3 Kg) were extracted with 50 % aqueous methanol in a soxhlet extractor under hot condition. The extract was distilled under reduced pressure using rotary vacuum evaporator (RII) to produce crude mass which further spread in petriplate and kept in desiccators. The solid crude mass was fractionated through column chromatography technique using 60-80 mesh silica gels as column material and different eluents of increasing polarity. Each fraction were tried to isolate solid compounds by technique of differential solubility. Most of the fractions were found to be low melting yellow fats and greenish oil. Five solid compounds were isolated. Elution of column with 10 % EtOAc with Pet-ether gave CJ1. Further elution of column with 10 % MeOH with chloroform produced CJ2 and CJ3. Elution with 17 % MeOH with $CHCl_3$ yielded CJ4 and with 70-75 % MeOH with $CHCl_3$ produced CJ5.

Stigmasterol glucoside: Pale white amorphous powder, m.p. 260 °C (decomposed), IR (KBr, ν_{max}, cm^{-1}): 3 408, 2959, 2360, 1582, 1165, 1073, 800; ^1H NMR: δ_H0.68 (s, H-18), 0.82 (d, *J* = 7Hz, H-27&29),0.85 (d, *J* = 6Hz, H-26), 0.87 (s, H-9), 0.91 (br, H-24), 0.93 (d, *J* = 6Hz, H-21), 1.01 (s, H-19), 1.03 (s, H-15), 1.06 (m, H-14&28), 1.13 (m, H-17), 1.15 (m, H-12), 1.48 (m, H-7), 1.54 (brs, H-14), 1.56 (m, H-25), 1.87 (br, H-16), 2.37 (m, H-1), 3.23 (m, H-2′), 3.35 (m, H-4′), 3.43 (m, H-3), 3.58 (m, H-3′), 3.76 (m, H-5′), 3.80 (m, H-6′), 4.39 (d, *J* = 8Hz, H-1′), 5.05 (m, H-23), 5.14 (m, H-22), 5.37 (br, s, H-6), ^{13}C NMR ($CDCl_3$: CH_2OD = 4:1): δ_C37.6 (C-1), 32.9 (C-2), 78.2 (C-3), 36.2 (C-4), 139.3 (C-5), 121.2 (C-6), 30.9 (C-7), 30.9 (C-8), 49.2 (C-9), 35.7 (C-10), 220 (C-11), 38.7 (C-12), 41.1 (C-13), 55.7 (C-14), 25.0 (C-15), 28.5 (C-16), 55 (C-17), 10.8 (C-18), (C-19), 35.1 (C-20), 18.7 (C-21), 137.3 (C-22), 128.9, 18.2 (C-23), 44.8 (C-24), 28.1 (C-25), 17.7 (C-26), 18.0 (C-27), 23.8 (C-28), 10.9 (C-29),100.1(C-1′), 72.5 (C-2′), 75.4 (C-3′), 72.5 (C-4′), 74.8 (C-5′), 60.6 (C-6′); Mass: *m/z* 397 [M + 1)-Glucose]$^+$.

***Cissus javanol*, CJ4 (Fig. 1):** White amorphous, m.p. 147 °C; IR (KBr, ν_{max}, cm^{-1}): 3390, 3227, 2959, 1695, 1614, 1471. ^1H NMR (CD_3OD): δ_H3.42, 3.46, 3.66, 3.68, 3.71, 3.73, 3.80, 3.83, 3.89 (OCH_3); 4.04, 4.06, 4.08, ^{13}C (CD_3OD): δ_C 60.9 (OCH_3), 62.6 (=CH_2), 71.8 (CH), 74.2 (CH), 75.6 (CH), 81.4 (CH), 83 (CH),111 (C3, OH), 117.3 (C6), 119.4 (C5, CH-OH), 142.2 (C4), 149 (C2, H_2C=C<), 152.3 (C7, >C=CH), 165.8 (CO), Mass: *m/z* 324 [M + Na + K].

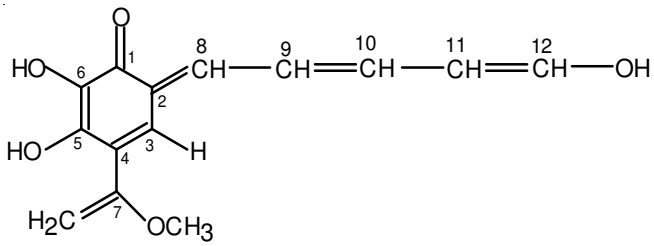

Fig. 1. Structure of *Cissus javanol*, CJ4

RESULTS AND DISCUSSION

Five compounds were isolated from the aqueous methanol extract of the leaves of *Cissus javana* DC. When the aqueous methanol extract was eluted with petroleum ether-ethyl acetate (90: 10 v/v), white fibrous crystalline, CJ1, was produced which was extracted and purified by treatment with acetone. The structure of CJ1 was determined by its IR, ^1H NMR, ^{13}C NMR spectral data. Peak at 3408 cm^{-1} in IR showed the presence of OH group. The δ_H 0.68, 0.82, 0.85, 0.93 and 1.01 in the ^1H NMR spectrum showed peaks due to H-18, H-27 & 29, H-26, H-21, H-19 respectively. The olefinic protons at H-22 and H-23 showed the peaks at δ_H 5.14 & 5.05. The protons correspon-

ding to the sugar moiety showed the peaks at δ_H 4.39, 3.23, 3.58, 3.35, 3.76 and 3.80 respectively. The ^{13}C NMR spectrum of CJ1 showed at δ_C10.8, 18.2, 17.7, 18 and 10.9 due to methyl groups at C-8, C-19, C-21, C-26, C-29 respectively. The olefinic C-atoms at C-5, C-6, C-22 and C-23 showed the peaks at δ_C 139.3, 121.2, 137.3 and 128.9 respectively. The mass spectrum at *m/z* 397 due to [(M + 1)-Glucose]. Thus, from all the above spectral data, the structure of compound, CJ1 was assigned as Stigmasterol glucoside which was confirmed by comparing the MS,^1H & ^{13}C NMR spectral data with those of reported in the literature and by taking the authentic sample.

The compound CJ2 was obtained by eluting the column with chloroform-methanol (90: 10 v/v) and by further purification through solvent treatment. The colorless CJ2 (m.p. 210 °C) showed peaks at 3352, 2957, 1460 cm^{-1}. The ^1H NMR spectrum of CJ2 showed the presence of six tertiary CH_3-groups on saturated Cs with the δ_H 0.71, 0.81, 0.86, 1.03, 1.27 and 1.52. The spectrum also showed δ_H 0.94 and δ_H 2.02 due to one secondary CH_3 protons on a saturated C and one olefinic CH_3 respectively. The ^1H NMR spectrum also exhibited two deshielded methine protons each bearing an OH group at δ 3.72 & 3.86; and the OH protons are observed at δ 4.73 & 5.38. Long range coupling of the olefinic proton at δ 4.41 (d) showed that possibility of an olefinic CH_3 was coupled with the olefinic proton. The presence of an onocerane skeleton was demonstrated by the appearance of an olefinic methyl at δ 2.02 & an olefinic proton at δ 4.41. Thus, from the spectral data, compound CJ2 was found to be Onocer-7-ene-3α, 21β-diol which was confirmed by comparing with the authentic compound.

The CJ3 was obtained by eluting the column with the solvent mixture MeOH: $CHCl_3$ (10: 90) v/v. It is a pale yellowish crystal, m.p. 297 °C. The strong absorption at 3408 cm^{-1} showed the presence of OH group. ^1H NMR spectrum showed signals of CH_3 groups at δ_H 0.68, 0.82, 0.85, 0.93, 1.01 due to H-18, H-27 & 29, H-26, H-21 and H-19 respectively. The olefinic protons at H-22 and H-23 showed signals at δ_H5.14 and 5.05 respectively. The ^{13}C NMR showed peaks at δ_C10.8, 18.2, 17.7, 18, 10.9 due to C-8, C-19, C21, C-26 and C-27 & 29 respectively. The olefinic carbon showed peaks at 139.3 (C-5), 121.2 (C-6), 137.3 (C-22) and 128.9 (C-23).

The compound CJ4 was obtained by eluting the column with chloroform-methanol (83: 17 v/v) mixture. On standing the concentrated fractions undisturbed for about 10 days, the white amorphous substance was obtained which was further washed with acetone methanol mixture. The highly pured compound, m.p. 147 °C, showed peaks at 3390, 3227, 2959, 1695, 1614 and 1471 cm^{-1}. The ^1H NMR spectrum of CJ4 showed peaks at 3.42, 3.46, 3.66, 3.68, 3.71, 3.73, 3.80, 3.83, 3.89 (OCH_3), 4.04, 4.06, 4.08. The ^{13}C NMR(CD_3OD) gave peak at δ_C 60.9 for -OCH_3 group and δ_C 62.6 for =CH_2 group. The peaks at δ 71.8, 74.2, 75.6, 81.4 and 83 were due to -CH groups. Peaks at 111 (CH-OH) for C-3, 117.3 (CH-OH) for C-6, 119.4 (CH-OH) for C-5, 142.2 for C-4, 149.4 (H_2C=C <) for C-2, 152.3 (> C=CH) for C-7 were also obtained. The δ 165.8 value showed the presence of CO group. The compound CJ4 showed fragmentation at *m/z* 324.

From the spectral data, compound CJ4 was found to be alcohol and is a new compound. Its name was assigned as *Cissus javanol*.

The CJ5, a greenish grey amorphous substance m.p. 200 °C was obtained from the fraction eluted with 30-70 v/v of chloroform-methanol mixture and by further purification. By comparing the spectral data with the authentic data, it was confirmed to be β-amyrin[olean-12(13)-en-3-one] which is a known compound. In addition to the above five compounds, waxy yellowish substances and greenish oil were also obtained.

The atomic absorption spectroscopic studies (Perkin Elmer, Analyst-AA200) revealed that the leaves of *Cissus javana* DC contain a high amount of calcium (2960 ± 0.07 mg/100 g). The leave also riches in iron (520 ± 0.08 mg/100 g) and magnesium (465 ± 0.02 mg/100 g). It was also found to contain manganese (63 ± 0.08 mg), zinc (26 ± 0.07 mg) and trace of copper (3 ± 0.02 mg) per 100 g of dried leaves.

The high calcium content of the leaf augmented its traditional usefulness in the healing up of bone fracture. The calcium content of the leaf also showed its antilithiatic property since calcium is a competitive inhibitor of oxalate in calcium oxalate stone formation. The high iron content of the leaf may be useful in anemic patient, in pregnant and lactating woman.

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