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Phytochemical screening, isolation of betulinic acid, trigonelline and evaluation of heavy metals ion content of *Doliocarpus dentatus*

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ABSTRACT

The stems of *Doliocarpus dentatus* (Kapadulla), a plant of the Guyana flora were screened for natural product. Phytochemical screening revealed the presence of sterols, triterpenes, coumarins and reducing compounds for the C_6H_{14} extract. From the CH_2Cl_2 extract, an off white solid crystallized and further chromatographic purification yielded betulinic acid. The EtOAc extract showed the presence of emodols, tannins, flavones, reducing compounds and alkaloid salt. Flash column chromatography furnished Trigonelline as one of the major fractions. Trigonelline has never been isolated from *Doliocarpus dentatus.* Emodols, Tannins, Flavones and other reducing compounds were detected in the CH_3CH_2OH extract. For the hydrolysed CH_3CH_2OH extract, positive tests were noted for anthrasenosides and coumarins. Metal ion determination via Atomic Absorption Spectroscopy revealed selective presence of metals in the CH_3CH_2OH and EtOAc extracts. The structural integrity of the two compounds isolated were established via ^{1}H NMR, ^{13}CNMR, DEPT-135, ^{1}H-^{1}H COSY, HMQC and HMBC.

Keywords: Phytochemical screening; Solvent type extracts; Hydrolysed ethanol extract; Betulinic acid; Trigonelline.

INTRODUCTION

Natural products, the secondary metabolites of plants and animals are of restricted occurrence and are classified into major groups such as sterols, triterpenes, flavones aglycones, emodols (anthracenosides aglycones), coumarins, coumarins lactone derivatives, tannins (gallic), reducing compounds, sterols glycosides, cardenolides, saponins and sapogenins etc. They vary widely in both type and concentrations in different parts of the plant and are found as minor components of plant tissues (Mann, et. al, 1986., Mann, J., 1994). Medicinally, isolated natural products after been subjected to clinical trials can be used as drugs for the treatment of cancer (Crow et. al, 2008), antimicrobial agents (Woldemichael, et. al, 2003; Jagessar, et. al, 2008, Prasad et. al, 2010)' antitumor (Flores, et. al, 2010), antioxidant agents (Manga, et. al, 2006; Kukac, et. al, 2008; Nile, et. al, 2010)

Many pharmaceutical drugs synthesized to date are due to the mimic of structures from isolated natural products, rather than to total imagination and

creativity of contemporary organic chemist. For example, *Cyanthiwigin F*, a complex *bis* molecule active against tumours was first isolated from the Jamaica sea sponge *Myrmekioderma styx (*Enquist, et. al, 2008). The diterpenoid taxol, first isolated from the bark of the yew tree *Taxus brevifolia* has yielded two approved drugs for breast and ovarian cancer (Hadlington, et. al, 2006) and tomatoes use saponins to defend themselves against fungus attack (Sanderson, 2005). These compounds are now synthesized by many Pharmaceutical companies worldwide.

Also, crude plant extracts can be subjected to chromatographic separation, leading to the isolation, purification and structural elucidation of new and unknown bioactive natural products whose medicinal activity can be compared with that of the solvent type crude extract (Aponte, et. al, 2008; Manga, et.al, 2006; Wafo, 2010). There are several reports on Phytochemical screening (Audu, et.al, 2007; Prasad, et.al, 2008; Rao, et. al, 2011)

Doliocarpus dentatus (Kapadulla), is a forested climber to 16m from the diverse flora of Guyana. The stems and bark are used as a tea and have aphrodisiac properties. The plant is often used as a tonic with other plants, i.e. Sasparilla (*Philodendron fragrantissimum*), Devil-doer (*Strychnos*), Cockshun (*Smilax Schomburgkiana*), Kupa-rope (*Clusia grandiflora*) and Monkey-ladder (*Bauhinia scala-simiae*). A liqueur is produced from these plants and sold in market. The watery sap from a freshly cut stem is drunk for the relief of stricture or simply as a thirst-quencher (White, et. al, 1992).

Present work deals with the phytochemical screening of the C_6H_{14}, CH_2Cl_2, EtOAc, hydrolysed and non- hydrolysed CH_3CH_2OH extract of *Doliocarpus dentatus* Also, the isolation and structural elucidation of betulinic acid, Trigonelline and the concentration of heavy metal ion content in the EtOAc and CH_3CH_2OH extracts are reported. *Doliocarpus dentatus* was chosen, because of its folkloric values and since prior to this work, there is no documentation of phytochemical screening and evaluation of heavy metal ion concentration of *Doliocarpus dentatus* extracts in the literature. Also, Trigonelline has never been isolated from *Doliocarpus dentatus.*

The isolation and biological activities of some other constituents are noted. For example, cytotoxic metabolites such as betulinic acid, nataloe-emodin, bisnordihydrotoxyferine,2',4'-dihydroxy-6'-methoxy-3,4-methylene dioxy dihydro chalcone,2',4'-dihydroxy-4,6'-dimethoxydihydrochalcone and 12- O-tetradecanoyl phorbol-13-acetate were isolated from Peruvian *Doliocarpus dentatus (*Aponte, et. al., 2008). Leishmanicidal triterpenes (betulinic acid, betulin and betulinaaldehyde) and lignans (as (+) pinoresinol, (+) medioresinol and (−) lirioresinol B were also isolated. The antiproliferative bioassay-guided fractionation of *Doliocarpus dentatus* is also reported (Sauvain, et. al, 1996).

MATERIALS AND METHODS

Instrumentation: Melting points were measured on a Geahaka model PF 1500 version apparatus and are uncorrected. ^1H and ^{13}C NMR, ^{13}C- DEPT 135 NMR, COSY, HMQC and HMBC spectra were recorded on a Bruker DRX-500 spectrophotometer using $CDCl_3$ as the solvent. Chemical shifts are quoted in δ ppm with reference to TMS internal standard and coupling constants (*J)* expressed in Hertz (Hz). HREIMS and EIMS were recorded on a KRATOS/AEI MS-50 spectrometer. Silica gel 60A (70-230 mesh, Merck) was used for flash column chromatography (CC). Solvents: C_6H_{14}, CH_2Cl_2, EtOAc and Na_2SO_4 were purchased from Sigma Aldrich Company (USA) and were redistilled prior to use. Analytical TLC analyses were done on precoated Kiesegel 60 F_{254} (Merck) plates and were 0.25 mm thick.

TLC plates were viewed under a UV lamp, (Spectroline Longlife Filter) and developed chromatograms were visualized via initial spraying with iodine.

Plant Material: *Doliocarpus dentatus* (Family-*Dilleniaceae*) stems were collected from a commercial planter in the interior (remote) area of Guyana, a country with a highly biodiversified forest. Stems were freed of visible fungal and bacterial infection. Products of microbial synthesis might result in added chemical composition of the plant material and inspection was important. Stems were stored in plastic bags, sealed, labeled and transported to the University of Guyana. It was identified by a Taxonomist at the Bio Diversity Centre of the University. A voucher specimen (# 000418) is deposited in the herbarium of the Bio Diversity centre. Stems were then subjected to aerial drying for a week and were later ground into a fine powder (250g) using a grinding mill, Arthur H. Thomas Co. model (# 750611).

Plant extracts: The extraction was done in extraction jars at ambient temperature using sequentially freshly distilled C_6H_{12}, CH_2Cl_2, EtOAc and CH_3CH_2OH. For each solvent type, three extractions were done and each extract was filtered and dried over anhydrous Na_2SO_4 and solvents removed in *vacuo* to yield viscous oils and semi solids. From the viscous CH_2Cl_2 extract, a solid crystallized out and was further recrystallised using CH_2Cl_2/Hexane yielding an off white solid. The physical nature of the C_6H_{14}, EtOAc and CH_3CH_2OH extract was oil, red brown solid and a black gummy material respectively.

***Isolation of betulinic acid (3β-hydroxy-20(29)-lupaene-28-ore-acid, $C_{30}H_{48}O_3$)*:** The solid which crystallised from the CH_2Cl_2 extract was subjected to crystalization/ recrystalisation from CH_2Cl_2/Hexane, yielding an off white solid which was further purified via flash column chromatography on silica gel using the eluent C_6H_{14}/EtOAc followed with gradient elution using increasing concentration of EtOAc. Solvents were removed in *vacuo* from the second major band, providing betulinic acid as a white crystalline solid with an R_f of 0.29 (C_6H_{14}/EtOAc), m.p: 295-296°C.

^{1}H NMR ($CDCl_3$, 500MHz) δ: 0.647 (s, CH_3), 0.761 (s, CH_3), 0.865 (s, CH_3), 0.894 (s, CH_3) 0.929 (s, CH_3), 1.643 (s, CH_3), 1.086-1.261 (m), 1.237-1.396 (m), 1.436-1.54 (m), 1.802 (m), 2.1 (m), 2.2 (m), 2.948 (m), 4.56 (brd; 1H), 4.689 (brd; 1H); **^{13}C NMR : ($CDCl_3$, 500MHZ)** δ:177.71 (C-28), 150.79 (C-20) , 110.13(C-30), 77.22(C-3), 55.87(C-17), 54.34(C-5), 50.37(C-9), 48.98 (C-19), 47.08(C-18), 42.46 (C-14), 40.8 (C-8), 38.962 (C-1), 38.705 (C-4), 38.047(C-13), 37.18(C-10), 34.36 (C-7), 32.16(C-16), 30.55(C-15), 29.67(C-21), 28.57(C-23), 27.62(C-2), 25.53(C-12), 20.90(C-11), 18.42(C-6), 16.19(C-24), 16.28 (C-25), 16.19 (C-26), 14.85(C-27); **DEPT 135** (**500MHz, $CDCl_3$**) δ:109.57 (CH_2), 77.22 (CH), 55.34 (CH), 50.37 (CH), 48.98 (CH),47.08 (CH), 42.46 (CH), 38.05(CH), 36.784 (CH_2), 32.16 (CH_2), 30.55 (CH_2), 29.67 (CH_2), 28.57 (CH_3), 27.62 (CH_2), 25.53 (CH_2), 20.90 (CH_2), 18.42 (CH_2), 16.41 (CH_3), 16.29 (CH_3), 16.19 (CH_3), 19.4 (CH_3) 14.85 (CH_3); HREIMS m/z [M+] 457.68 (calcd for $C_{30}H_{48}O_3$, 457.69

Isolation of Trigonelline, $C_7H_7NO_2$. The EtOAc extract was purified via flash column chromatography on silica gel using CH_2Cl_2/EtOAc/Hexane as the eluent. Solvents removal from fractions emanating from the fifth band yielded Trigonelline as a white solid. R_f :0.05 (CH_2Cl_2/EtOAc/Hexane); m.p: 258 –259 °C (Hydrochloride); **^{1}H NMR (500MHz**) δ: 4.44 (s, H-7), 8.08 (t, J = 7.6 Hz; H-6), 8.86 (d, J = 6.0Hz, H-5), 8.89 (d, J = 8Hz, H-4), 9.20 (s, H-2); **^{13}CNMR (500MHz, $CDCl_3$)** δ: 53.64 (C-7), 128.856 (C-4), 140.312 (C-3), 146.359 (C-5), 147.215 (C-6), 147.96 (C-2), 167.203 (C-8), **DEPT 135:** 146.52 (CH), 145.75 (CH), 144.96 (CH), 127. 454 (CH), 48.06 (CH_3); **^{1}H-^{1}H COSY ($CDCl_3$, 500MHz):** H-2/H-4.H-5; H-4/H-

5, H-6; H-5/H-4, H-6; H-6/H-4, H-5; **HSQC**: H-7/C-7; H-6/C-6; H-5/C-5; H-4/C-4; H-2/C-2; **HMBC correlations**: H-7/C-6,C-5; H-5/C-3,C-4,C-6, H-4/C-6,H-2/C-6, H-2/C-2

***Elemental analyses*:** 1.0g of the dried solvent type extracts was dissolved in 10ml of the requisite solvent. This was followed with the addition of 1ml of 10% HNO_3. It was shaken to ensure a uniform mixture and then made up to 100ml mark. Appropriate working standard solutions were prepared for each element. The levels of requisite metal elements (K, Cu, Fe, Mn, Co and Zn) were determined using Atomic Absorption Spectrophotometry (AAS) method on a Varian AAS 50 spectrophotometer. First the sample solution was aspirated by a pneumatic nebulizer, transformed into an aerosol, which was introduced into a spray chamber, where it was mixed with the flame gases and conditioned in a way that only the finest aerosol droplets (< 10μm) entered the flame. On top of the spray chamber, a burner head produces a flame that is laterally long (usually 5–10cm) and only a few mm deep. The radiation beam passed through this flame at its longest axis, and the flame gas flow-rates was adjusted to produce the highest concentration of free atoms. The burner height was adjusted, so that the radiation beam passed through the zone of highest atom cloud density in the flame, resulting in the highest sensitivity. The concentration (C) of each analyte in the solutions aspirated was recorded. (Harris, D.C., 1996)

Phytochemical screening for secondary metabolites: On each solvent type extract, test for alkaloids, Saponin glycosides, Cardenolides, Bufadionolides, Flavonoids, Tannins, Polyphenolic compounds, Anthraquinones, Cyanogenic glycosides, Carbohydrates, Fixed oils, Fats, and Volatile oils were carried out using standard Phytochemical methods (Harbourne, et. al, 1988).

***Glycosides*:** A small amount of alcoholic extract was dissolved in 1ml of water and a few drops of aqueous sodium hydroxide solution were added. A yellow colour was taken to signify the presence of glycosides

Tannins: About 0.5g of extract was dissolved in 5 to 10ml of distilled water and was filtered. A few drops of a 5% $FeCl_3$ solution were added to the filtrate. A blue, blue-black, green, or blue-green colour or a precipitate was taken as an indication of the presence of tannins

***Flavonoids*:** A few drops of concentrated hydrochloric acid were added to a small amount of an alcoholic extract of the plant material. Immediate development of a red colour was taken as an indication of the presence of flavonoids.

Sterols and triterpenes

***Liebermann-Burchard Reaction*:** 10ml of the extract was placed in a test tube and evaporated to dryness on a water bath. The residue was dissolved in 1ml of acetic anhydride and 1ml of chloroform. The solution was then transferred to two clean dry test tubes; one served as the reference tube. 1-2ml of conc. H_2SO_4 was added to the other tube using a teat pipette. A violet ring was formed at the two liquids, with the supernatant becoming violet this indicates the presence of sterols and triterpenoids.

***Carotenoids:* (*Carr-Price Reaction*):** 10ml of the extract was added to a test tube and was evaporated to dryness on a water bath. This was followed with the addition of 2-3 drops of saturated $SbCl_3$ in $CHCl_3$ to the residue. A blue-green colour eventually changing to red indicates the presence of carotenoids.

***Flavone Aglycones: Shibata's Reaction or Cyanidin test*:** 3ml of the extract was evaporated to dryness in a water bath. The residue was then dissolved in 1-2ml of 50% CH_3CH_2OH while heating. A piece of magnesium ribbon and 4-5 drops of concentrated HCl were added. A red or orange colour indicates the presence of Flavone Aglycones.

Emodols (Anthracenoside and Anthracenoside Aglycone): Borntrager's Reaction: 1ml of 25% NH_3 was added to 3ml of the extract in a test tube. The mixture was then shaken well. A red colour indicates the presence of Emodols. To 2ml of the extract in a test tube, 1-2ml of 25% NH_3 was added while it was been shaken. A cherry-red colour indicates the presence of anthracenosides.

Test for Coumarin and derivatives: 5ml of ether extract was evaporated to dryness. The residue was dissolved in 1-2ml of water by heating. It was then divided in two equal portions. To the non-reference tube, 0.5ml of 10% NH_3 was added and then the tubes were viewed under UV light. The presence of blue-green fluorescence indicated the presence of coumarin. 4-5 drops of hydroxylamine hydrochloride solution and alcoholic KOH were added to the non-reference test tube until the pH was approximately 8-9. The resulting solution was evaporated to dryness. The residue was dissolved and the pH was adjusted to 3-4 by adding 10% HCl add 1-2 drops 3% $FeCl_3$ while being observed. A fast disappearing violet colour indicates the presence of coumarin derivatives.

Alkaloids: 10ml of the extract was evaporated to dryness. The residue was then dissolved in 1.5ml of 2% HCl. The solution was divided into two equal portions, one served as a reference. 2-3 drops of Mayer's reagent was added to the non-reference tube. The development of opalescence or a yellowish white precipitate indicates the presence of alkaloids.

Mayer's reagent: 1.35g of mercuric chloride was dissolved in 60ml of water, 5g of potassium iodide was added to 10ml of water and diluted to 100ml. 0.85g of basic bismuth nitrate was dissolved in a mixture of 40ml of water and 10ml of acetic acid. 8g of potassium iodide dissolved in 20ml of water and homogenize was added. The solution obtained was kept in a dark bottle for 2-3 months.

Non-Hydrolysed Ethanol extract

Tannins: 1-2 ml of water was added to 0.5-1.0ml of the extract and then 2-3 drops diluted (3% $FeCl_3$) solution was added to the mixture. A blackish-blue colour indicated the presence of Gallic tannins, while a greenish-black colour indicates the presence of catechol tannins.

Reducing compounds: 1-2ml of water was added to 0.5ml of the extract the 0.5-1.0ml of Fehling solution (1 and 2) were added. The mixture was then heated in a water bath. A brick red precipitate indicates the presence of reducing compound.

Alkaloids salt: 20 ml of the extract was evaporated to dryness and 5-10ml of 10% HCl was added to the residue. 10% NH_3 was then added until pH of 8-9 was achieved. The solution was placed into a separatory funnel and extracted with a polar solvent. The resulting extract was evaporated to dryness. The residue was then dissolved in 1.5ml of 2% HCl and divided into two portions; one was a reference. 2-3 drops of Mayer's reagent was added to the non-reference tube. Development of opalescence or a yellow-white precipitate indicates the presence of alkaloid salts.

Hydrolysed ethanol extract: 2ml of 10% HCl was added to the 25ml extract to hydrolyze the alcohol extract. The solution was refluxed for 30 minutes and then allowed to cool. 10-12ml of ether was extracted three times and the extracts were combined. A spatula full of anhydrous Na_2SO_4, filter was added and left for the following test.

***Cardenolides (Cardiac Glycosides and Aglycones: The Kedee's test*:** 4ml of the ether extract was evaporated to dryness. The residue was dissolved in 1-2ml methanol. 1-2ml of alcoholic KOH was added to the mixture. 3-4 drops of 1% alcoholic 3, 5-dinitrobenzene was added and the solution was heated. A disappearing violet colour indicates the presence of Cardenolides.

Saponins: 2ml of the ether extract was evaporated to dryness. The residue was dissolved in 1ml water and shaken vigorously. The presence of saponins was indicated by persistent foam (1cm in test tube).

Flavanosides: (Shibata's Reaction): 5ml of ether extract was evaporated to dryness. The residue was dissolved by heating in 1-2ml of 50% methanol. Metallic magnesium was added to the mixture followed with 5-6 drops conc. HCl. The development of an orange colour indicates the presence of flavanones. (Flavanols gave a characteristic red colour).

RESULTS AND DISCUSSION

Phytochemical screenings were done on the C_6H_{12}, CH_2Cl_2, EtOAc, CH_3CH_2OH extracts using standard phytochemical methods. Chemical screening involves the separation of various classes of chemicals or compounds via successive and selective extraction of different plant parts with the above solvents of increasing polarity. The more polar CH_3CH_2OH solvent extracted the more polar natural products. Different parts of the plant (stems, twigs, roots and bark) were screened, because natural products vary in both type and concentration in different parts of the plant. Only the stems of *Doliocarpus dentatus* were screened for natural products.

It was noticeable that from the CH_2Cl_2 extract, a solid crystallized out. Hence, the extract was subjected to crystallization/recrystalisation using CH_2Cl_2/Hexane, yielding an off white solid which was further purified via flash column silica gel column chromatography using CH_2Cl_2/EtOAc as the eluent and was obtained as the second major band with an R_f value of 0.29. The purified solid was found to be betulinic acid, a lupene type pentacyclic triterpene carboxylic acid, Fig 1.0. Phytochemical analysis also confirms the steroidal nature of the compound and the presence of the carbonyl moiety. It was characterized using modern spectroscopic techniques: ^{1}H NMR, ^{13}C NMR, DEPT-135, ^{1}H-^{1}H COSY, HMBC and HMQC. The ^{1}HNMR shows diagnostic peaks for the six methyls and these resonate at 0.647, 0.761, 0.865, 0.894, 0.929 and 1.643 ppm. In addition, multiplets are seen at 1.086-1.261, 1.237-1.396, 1.436-1.54, 1.802, 2.1, 2.2 and 2.948 ppm and these arise from either CH or CH_2 protons. Two distinct broad doublets are seen at 4.69 and 4.56 ppm and these arise from terminal alkene protons H-30. ^{13}C NMR spectrum revealed the presence of thirty signals, arising from 30 carbons of the triterpene. Dept-135 NMR indicates the presence of 11 CH_2 signals and six CH signals which are consistent with the structure. The spectroscopic details are in accordance with literature (Sholichin, et. al., 1980) and no further elucidation is required here. Phytochemical analysis also confirms the steroidal nature of the compound and the presence of the carbonyl moiety. Phytochemical tests were also done on the CH_2Cl_2 filtrate and reveal mainly compound of a steroidal nature. Betulinic acid has been shown to exhibit a variety of biological activities such as the inhibition of human immunodeficiency virus (HIV) replication in H9 lymphocyte cells, blockage of HIV type 1 entry into cells, and inhibition of DNA polymerase β, anti-cancer, anti-protozoan effects (Yogeeswari, et.al., 2005; Fulda, 2008; Kumar, et. al., 2010). It also induces apoptosis in melanoma cells (Kouzi, et. al., 2000)

The EtOAc extract revealed the presence of Emodols, Tannins, Flavone, reducing compounds and alkaloid salts. Trigonelline, Fig. 2.0 was isolated as a white crystal following solvent removal from one of the chromatographic band after flash column chromatography using EtOAc/Hexane followed via gradient elution with EtOAc/CH_2Cl_2. The spectral data are presented in Table, 2.0. The ^{1}H NMR spectrum for Trigonelline revealed a singlet for the H-2 protons at 9.20 ppm. H-4 proton is split

by H-5 to yield a doublet (J = 8Hz) which is further split by H-6 to yield a doublet of doublet (J = 8Hz). H-6 protons resonate as a virtual triplet at 8.060 ppm. An H-7 proton is seen as a singlet at 4.44 ppm. ^{1}H-^{1}H COSY experiment indeed revealed that H-4 proton show cross peaks which are linked with those of H-5 and H-6. ^{13}CNMR indicates that there are seven signals resulting from seven different types of carbon. These resonate at 167.20, 147.96, 147.22, 146.359, 140.31, 128.85 and 53.64 ppm. Dept-135 established the resonance at 146.52, 145.748, 144.96 and 127.45 ppm to be CH whereas the resonance at 167.20 ppm is due to the carbonyl moiety. HMQC established proton-carbon connectivity and revealed the following: 53.64 (C-7), 128.856 (C-4), 140.312 (C-3), 146.359 (C-5), 147.215 (C-6), 147.96 (C-2), 167.203 ppm (C-8). Trigonelline, an alkaloid which is widely distributed in terrestrial plants as well as in marine invertebrates has been previously isolated from the algae, *Pterocladia capillacea* (Wu, et. al., 1997). However, it has never been isolated from *Doliocarpus dentatus*. It is a zwitterion formed by the methylation of the nitrogen atom of niacin (vitamin B_3) and is a product of niacin metabolism that is excreted in urine. It has been proven to have anti-diabetic activities (Yoshinari, et. al., 2009). The content and concentrations of trigonelline have been studied in leaves and fruits of *Coffea arabica* at different stages of growth (Zheng, et al., 2004).

A portion of the CH_3CH_2OH extract was hydrolysed. Hydrolysis of the CH_3CH_2OH extract will result in the conversion of functional groups such as esters into carboxylic acids, carbonyl groups into acetals. The hydrolysed CH_3CH_2OH extract revealed the presence of steroid, anthracenosides and coumarins. The non-hydrolysed ethanol extract revealed the presence of emodols, tannins, flavones and reducing sugars.

Phytochemical screening also indicates that natural products show different propensity for solvent type extract. For example, sterol and coumarins are present in the solid that crystallized out of the CH_2Cl_2 extract but not in the EtOAc extracts. Likewise alkaloid salts are present in the EtOAc extract but not in the non-hydrolysed CH_3CH_2OH extracts. Triterpenes are present in the hexane, CH_2Cl_2 extract and the CH_2Cl_2 filtrate but not in the EtOAc and CH_3CH_2OH extract.

TLC analyses indicate the presence of seven and five components for the EtOAc and C_2H_5OH extract respectively. The EtOAc and C_2H_5OH extracts were developed using the eluents: EtOAc/Hexane and C_2H_5OH/Hexane respectively. The R_f value for each compound is presented in Table 1.0.

The EtOAc and CH_3CH_2OH extract were analysed for heavy metals such as K, Cu, Fe, Mn, Co and Zn. The concentration of these metals varies and in some cases weren't detected. For example, Mn weren't detected in both EtOAc and CH_3CH_2OH extract. Also, Fe was detected the EtOAc extract but not in the CH_3CH_2OH extract. Fe is a component of metalloenzymes and is involved in important biocatalytic processes such as the fixation of nitrogen, methane biogenesis, oxidation, oxygen storage and utilization in higher organisms. Zn wasn't detected in the CH_3CH_2OH extract but in the EtOAc extract. In general, the concentration of transition metals was higher in the EtOAc extract than in the CH_3CH_2OH extract. For example, the concentrations of Cu and Fe in the EtOAc extract were 9.68 and 5.36mg/L respectively. For the CH_3CH_2OH extract there were 4.36mg/L and not detected respectively. A significantly high concentration of Co and Zn were found in the EtOAc extract in comparison to the CH_3CH_2OH. For example, the concentration of CO and Zn in the EtOAc extract was 806 and 750mg/L respectively. For the CH_3CH_2OH extract, these were 1.37mg/L and not detected (ND) respectively. Interestingly, the concentration of K, a non transition metal was 358mg/L in the

CH_3CH_2OH extract compared with 90.1 mg/L in the EtOAc extract.

Heavy metals studied above viz Cu, Fe, Mn, Co and Zn are important micronutrients of plants, whereas K is an important macronutrient. These elements play important roles in the plant biochemistry and affect the surrounding environment. 9% of eukaryotic proteins bind various metals and 40% of all enzyme catalysed reactions involve metals such as Mg, Zn, Fe, Mn, Ca, Co, Cu, Ni, Mo, W, Na, K and V (Kemsley, 2012). Copper is necessary for many enzyme processes, proper photosynthesis, manufacture of lignin (cell walls) and in grain production. Manganese is necessary for building the chloroplasts. Manganese deficiency may result in coloration abnormalities, such as discolored spots on the foliage. Iron is necessary for photosynthesis and is present as an enzyme cofactor in plants. Iron deficiency can result in interveinal chlorosis and necrosis. Iron is not the structural part of chlorophyll but very much essential for its synthesis. Potassium as macronutrients regulates the opening and closing of the stomata via a potassium ion pump. Potassium deficiency may cause necrosis or interveinal chlorosis. K^+ is highly mobile and can aid in balancing the anionic charges within the plant. It serves as an activator of enzymes used in photosynthesis and respiration. Potassium deficiency may result in higher risk of pathogens, wilting, chlorosis, brown spotting, and higher chances of damage from frost and heat.

Zinc a micronutrients is essential for optimum crop growth. Their deficiency causes various adverse effects on growth and yield of crops. It is also involved in formation of chlorophyll, carbohydrates, in several dehydrogenises, proteinese and peptidase enzymes. It promotes growth hormones (auxin) and starch formation. It also responsible for the biosynthesis of cytochrome: a pigment and maintain plasma membrane integrity and synthesis of leaf cuticle. Cobalt has proven to be beneficial to at least some plants, but is essential in others, such as legumes where it is required for nitrogen fixation for the symbiotic relationship it has with nitrogen-fixing bacteria. Protein synthesis of *Rhizobium* is impaired due to Cobalt deficiency.

CONCLUSION

Natural products, betulinic acid and Trigonelline were isolated from the CH_2Cl_2 and EtOAc extract of *Doliocarpus dentatus* respectively. Phytochemical screening of the solvent type extract showed variation in Phytochemicals and heavy metal ion concentration (mg/L) in accordance with the solvent type extracts.

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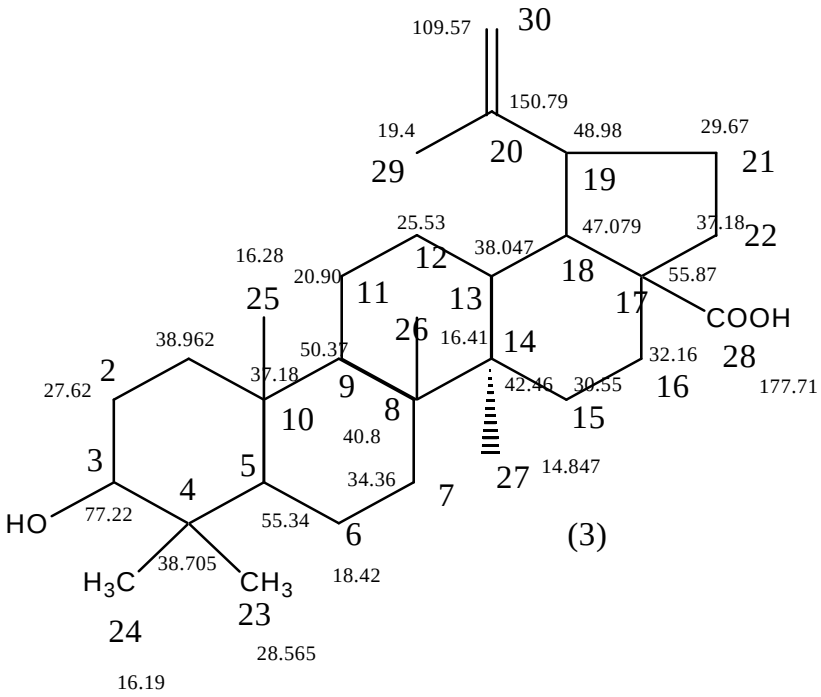

Figure-1: betulinic acid with chemical shift assignment

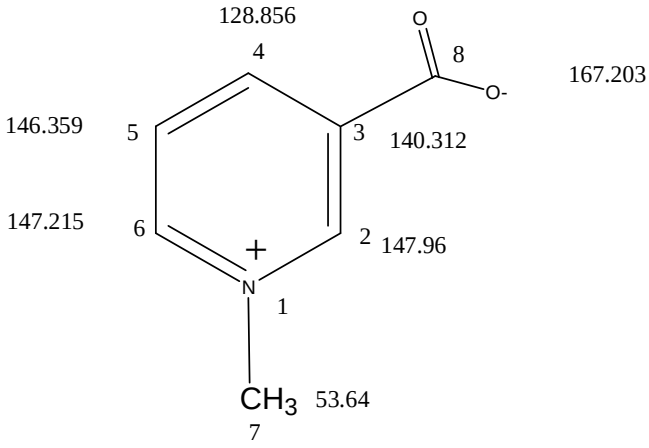

Figure- 2: Trigonelline with chemical shift assignment

Table-1: Summary of Phytochemical screening analyses.

Compounds	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
n-C_6H_{14}	-	-	-	-	+	+	-	-	-	-	+	+
CH_2Cl_2 Solid	-	-	-	-	+	+	-	-	-	-	+	+
CH_2Cl_2 Extract Filtrate	-	-	-	-	+	-	-	-	-	-	-	+
CH_3CH_2OHExtract (Non- hydrolysed)	-	+	+	+		+	-	-	-	-	-	-
Hydrolysed	-	-			+		-	+	-	-	+	-
EtOAC		+	+	+		+				+	-	-

- A : Test for Carotenoidl; B : Test for Emodols; C : Test for Tannins; D : Test for Flavones; E : Test for Steroid; F: Test for Reducing Compound; G : Test for Saponins; H : Test for Anthracenosides; I : Test for Cardenolides; J : Test for Alkaloid Salt; K : Test for Coumarin; L: Triterpenes

Table-2: Metal ion concentration (mg/L) for EtOAc and CH_3CH_2OH extract using Atomic Absorption Spectroscopy (AAS).

Sample Description	**A**	**B**	**C**	**D**	**E**	**F**
EtOAc Extract	90.1	9.68	5.36	ND	806	750
CH_3CH_2OH Extract	358	4.36	ND	ND	1.37	ND

- A: Potassium (K).; B: Copper (Cu); C: Iron (Fe); D: Manganese (Mn); E: Copper (cu); F: Zinc (Zn).
- ND: Not detected

Table-3: R_f values for components of EtOAc and C_2H_5OH extract using eluents EtOAc/Hexane and EtOAc/C_2H_5OH) respectively.

EtOAc Extract	**C_2H_5OH Extract**
0.051	0.07
0.025	0.103
0.103	0.538
0.21	0.846
0.62	0.897
0.77	-------
0.89	------

