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Research Article

Secondary Metabolites from the Heart Wood of *Combretum albidum* G Don.

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ABSTRACT
The liana *Combretum albidum* G. Don is an extensive woody climber occupying the canopy of host tree, belonging to the family combretaceae and its distribution is restricted in semi evergreen and deciduous forests, along river banks of peninsular India and Sri Lanka. Phytochemical exploration of a heartwood extract from *Combretum albidum* afforded five triterpenoids namely ursolic acid, oleanolic acid, betulinic acid, arjunolic acid and betulin. The heart wood of the plant also yields beta sitosterol, gallic acid and ellagic acid as other constituents. The compounds oleanolic acid, betulinic acid, arjunolic acid, betulin and ellagic acid were isolated and reported for the first time from this plant. The compounds ursolic acid, beta sitosterol and gallic acid isolated were characterized for the first time from this plant using spectroscopic methods. The structures of all compounds were confirmed on the basis of spectroscopic and available literature data.

Keywords: *Combreum albidum*, local medicinal plant, five triterpenoids, beta sitosterol, gallic acid, ellagic acid.

INTRODUCTION

The *Combretum albidum* G. Don belonging to the family combretaceae, commonly known as Buffalo calf in English, Karalankody in Malayalam and Vragay in Tamil. Its distribution is restricted in semi evergreen and deciduous forests, along the river banks of peninsular India and Sri Lanka[1]. Decoction of its fruit is reported as a remedy for the treatment of diarrhea, dysentery[2] and for cough cure[3]. Stem bark aqueous extract is reported as a remedy for jaundice[1]. Leaf of the plant is reported to cure wounds and cuts[4] and its wiry stem, seed oil, root reported to cure eye problems, eczema and malarial fever[5]. In traditional practice a number of such plants have been used against various ailments, which are very effective, but the use and therapeutic efficacy of many of those plants are not yet been documented or scientifically validated. Herbal drugs play an important role in health care programs especially in developing countries. However obstacle behind the acceptance of alternative medicines in developed countries is the lack of documentation and stringent quality control. Therefore, the documentation of chemical constituents of the raw materials used in herbal medicine is very essential for the worldwide acceptance of this system of medicine. Though the Pharmacognostic standardization, physicochemical analysis and preliminary phytochemical studies of the plant were reported by several researchers[6-8], no systematic investigation has been carried out on the chemical constituents of the plant. Tentative identification of ursolic acid, beta sitosterol and gallic acid using TLC and qualitative assay is the only work reported from the plant about its chemical constituents[9]. Hence the present investigation deals with the exploration of bioactive constituents, its isolation and characterization from the heart wood of *C albidum*.

MATERIALS AND METHODS

General

UV-Visible spectrum was measured using Shimadzu 1700, Japan spectrophotometer, NMR studies were conducted on Bruker 400 MHz spectrometer, and HRMS studies were conducted using WATERS Acquity HPLC coupled to Xevo G2-QTOF and IR analysis were done on Shimadzu Prestige-21. HPLC studies were carried out on Shimadzu LC-20AD equipped with Prominence degauzzer DGU-$20A_5$, PDA detector equipped with Phenomenex Luna 5µ C18 (2) column of dimensions 250 X 4.6 mm. Flash chromatographic isolation carried out using automated Yamazen AI580S Flash chromatography system equipped with Universal premium column of size 5L. All the authentic samples were procured from sigma Aldrich, Bangalore. All the solvents used were double distilled and dried before use.

Plant materials

The stem bark, heart wood and leaf of the plant *C. albidum* were collected from Vavanur, Palakkad district, Kerala. The plant was authenticated at Taxonomy Division, Centre for Medicinal Plants Research (CMPR), Arya Vaidya Sala, Kottakkal, Kerala, India. The voucher specimens and raw drugs have been deposited at CMPR herbarium and raw drug museum respectively for future reference.

Preparation of extract

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Shade dried powdered drug samples (4 kg) extracted with methanol by cold maceration technique for 48 hrs. Filtered, maximum solvent removed by distillation and the extract dissolved in ethyl acetate and insoluble were separated by filtration. Then the ethyl acetate removed by distillation and final traces of solvent under vacuum in a Rota vapor to get the extract.

Isolation of Chemical constituents

The ethyl acetate soluble extract loaded over a silica gel (1000g) of mesh size 200-400 in a cylindrical column of 1500 mm height and 55 mm dia. The column then eluted with hexane, hexane containing 5%, 10%, 20%, 30%, 50% acetone followed by ethyl acetate, ethyl acetate containing 10%, 20%, 40%, 60% methanol and finally with 100% methanol. Totally 154 fractions of 1000 ml were collected. The fractions 1-8, 9-11, 12-19, 20-30, 31-35, 36-52, 53-60, 61-72, 73-78, 99-104 were pooled based on the TLC analysis. Combined fractions were distilled to remove the solvent and finally under vacuum to remove the final traces of solvent.

Fraction 1-8

It was chromatographed to remove the oily constituents using hexane containing 5% ethyl acetate and 20% ethyl acetate. The fraction eluted with 20% ethyl acetate yielded a needle shaped crystal of compound 1.

Fraction 9-11

The fractions 9-11 containing multi components were purified by preparative HPLC on a ODS column to yield compounds 2, 3, 4 and 5 using methanol and water as a mobile phase.

Fraction 53-60

Fraction 53-60 purified over flash chromatography using hexane containing 20% ethyl acetate which yielded a white solid compound 6 on keeping in acetone.

Fraction 73-78

Fraction 73-78 on TLC screening, showed a major compound having absorbance in 254nm and 366nm. The compound isolated through flash chromatography using silica column with the solvent system Toluene and ethyl acetate (50:50) yielded a buff colored amorphous powdery material. This was recrystallized using hot water to get a white colored amorphous powder.

Fraction 99-104

On TLC screening, it showed a major compound having absorbance in 254nm and 366nm. The compound isolated through flash chromatography using silica column with the solvent system ethyl acetate and methanol (80:20) which gave a buff colored amorphous powdery material. The powdery material on recrystallization with aqueous methanol yielded a pale white colored amorphous powder. The NMR, IR, Mass, TLC and HPLC of the isolated compounds were compared with the reported values and with authentic samples (Sigma Aldrich.)

RESULTS AND DISCUSSION

The ethyl acetate soluble part of methanol extract of heart wood was purified and eight compounds were obtained

Beta-sitosterol (1)

The crystalline material was further purified by recrystallization from methanol to get a colorless needle shape crystal. M.P 135-137 ºC reported value in literature is 135-137 ºC [10]. IR υmax (cm^1): 3373.6, 2940.7, 2867.9, 1457.3, 1641.6, 1381.6, 1038.7, 881.6. ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.69 (3H, *s*, Me-18), 0.81 (3H, *d*, Me-27), 0.83 (3H, *d*, Me-26), 0.85 (3H, *t*, Me-29), 0.92 (3H, *d*, Me-21), 1.01 (3H, *s*, Me-19), 3.52 (1H, *m*, H-3), 2.25 (2H, *m*, H-4), 5.35 (1H, *m*, H-6).

^{13}C-NMR (100 MHz, $CDCl_3$) : δC 12.0 (C-18), 12.2 (C-29), 19.0 (C-21), 19.2 (C-27), 19.6 (C-19), 20.0 (C-26), 21.3 (C-11), 23.2 (C-28), 24.5 (C-15), 26.2 (C-23), 28.4 (C-16), 29.3 (C-25), 31.8 (C-2), 32.1 (C-7), 32.1 (C-8), 34.1 (C-22), 36.3 (C-20), 36.7 (C-10), 37.4 (C-1), 39.9 (C-12), 42.5 (C-13), 42.5 (C-4), 46.0 (C-24), 50.3 (C-9), 56.2 (C-17), 56.9 (C-14), 72.0 (C-3), 121.9 (C-6), 140.9 (C-5). The spectral comparison with literature data[11] and TLC/HPLC of the compound with the authentic sample showed that the compound is beta sitosterol.

Beta sitosterol is well-known natural sterol in composition of known herbal drugs for treatment of benign prostatic hyperplasia and prostate cancer. It also acts as a non-enzymatic antioxidant in cells making it effective anti-diabetic, neuroprotective and chemoprotective agent as well[12].

Betulin (2)

The compound (2) was recrystallized from methanol ant its melting point was found to be 256 ºC and reported for betulin is 256-257 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) : δH 0.76 (3H, *s*, Me-24), 0.82 (3H, *s*, Me-25), 0.96 (3H, *s*, Me-26), 0.97 (3H, *s*, Me-27), 1.01 (3H,*s*, Me-23), 1.67 (3H, *s*, Me-30), 2.37 (1H, *m*, H-19), 3.18 (1H, *dd*, H-3), 3.34 (1H, *d*, Ha-28), 3.78 (1H, *d*, Hb-28), 4.75, 4.67 (each 1H, H2-29).

^{13}C NMR (100 MHz, $CDCl_3$) : δC 14.8 (C- 27), 15.6 (C-26), 16.0 (C-24), 16.1 (C-25), 18.3 (C-6), 19.1 (C-30), 20.9 (C- 11), 25.2 (C-12), 27.0 (C- 15), 27.2 (C-2), 28.0 (C- 23), 29.2 (C-16), 29.7 (C-21), 34.0 (C-7), 34.2 (C-22), 37.2 (C-10), 37.3 (C-13), 38.6 (C-1), 38.8 (C-4), 41.0 (C-8), 42.8 (C-14), 47.8 (C- 19), 47.8 (C-17), 48.8 (C-18), 50.5 (C-9), 55.3 (C-5), 60.6 (C- 8), 79.0 (C-3), 109.7 (C-29), 150.5 (C-20). The spectral comparison with literature data[13] and TLC/HPLC of the compound with the authentic sample showed that the compound is betulin. Betulin is reported to have anti-inflammatory and antitumor activity[14].

Betulinic acid (3)

The compound (3) recrystallized from methanol and its melting point was found to be 284~286 °C, value reported in literature for betulinic acid is 283-285 ºC[15]. IR νmax (cm^{-1}): 3430, 3250, 2939, 2864, 1680, 1640, 1446, 1372, 1190. ^{1}H-NMR (400 MHz, $CDCl_3$) : δH 0.77 (3H, *s*, Me-25), 0.91 (3H, *d*, Me-30), 0.93 (3H, *d*, Me-29), 0.98 (3H, *s*, Me-24), 1.08 (3H, *s*, Me-26), 1.14 (3H, *s*, Me-27), 1.19 (1H, *m*, Ha -22), 1.25 (3H, *s*, Me-23), 2.00 (1H, *dd*, Hb -22), 2.18 (1H, *d*, H-18), 3.21 (1H, *dd*, H-3), 5.28 (1H, *t*, H-12).

^{13}C-NMR (100 MHz, $CDCl_3$) : δC 14.8 (C-25), 15.2 (C-24), 16.4 (C-26), 16.6 (C-11), 18.3 (C-6), 22.8 (C-30), 22.9 (C-29), 23.1 (C-27), 24.1 (C-16), 27.5 (C-2), 28.0 (C-23), 29.5 (C-15), 30.5 (C-21), 33.1 (C-7), 36.9 (C-10), 36.9 (C-22), 38.7 (C-4), 39.2 (C-19), 39.2 (C-20), 39.2 (C-1), 39.6 (C-8), 41.7 (C-14), 47.8 (C-9), 78.5 (C-3), 47.7 (C-17),

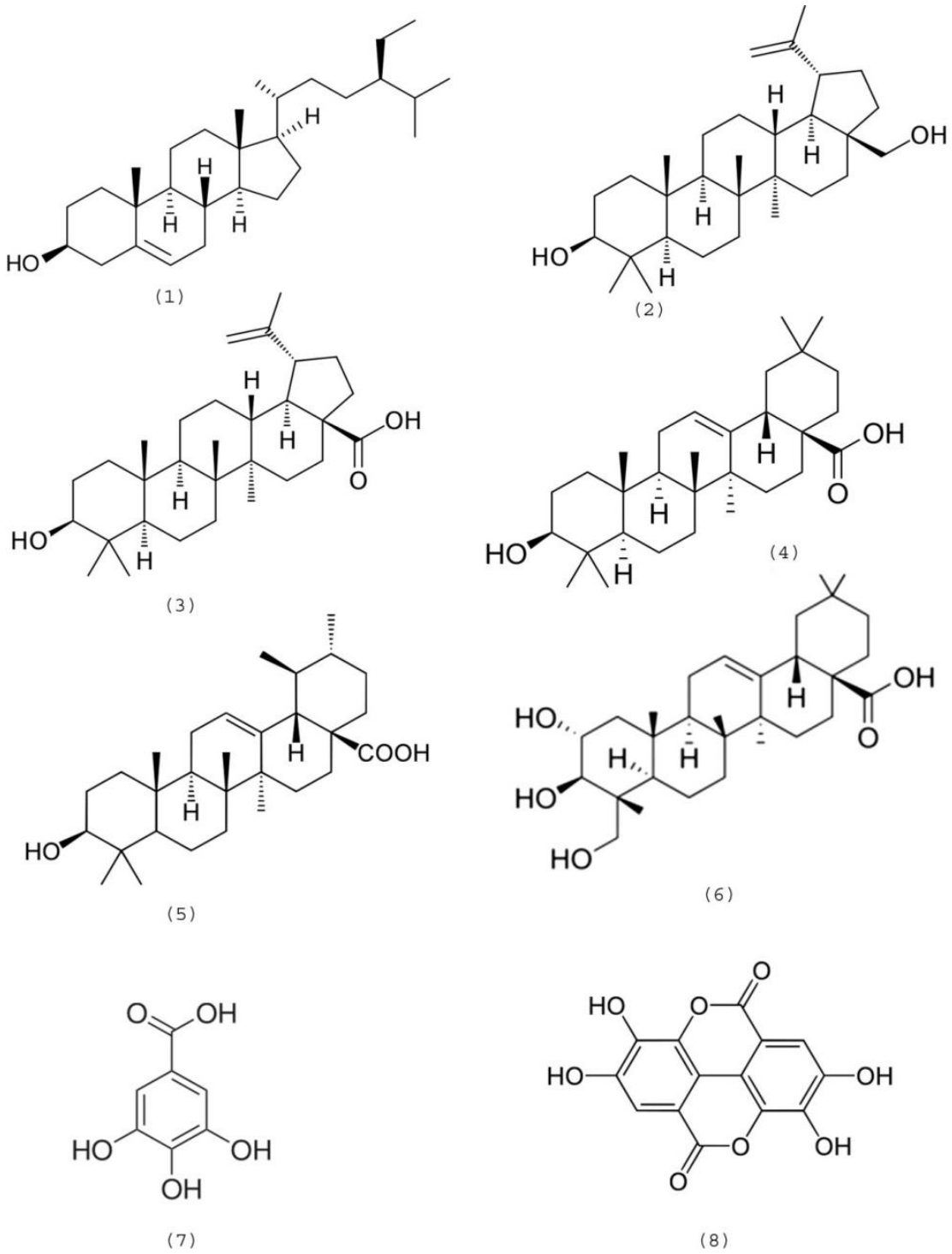

Fig.1: Structures of Isolated Chemical Constituents

53.1 (C-18), 55.5 (C-5), 125.7 (C-12), 138.4 (C-13), 180.4 (C-28). HRMS (M^+H^-) found out as 455.3526, calculated for $C_{30}H_{48}O_3$ is 456.3603. The spectral comparison with literature data[16] and TLC/HPLC of the compound with the authentic sample confirmed that the compound is betulinic acid. Betulinic acid exhibits a variety of biological and medicinal properties such as inhibition of human immunodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antinociceptive, anti-HSV-1, and anti- cancer activities[17].

Oleanolic acid (4)

The compound (4) recrystallized from methanol and its melting point was found to be 306~308 °C reported value in literature for oleanolic acid is 305-306 °C [18]. IR νmax (cm-1): 3429, 2926, 1685, 1450. ^{1}H-NMR (400 MHz, $CDCl_3$) : δH 0.77 (3H, *s*, Me-25), 0.91 (3H, *d*, Me-30), 0.93 (3H, *d*, Me-29), 0.98 (3H, *s*, Me-24), 1.08 (3H, *s*, Me-26),

1.14 (3H, *s*, Me-27), 1.19 (1H, *m*, Ha -22), 1.25 (3H, *s*, Me-23), 2.00 (1H, *dd*, Hb -22), 2.18 (1H, *d*, H-18), 3.21 (1H, *dd*, H-3), 5.28 (1H, *t*, H-12).

^{13}C-NMR (100 MHz, $CDCl_3$): δC 14.8 (C-25), 15.2 (C-24), 16.4 (C-26), 16.6 (C-11), 18.3 (C-6), 22.8 (C-30), 22.9 (C-29), 23.1 (C-27), 24.1 (C-16), 27.5 (C-2), 28.0 (C-23), 29.5 (C-15), 30.5 (C-21), 33.1 (C-7), 36.9 (C-22), 36.9 (C-10), 38.7 (C-4), 39.2 (C-19), 39.2 (C-20), 39.2 (C-1), 39.6 (C-8), 41.7 (C-14), 47.7 (C-17), 47.8 (C-9), 53.1 (C-18), 55.5 (C-5), 78.5 (C-3), 125.7 (C-12), 138.4 (C-13), 180.4 (C-28). HRMS (M^+H^-) found as 455.3528, calculated for $C_{30}H_{48}O_3$ is 456.3603. The spectral comparison with literature data[11] and TLC/HPLC of the compound with the authentic sample showed that the compound is oleanolic acid.

Ursolic acid (5)

The compound (5) recrystallized from methanol to get as a white amorphous powder and its melting point was found to be 280~283 °C. The reported melting point of ursolic acid is 283-285 ºC [15]. IR νmax (cm^{-1}): 3424, 2924, 1686, 1451. ^{1}H-NMR (400 MHz, $CDCl_3$) : δH 0.76 (3H, *s*, Me-26), 0.78 (3H, *s*, Me-24), 0.84 (3H, *s*, Me-25), 0.87 (3H, *s*, Me-29), 0.93 (3H, *s*, Me-30), 0.96 (3H, *s*, Me-23), 1.25 (3H, *s*, Me-27), 2.82 (1H, *dd*, H-18), 3.21 (1H, *dd*, H-3), 5.24 (1H, *t*, H-12).

^{13}C-NMR (100 MHz, $CDCl_3$) : δC 14.7 (C-24), 15.1 (C-25), 16.5 (C-26), 18.3 (C-6), 22.7 (C-11), 22.8 (C-16), 23.3 (C-30), 25.2 (C-27), 26.7 (C-2), 27.7 (C-15), 28.8 (C-23), 30.4 (C-20), 32.3 (C-22), 32.6 (C-7), 32.8 (C-29), 33.7 (C-21), 37.0 (C-10), 38.6 (C-1), 39.2 (C-4), 39.6 (C-8), 41.5 (C-18), 42.0 (C-14), 46.1 (C-19), 46.7 (C-17), 48.1 (C-9), 55.5 (C-5), 78.5 (C-3), 122.4 (C-12), 144.1 (C-13), 180.4 (C-28). HRMS (M^+H^-) found as 455.3529, calculated for $C_{30}H_{48}O_3$ is 456.3603. The spectral comparison with literature data[11] and TLC/HPLC of the compound with the authentic sample confirmed that the compound is ursolic acid.

Oleanolic acid and ursolic acid are triterpenoid compounds that exist widely in medicinal herbs and other plants. Both oleanolic acid and ursolic acid are effective in protection against chemically induced liver injury in laboratory animals. Oleanolic acid has been marketed in China as an oral drug for human liver disorders. The mechanism of hepato protection by these two compounds may involve the inhibition of toxicant activation and the enhancement of the body defense systems. Oleanolic acid and ursolic acid have also been long-recognized to have properties like antioxidant, anti-inflammatory and antihyperlipidemic etc[19].

Arjunolic acid (6)

The white powdery mass recrystallized from methanol showed the following spectral characteristics. Mp: 338-340 °C reported in literature is 338 ºC [20]; IR υmax (cm^{-1}) 3395, 2929, 1496, 1699, 1290, 1049. ^{1}H-NMR (400MHz, DMSO-d6) δH: 0.71 (3H, *s*, CH_3), 0.87 (3H, *s*, CH_3), 0.92 (3H, *s*, CH_3), 1.10 (3H, *s*, CH_3), 1.98–0.74 (terpenoid protons, 26H), 2.74 (1H, *dd*, CH), 3.04 (1H, *d*, OCH), 3.17 (1H, *d*, OCH), 3.31 (2H, *d*, OCH_2), 4.05–4.50 (broad peak, 3H, OH), 5.17 (1H, *m*, HC), 11.97 (1H, *s*, COOH).

^{13}C-NMR (100 MHz, DMSO) δC: 14.3 (C-24), 17.3 (C-25), 17.5 (C-26), 18.5 (C-6), 23.6 (C-11), 23.7 (C-30), 23.9 (C-16), 26.1 (C-27), 28.2 (C-15), 30.9 (C-20), 32.8 (C-7), 33.2 (C-22), 33.2 (C-29), 34.1 (C-21), 38.4 (C-10), 39.8 (C-8), 41.9 (C-18), 42.2 (C-14), 43.6 (C-4), 46.3 (C-19), 46.6 (C-17), 47.6 (C-1), 47.9 (C-5), 48.1 (C-9), 66.4 (C-23), 68.8 (C-2), 78.2 (C-3), 122.4 (C-12), 144.8 (C-13), 180.2 (C-28); HRMS (M^+H^-) found as 487.3429, calculated for $C_{30}H_{48}O_5$ is 488.3501. The spectral comparison with the available literature data [21] and TLC/HPLC of the compound with the authentic sample showed that the compound is arjunolic acid.

The multifunctional therapeutic application of arjunolic acid has already been documented by its various biological functions including cardio protective, hepato protective, antioxidant, anti-fungal, anti-bacterial, anticholinesterase, antitumor, antiasthmatic, wound healing and insect growth inhibitor activities[22].

Gallic acid (7)

The compound gave a bluish green color with ferric chloride suggesting being a phenolic compound. The melting point of isolated compound was found to be 250-253 ºC, melting point reported for gallic acid is 251-253 ºC [23]; UV λ max 215, 271 nm (methanol); IR υmax (cm^{-1}): 3367, 3064, 2654-2907, 1702, 1618, 1541, 1246, 1026. ^{1}H NMR: (400MHZ, $CDCl_3$): δH 3.37-3.86 (4H, *d*, OH), 6.96 (2H, *s*, Ar-H), 9.21 (1H, *s*, COOH). ^{13}C NMR (100MHZ, $CDCl_3$): δC 109.042, 120.75, 145.65 (Ar-C), 167.84 (COOH), 178.29 (Ar-C). The spectral comparison with the available literature data [23] and TLC/HPLC of the compound with the authentic sample showed that the compound is gallic acid. Gallic acid has several biological activities including antioxidant, antityrosinase, antimicrobial, antiinflammatory and anticancer activities[24].

Ellagic acid (8)

This also gave a bluish green color with ferric chloride suggesting the compound to be phenolic in nature. The melting point of compound (8) was found to be 317 ºC, the melting point reported for ellagic acid is 317 ºC[23]; UV λ max 253, 364 nm (methanol); IR υmax (cm^{-1}): 3556, 1699, 1618, 1508, 1192. ^{1}H NMR: (400MHZ, $CDCl_3$): δH 7.5 (2H, *s*, aromatic H), 2.7 - 4.4 (4H, 4OH). ^{13}C NMR (100MHZ, $CDCl_3$): δC 110.07 (C6andC13), 112.21 (C5andC12), 136.30 (C2), 140.07 (C3andC10), 148.03 (C4andC11), 159.00 (C7andC14). The spectral comparison with the available literature data[23] and TLC/HPLC of the compound with the authentic sample showed that the compound is ellagic acid. Ellagic acid has a variety of benefits for their anti-mutagenic, antimicrobial, antioxidant properties and inhibitors of human immunodeficiency virus (HIV) [25].

Thus the isolated compounds were confirmed as β-sitosterol(1), betulin(2), betulinic acid(3), oleanolic acid(4), ursolic acid(5), arjunolic acid(6), gallic acid(7) and ellagic acid(8) (Fig-1) using different analytical and spectral methods like UV, IR, ^{1}HNMR, ^{13}CNMR and Mass.

CONCLUSION

The compounds betulin, betulinic acid, oleanolic acid, arjunolic acid and ellagic were reported for the first time from the plant. The isolated compounds were well known for its hepato protective activity and clinically documented by several literatures. The practice of tribes in the area of Chinnar to cure jaundice with stem bark and stem of *C. albidum* is proven to be beneficial with the presence of hepato protective constituents in the plant. The plant can also be used for other biological activities like cardioprotective, antibacterial, antimalarial, anticancer etc., as the isolated compounds are well known for these properties too. However to the best of our knowledge this is for the first time that the chemical constituents were isolated and reported with sufficient spectroscopic data from the plant.

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