Investigation of Antitumor Activity *in vitro* and Isolation of some Organic Constituents from *Dregea volubilis* Benth. (Gway-Tauk) Fruits

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Abstract

The preliminary phytochemical investigation: alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, steroids, saponins, terpenoids and tannins were found in dry fruits of *Dregea volubilis* Benth. (Gway-tauk).Whereas, starch was not observed in this sample. Terpenoid compound was possessed as a major constituent in *D. volubilis.* Benth. According to the physiochemical analysis, 7.74 % of moisture, 7.01 % of ash, 9.75 % of protein, 44.31 % of fiber, 2.72 % of fat, 28.47 % of carbohydrate and 179 kcal/100 g of energy values were found by AOAC method respectively. The collected sample was found to contain Ca (112.18 ppm), Mg (104.27 ppm), Fe (14.34 ppm) determined by atomic absorption spectrophotometry method. Other toxic elements: Cd, Cu, Mn, As and Pb were not detected in this sample. It was possessed that methanol extract was exhibited the most potent antimicrobial activity against *Pseudomonas aeruginosa*. Antioxidant activity of the watery, ethanol extracts and one isolated compound were evaluated by DPPH assay method. The IC_{50}value, the radical scavenging activity of one isolated compound was found more potent than that of ethanol extract and watery extract. By the acute toxicity test *in vivo* there was no toxic effect in Gway-tauk fruits. Otherwise, the antitumor activity of 12.5μg/disc of petroleum ether extract *D. volubilis.* Benth. fruits was observed in the prevention of tumor formation by potato crown gall method. Furthermore, from the separation of silica gel column chromatographic method, one terpenoid compound: (0.25%, 288ºC, R_f = 0.43) was isolated from petroleum ether extract of *D. volubilis* Benth. The one isolated compound terpenoid was identified by UV visible and FTIR spectroscopy. The isolated compound from the fruit of Gway-tauk plants grown in Loikaw City, Kayah State area is being reported for the first time and it can be used in essential basic work in exploring valuable natural source from Myanmar herbal plants for development of new antioxidant and antitumor drug.

Keywords: *Dregea volubilis*. , terpenoid, UV, antioxidant, antitumor

Introduction

Dregea volubilis Benth. (Gway-tauk) Fruits

D. volubilis Benth. expresses the family of *Asclepiadaceae* and is a perennial herb with densely lenticulate branches (Moulisha *etal*, 2009). It is a hoary, stout, smooth, woody vine and commonly used in traditional treatment of different kinds of diseases (Karthika, 2012). Leaves are rounded, ovate, 7.5 to 15 centimeters long, rather leathery at the base, and tip. The fruits are generally double, broadly lanceolate, 7.5 to 10 centimeters long, turgid, and velvety until mature (Bharathamma, 2015). It has been reported that the leaves are much employed as an application to boils and abscesses. The roots and tender stalks are considered emetic and expectorant (Venkateson, 2013). Subsequently, (Purushoth,2012) has been reported that the fruits of *D.volubilis* Benth. are treatment of sore throat, carbuncles, eczema, asthma, emetic, expectorant, febrifuge, eye-disease activity and antidote for poison. That's why, in this study the phytochemical constituents, physiochemical analysis, elemental analysis of fruits of *D.volubilis* Benth. was conducted. Moreover, antibacterial , antioxidant, acute toxicity and antitumor activity of fruits from *D.volubilis* Benth. were studied by different methods. Then, some organic constituents of of *D.volubilis* Benth. were separated by column chromatographic technique.

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Materials and Methods

Firstly, the sample fruits of *D.volubilis* Benth. were collected from Mine-Lone Quarter, Kayah State, Loikaw City. The sample was identified at the Department of Botany, Yangon University. These collected fruits were cleaned and cut into small pieces and air-dried and ground to produce finely powdered using blender. Then, these powdered samples were kept in air-tight glass containers for detecting some biological activities and separations (Figure 1). The phytochemical constituents such as α-amino acids, carbohydrate, flavonoids, glycosides, saponin, starch, steroids, alkaloids, terpenoids tannins and phenolic compounds were carried out in *D.volubilis* Benth. by using the test tube method (Harborne,1984). The some of the nutritional values: moisture content, the ash content, the fat content, the carbohydrate contents and the protein content were determined by micro Kjeldahl method. Moreover, the fiber content and the energy value were determined by AOAC method (AOAC,1990). The some elemental analysis in *D.volubilis* Benth. was detected by using atomic absorption spectrometry (AAS).

In the determination antibacterial activity, agar well diffusion method was conducted for different extracts of *D.volubilis* (Balouiri, 2016). In this antibacterial activity test, the nutrient agar (20-25 ml) was boiled and poured into the test tube and plugged with cotton wool and sterilized at 121°C for 15 minutes in autoclave (Finegold, 1978). After autoclaving, the tubes were cooled down to 30-35°C and poured into sterilized petridish and 0.1-0.2 mL of test organisms were also added into dishes. They were allowed to set the agar for 2-3 hours. After the agar was set, 10 mm agar wells were made by the help of sterilized agar well cutter. After that, about 0.2 mL of sample namely PE, EtOH MeOH and H_2O solution of *D.volubilis* Benth. were introduced into the each agar well and incubated at 37°C for 24 hours. The inhibition zone appeared around the agar well indicated that the presence of antimicrobial activity.

Furthermore, the antioxidant activity of 95 % ethanol, watery extracts and isolated compound were carried out by DPPH (1, 1-Diphenyl, 2-Picryl Hydrazyl) radical scavenging assay using UV visible spectrophotometer (Halliwell, 2012). Then, IC_{50} (50 % oxidative inhibitory concentration) values were also calculated by linear regressive excel program (Kahkonen, 1999). Besides, the information on the hazardous properties of a chemical limited test of OECD guideline 425 was used for acute toxicity test. Because this procedure was needed few animals than the other acute toxicity testing methods (OECD, 2000) (Figure 2 and 3).

In the section of antitumor activity test, the tumor producing bacteria *Agrobacterium tumefaciens* was isolated from gall tissues of leaves of *Sandoricum koetjape* Merr.(Thitto). It was cultured for the Potato Crown Gall (PCG) test and used petroleum ether extract of *D. volubilis* Benth. for the antitumor activity test (Ferrigni, 1982). Firstly, in the preparation of agar medium, (YEP) yeast extract peptone agar medium, the meat extract of (0.5g), yeast extract (0.1g), peptone (0.1g) and sucrose (0.5g) were mixed with 100 mL of distilled water and adjusted at pH 7.2. Then, the agar powder (1.5 g) were added and formed YEP agar medium. It was put into sterilized conical flask and plugged with cotton wool and then autoclaved at 121°C for 15 min. After autoclaving, the test tubes were sloped at about 15° from the horizontal position and allowed to solidify. Then, in the isolation of tumor producing bacteria: *Agrobacterium tumefaciens* were extracted from The leaf of *Sandoricum koetjape* Merr. (Thitto) with Tween 80 or polyoxyethylene (20) sorbitan monooleate. 1mL of the extract solution was made up to 100 mL with distilled water to obtain 10^2 dilutions. Then 1 mL of this 10^2 dilution solution was made up to 9 mL with distilled

water and 10^3 dilution solution was obtained. Similarly, the serial dilution as 10^4, 10^5 and 10^6 were made. And then 0.1 mL of each dilution solution was transferred to the petridish with a sterilized disposable pipette near the burner. About 20 mL of YEP agar medium (after autoclaving, cool down to 40°C) was poured into the petridish containing 0.1 mL of each diluted solution and incubated at 27 °C. The colonies of bacteria were seen on YEP agar medium after 24 h. The creamy colour of bacteria colonies was transferred into YEP agar slant with inoculation loop near the flame of a spirit burner and incubated again at 27 °C for 24 h to get pure culture (Figure 4,5,6,7,).

(a)

(b)

Figure 1(a). Fruits of *D.volubilis* Benth.
(b). Plant of *D.volubilis* Benth.

Figure 2. Dose the ethanol extract of to albino rats

Figure 3. Dose the watery extract of to albino rats

Figure 4. Tumor on the leaves of *Sandoricum koetjape* Merr. (Thito)

Figure 5. Bacteria culture on YEP agar medium

Figure 6. The bacteria sub-culture transferred on the YEP slant

Figure 7. Isolated bacteria under microscope

In this procedure for antitumor activity, the fresh potato tubers with diseases free were collected from Demoso Township,Loikaw District in the eastern part of Kayah State in Myanmar. It was brought into the laboratory within 48 hours. The suitable sizes of tubers were surface-sterilized in solution of 50 % sodium hypochlorite (Clorox) for 20 min. The edges of tubers were removed and immersed again in more Clorox for 10 min. A core of the tissue with 0.5 cm thick was made from each tuber provided by 1.5 cm wide cork borer. The four tuber discs were transferred to 1.5 % of agar plates (1.5 g of Difco agar was dissolved in 100 mL of distilled water, autoclaved and used in 20 mL poured into each petri dish). This procedure was carried in the clean bench in the sterile room. Then, 8 mg of sample was dissolved in 2 mL of dimethyl sulphoxide (DMSO); this solution was filtered through Millipole filters (0.22 μm) into sterile tube. This aseptic tested sample solution (0.5 mL) was added to 1.5 mL of sterile distilled water and 2 mL of broth culture of *A. tumefaciens* stain (48 h culture containing 3-5 x10^9 cells/mL). The controls discs were created in 0.5 mL of DMSO, 1.5 mL of sterile distilled water and 2 mL of broth culture of *A. tumefaciens* (from the same 48 h culture). 1 drop (0.05 mL) from sterile tubes with a sterile disposable pipette was used to inoculate each potato disc, spreading it over the disc surface. The incubating plates were tightly closed with tape and not to lose the moisture at room temperature for 12 days. After that, one drop of Lugol's solution (I_2-KI) was put onto the incubating plate and the antitumor could be seen under a microscope and compared with control (Figure 8).

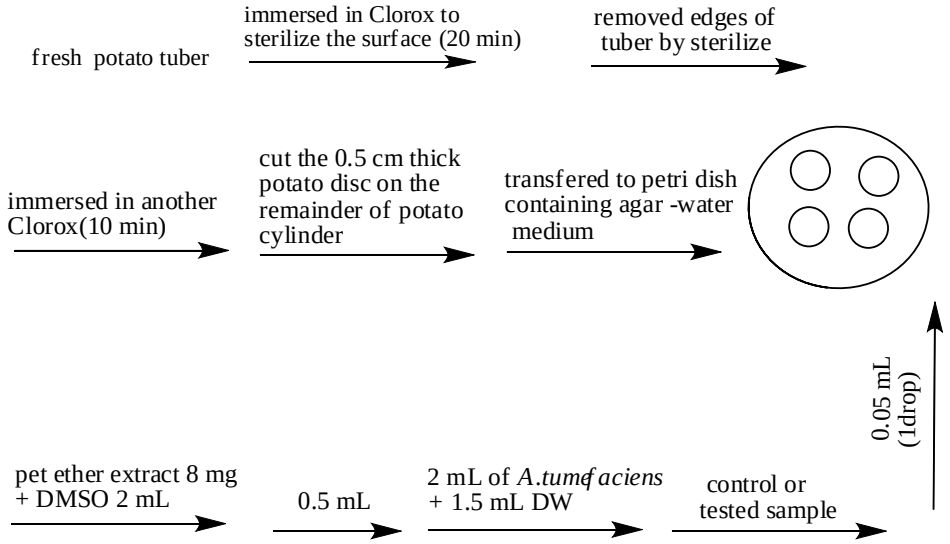


Figure 8. Procedure for screening of antitumor activity by Potato Crown Gall (PCG)

In the isolation of the PE extract of *D.volubilis* Benth. was separated by silica gel column chromatographic techniques. A total 60 fractions of various solvent system (compound A, compound B, compound C) were collected. All the collected fractions were checked on TLC by spraying with 5 % H_2SO_4 followed by heating. Among them, the fraction of (F_{28-34}), compound A, (PE:EtOAc, 30:1), the fraction of (F_{40-45}) compound B, (PE:EtOAc, 25:1), were observed not clear on TLC under UV shorter and longer wavelength. Moreover, the fractions of F_{53-57} (PE:EtOAc, 9:1), showed one spot on TLC and provided as the compound C. The R_f value of the isolated compounds were calculated on TLC. After that The isolated compound was characterized by some colour test such as 5 % H_2SO_4, Libermann Burchard, anisaldehyde/sulphuric acid, 5 % $FeCl_3$ and Mg/HCl. A chromatogram was prepared by developing in PE: EtOAc (30:1, 25:1, 9:1v/v) solvent system. Then the chromatogram was sprayed with 5 % H_2SO_4 followed by heating, Libermann Burchard followed by heating, anisaldehyde/sulphuric acid followed by heating, 5 % $FeCl_3$ and treated with Mg / HCl in test tube method. The observed colouration were denoted. The pure isolated compound C was identified by modern spectroscopic techniques such as UV-visible and FT IR spectroscopy (Markan, 1982).

Results and Discussion

According to phytochemical test, glycosides, flavonoids, alkaloids, α-amino acid, carbohydrates, phenolic compounds, saponins, steroids, tannins and terpenoids were found in *D.volubilis* Benth. Whereas, starch was not observed in collected sample. In this sample, the fiber (44.31%) was observed the highest amount. In addition, protein (9.75 %) and carbohydrate content (28.47 %) were higher than other nutrient, moisture (7.74 %) and ash (7.01 %). The fat content (2.72 %) was the lowest amount in *D.volubilis* Benth. fruits. The energy value was observed 179 kcal/100g from *D.volubilis* Benth. The mineral elements present in dried powder of fruits from *D.volubilis* Benth. was determined by Atomic Absorption Spectrometer (AAS). Ca, 112.18 ppm and Mg, 104.27 ppm were found as major amounts but Fe, 14.34 ppm as trace elements and Cd, Cu, Mn were not detected in it. The MeOH extract (inhibition zone diameter 15 mm) was exhibited the most potent antimicrobial activity against *Pseudomonas aeruginosa*. The remaining extracts were exhibited antimicrobial activity against on six strains of microorganisms. The results of inhibition zone diameters are mentioned in Table 1 and Figures 1 and 2.

Table 1. Inhibition Zone Diameter of Various Extracts of *D. volubilis* Benth. Against Six Microorganism by Agar Well Diffusion Method

No.	Microorganisms	Inhibition Zone Diameter (mm)			
		PE	EtOH	MeOH	H_2O
1	*Bacillus subtilis*	13	14	13	12
2	*Staphylococcus aureus*	13	13	13	12
3	*Pseudomonas aeruginosa*	13	13	15	12
4	*Bacillus pumilus*	13	14	13	13
5	*Candida albicans*	13	13	13	13
6	*Escherichia coli*	13	14	13	11

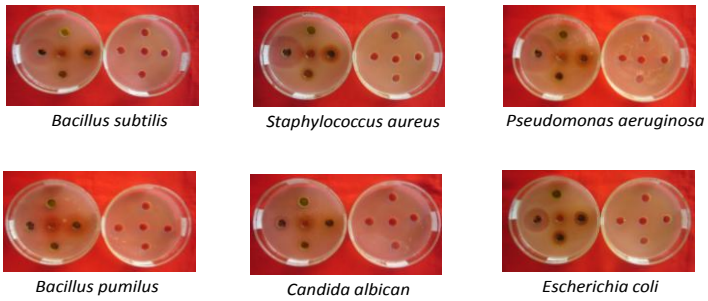


Figure 1. Inhibition zone diameter of crude extract against six microorganisms

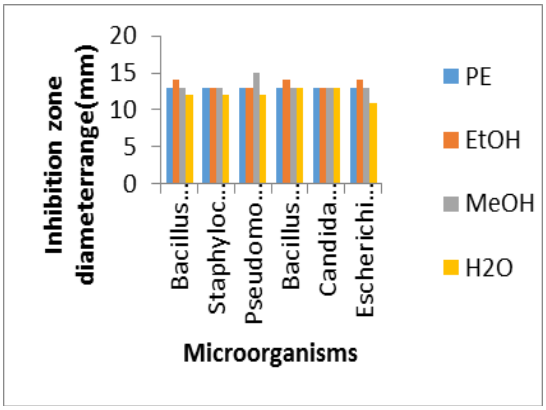


Figure 2. Comparison of inhibition zone diameters for crude extracts of *D. volubilis* Benth.

In the determination of antioxidant activity, the lower the IC_{50} values, the higher the antioxidant activity of the sample. By using DPPH free radical scavenging assay, the compound C was found the most potent antioxidant activity than 95% ethanol and watery extracts. The results of antioxidant activity are shown in Table 2 and Figure 3 and Figure 4.In acute toxicity, there is no lethality at the dose of 5000 mg/kg body weight of the extracts. It can be denoted that the ethanoic and watery extracts of *D.volubilis* Benth. were supposed to be practically nontoxic.

In antitumor activity of PE extract of *D.volubilis* Benth. fruits was detected by Potato Crown Gall test with the isolated bacterium *Agrobacterium tumefaciens*. The broth cultures containing $5x10^9$ cells /mL of the potato disc were inoculated for 48 hour. The test sample of PE extract was dissolved in DMSO, diluted and mixed with the bacterial culture for inoculation. After that, the tumors were appeared on potato discs and checked by staining the knob with one drop of Lugol's (I_2=KI) solution onto the agar. From this observation, PE extract of *D.volubilis* Benth. was possessed the prevention of tumor formation with the doses of 12.5 µg/disc. The results of antitumor activity are shown in Figure 5.

In the isolation of some organic constituents from petroleum ether extract (2g) of *D. volubilis* Benth. was separated by silica gel column chromatogyphy, the solvent system in the ratio of petroleum and ethyl acetate (30:1), (25:1) and (9:1) v/v were successively used to elute

the isolated compound. In this separation a total of 60 fractions ($3cm^3$/fractions) were collected. The fraction of (F_{28-34}), (PE:EtOAc, 30:1), compound A, the fraction of (F_{40-45}), (PE:EtOAc, 25:1), compound B, were observed not clear on TLC check under shorter and longer wavelength. However, the fractions of F_{53-57} (PE:EtOAc, 9:1), showed the similar TLC behavior provided the compound C, yellow colour crystal (0.25 %). The isolated compound C was recrystallized by acetone.

In the classification of isolated compound c *D.volubilis* Benth. by colour reaction test, the isolated compound C, was observed that brown color on TLC by spraying with 5 % H_2SO_4 and heating. After that, the pink colour was observed testing with vanillin sulphuric acid and heating. Moreover, the violet colour was observed anisaldehyde sulphuric acid and heated on TLC. Compound C, from *D. volubilis* Benth. Was classified as terpenoid compound due to pink colouration occurred when it treated with Libermann Burchard reagent in test tube. There was no colouration on TLC by spraying with 5 % $FeCl_3$ followed by heating and Mg/HCl in test tube. In addition the isolated compound C was observed as a yellow color with bromothylmol blue solution test in test tube. The above results confirmed that, the isolated compound C, from *D. volubilis* Benth. as a terpenoid compound as shown in Table 3, 4 ,5 and Figure 6.

Table 2. Oxidative Percent Inhibitions and IC_{50} Values of Crude Extracts of *D. volubilis* Benth. and Standard BHT

Sample	% Inhibitions (Mean ± SD) in various Concentrations (μg/ml)								IC_{50} (μg/ml)
	3.125	6.25	12.5	25	50	100	200	400	
95 % EtOH	38.095 ± 3.863	36.395 ± 2.567	30.272 ± 7.167	27.347 ± 18.395	37.551 ± 4.081	54.762 ± 4.248	52.381 ± 5.802	59.372 ± 15.587	86.17
Watery	20.748 ± 0.601	35.034 ± 0.601	41.497 ± 1.202	64.966 ± 0.601	80.952 ± 0.601	94.217 ± 1.202	93.197 ± 0.601	93.06 ± 1.323	17.04
Isolated Compound C	20.748 ± 0.601	52.262 ± 0.601	39.532 ± 1.202	21.428 ± 0.601	25.17 ± 0.601	25.489 ± 0.601	24.499 ± 0.601	25.987 ± 0.531	7.68
Standard BHT	43.301 ± 1.40	53.582 ± 2.49	65.53 ± 1.132	74.82 ± 0.621	83.321 ± 0.782	87.412 ± 2.372	91.516 ± 1.113	94.702 ± 0.692	3.16

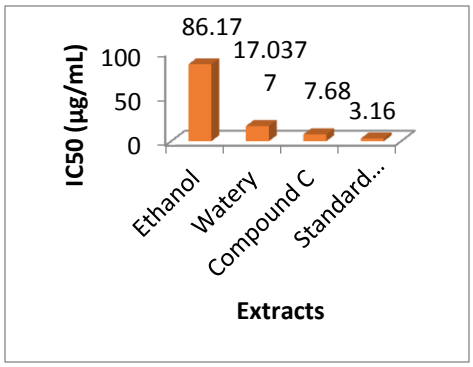


Figure 3. A bar graph of IC_{50} (µg/ml) Values of different concentration of watery, EtOH extracts and isolated compound C from *D. volubilis* Benth.

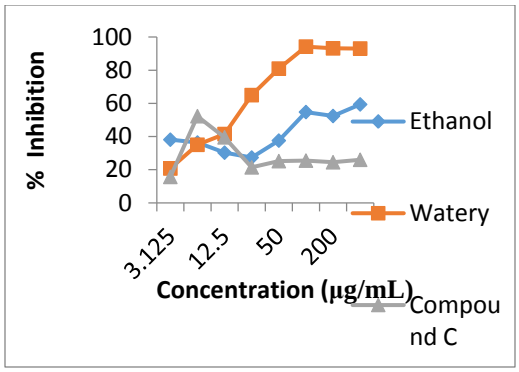
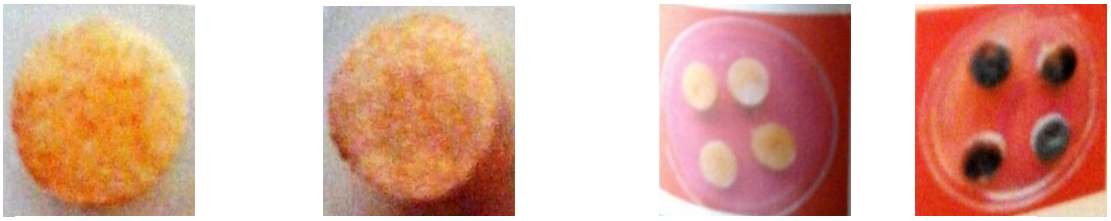


Figure 4. DPPH radical scavenging activity of different concentration of watery, EtOH extracts and isolated compound C from *D. volubilis* Benth.



(a) (b) (c) (d)

Figure 5. Antitumor activity of *D. volubilis* Benth. by Potato Crown Gall Test
(a) Control potato disc without test sample
(b) Potato disc containing test sample
(c) Before treating with Lugol's solution
(d) After treating with Lugol's solution

Table 3. Results of Colour Tests on TLC of Isolated Compound C from of *D. volubilis* Benth.

No.	Spraying reagent	Observation on the test of isolated compound
1	5% H_2SO_4, Δ	brown color on TLC
2	Vanillin+H_2SO_4, Δ	pink color on TLC
3	Anisaldehy de+H_2SO_4, Δ	violet color on TLC
4	1% $FeCl_3$	no color change
5	I_2vapour	pink color on TLC

Table 4. Classification of Isolated Compounds C from of *D. volubilis* Benths.

No.	Reagent tested					
	Acetic anhydride and H_2SO_4 in $CHCl_3$	Mg and conc:HCl in EtOH	1% $FeCl_3$in EtOH	10% lead acetate in EtOH	Bromo thylmol blue	Remarks
C	Pink	-	-	-	Yellow	terpenoid

Table 5. Yield Percents, R_f Values and Melting Points of Isolated Compound C from of *D. volubilis* Benths.

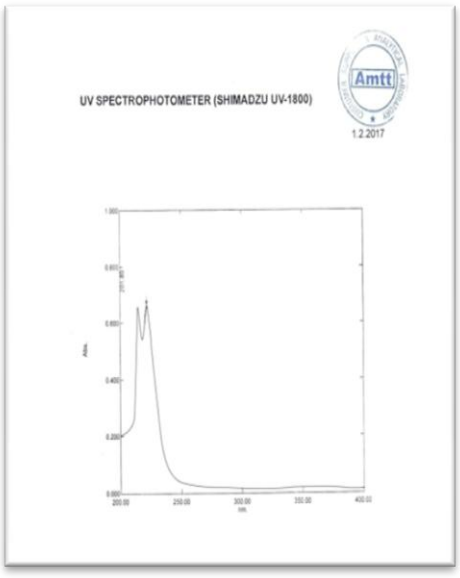
Isolated Compound	Yield (%)	R_f	mpt°C	Appearance
C	0.25	0.43 (PE:EtOAc, 9:1)	286-288 (PE/EtOAc)	flat shaped yellow crystal

Figure 6. Appearance of crystal C from *D.volubilis* Benth.

In the identification of isolated compound C from petether extract of *D.volubilis* Benth. the UV spectrum of compound 'C' in MeOH is shown in Table 6 and Figure 7. According to UV spectrum the major absorption bands were found to be 214 and 226 nm. This information pointed out that compound C contained double bond conjugation system in it (Markham, 1982).

The functional groups present in compound "C" were also studied by FTIR spectroscopy as shown in Table 7 and Figure8. The present of O-H stretching of alcoholic and carboxylic O-H group could also be confirmed with the peak appeared at 3430 $cm^{-1}$and the bands at 1690 cm^{-1}, suggested the stretching vibration of C=O in carboxylic acid.

The characteristic bands at 2988, 1400, 1460 and 1040 cm^{-1} also showed the presence of C-H stretching and bending of CH_2 , CH_3 group, C=C stretching of alkene and C–O stretching of alcohol and carboxylic group. In addition the appearance of crystal C, TLC chromatogram and physical properties are described in Table 8 .



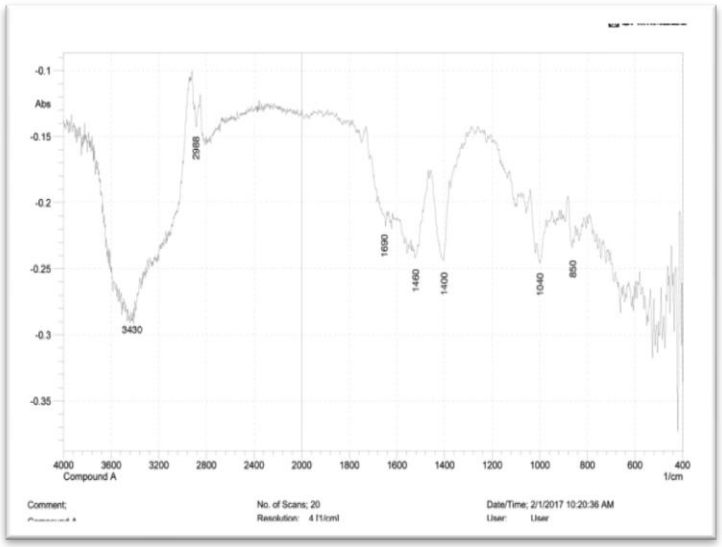


Figure 7. UV-visible spectrum of isolated Compound C from *D.volubilis* Benth in MeOH

Figure 8. FT IR spectrum of isolated compound C from PE extract of *D.volubilis* Benth.

Table 6. UV-Visible Spectral Data of isolated compound C from *D. volubilis* Benth. In MeOH

Reagent	Observed λ_{max} (nm)	Remark
MeOH	214, 226 (π-π*)	double bond conjugation

Table 7. FT IR Spectral Data of Isolated Compound C from *D. volubilis* Benth.

Wave Number (cm^{-1})	Band Assignment
3430	O-H stretching of alcoholic and carboxylic O-H group
2988	C-H stretching of CH_2 and CH_3 group
1690	C=O stretching of frequency of unsaturated carboxylic acid
1460	C=C stretching of alkene
1400	C-H bending of methyl group
1040	C-O stretching of alcohol and carboxylic group
850	out -of- plane bending of C-H group

Table 8. Some Physical Properties of Isolated Compound C from *D. volubilis* Benth.

Experiment	Observation	Remark
Melting point/^oC	226-228	recrystallized from acetone
R_f	0.43	PE:EtOAc (9:1/v/v) on TLC
UV	active	conjugated double bond
5% H_2SO_4, Δ	brown on TLC	terpenoid
Libermann Buchard, Δ	pink on TLC	terpenoid
Vanillin, H_2SO_4, Δ	pink on TLC	terpenoid
Anisaldehyde, H_2SO_4, Δ	violet on TLC	terpenoid
I_2vapour	yellow on TLC	terpenoid
10% $KMnO_4$ solution test	decolourized	C=C present

1% $FeCl_3$solution test	no colour change	phenolic OH absent
Libermann Buchard	pink in $CHCl_3$	terpenoid
Bromothymol blue solution test	yellow colouration	carboxylic acid

Conclusion

In the overall assessments of the research work, the preliminary phytochemical investigation indicated that alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, steroids, saponins, terpenoids and tannins were found in *D. volubilis* Benth. The nutritional values as 28.47% of carbohydrates, 9.75% of protein, 44.31 % of fiber, moisture 7.74 % and ash 7.01 % were found in it. The fat content (2.72%) was occurred the lowest amount in *D. volubilis* Benth. fruit. The energy value was observed to be 179 kcal/100g in collected sample. According to qualitative elemental analysis carried out by AAS spectrometry, Ca and Mg were occurred as major components. In *in vitro* antibacterial activity by using agar well diffusion method, MeOH extract of *D. volubilis* Benth. fruit was found the most potent antimicrobial activity 15 mm against on the microorganism *Pseudomonas aeruginosa*. The IC_{50} values of compound C (7.68μg/mL) was observed the most potent antioxidant activity than watery extract 17.04 μg/mL and 95% ethanol extract 86.17μg/mL by using linear regressive excel program. In acute toxicity test, there is no lethality at the dose of 5000 mg/kg of the both watery and ethanol extracts. From the determination of antitumor activity, *D.volubilis* Benth was possessed the prevention of tumor formation with the concentration of 12.5 μg/disc of petroleum ether extract by the aids of Potato Crown Gall Test. In order to find out some organic constituents from petroleum ether extract of *D. volubilis* Benth. fruit, under silica gel column chromatographic technique using PE/EtOAc solvent system with various ratios was carried out. One of the pure terpenoid compound **C**: (0.25%, R_f=0.43, mp=288 ºC) was isolated from PE extract *D. volubilis* Benth. fruit. This compound C can be denoted that terpenoid compound using by modern spectroscopic methods: UV and FTIR spectroscopy. Consequently, it can be deduced that *D. volubilis* Benth. fruit may be used as antioxidant in reducing of oxidative stress, some aged related orders, some diseases related to bacterial infection, and new antitumor drug in Myanmar Traditional medicine for human bodies.

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