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Identification and Characterization of Phytochemicals and Polyphenolic Compounds in Ethylacetate Fraction of *Desmodium velutinum* (Wild.) DC. Methanol Root Extract Using High-performance Liquid Chromatography (HPLC)

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Abstract. This present study evaluated the phytochemical and bioactive constituents of *Desmodium velutinum* root. This was carried out using standard methods and HPLC. The extraction of 1000 g of *Desmodium velutinum* root with methanol (80%) produced an extract with a percentage yield of 3.45%. The fractionation of 40 g of *Desmodium velutinum* root methanol extract using ethylacetate gave a percentage yield of 15%. Results of the quantitative phytochemical analysis showed alkaloids (6.82 ± 2.31 mg/g), flavonoids (1.03 ± 0.01 mg/g), tannins (3.82 ± 0.03 mg/g) and phenols (5.12 ± 0.06 mg/g), while saponin and steroids were not detected. The high performance liquid chromatography analysis of the ethylacetate fraction of *Desmodium velutinum* root methanol extract showed the concentration of various constituents of flavonoids, phenols and tannins. The flavonoids include chlorogenic acid (0.6052 µg/g), catechin (1.1424 µg/g), rutin hydrate (0.1253 µg/g), caffeic acid (0.1725 µg/g), quercetin (0.3689 µg/g) and an unidentified flavonoid compound with retention time, 0.065 min. The phenols identified were ascorbic acid (0.1482 µg/g), *p*-coumaric acid (0.0174 µg/g), rutin (0.0205 µg/g), ferulic acid (0.5782 µg/g), apigenin (0.2344 µg/g) and an unidentified phenolic compound with retention time, 15.907 min, while the tannins identified were ellagitannin (0.2214 µg/g) and gallotannin (0.1396 µg/g) as well as eight (8) unidentified tannic compounds with different retention time. This study reveals that ethyl acetate fraction of *Desmodium velutinum* methanol root extract contains bioactive compounds and this validate its possible option as therapeutic agent or drug formulation, as it could aid in the prevention and alleviation of cancer, ulcer and other health complications.

Keywords: bioactive compounds, tannin, alkaloids, polyphenols, secondary metabolites

Introduction

The use of plant drugs for health care delivery over the centuries, disease remedies from plant sources for mankind are as old as human history and all are still in use to date. It is estimated that about 75% of useful bioactive plant-derived pharmaceuticals used globally are discovered by systemic investigation. In recent times, numerous medicinal plants are large number of secondary metabolites and essential oils of traditional and therapeutic importance. So, the used of cure several diseases in developing countries (Vilena-Tejada *et al*., 2021). The aromatic medicinal plants contain desirable drugs are isolated from various types of plant parts like roots, leaves and stem which explain why several local people still depend on the medicinal plants for their primary healthcare and treatment of various diseases (Chaachouay *et al*., 2021). The important advantages claimed for therapeutic uses of medicinal plants in various ailments are their safety being economical, effective and their easy availability (Jahan *et al*., 2019). The most important ingredients present in plants include alkaloids, terpenoids, steroids, phenols, glycosides, tannins (Kumar *et al*., 2021). The plant *Desmodium velutinum* is used traditionally for treatment of several diseases like jaundice, rheumatism, puerperal fever, paralysis, oedema, filarial and post-natal care to avoid secondary complications. It also provides general support to the body during periods of influenza, cough, cold, neuralgia and headache, it is also used as a dietary supplement (Okoubi and Eze-Steven, 2019). Findings from previous research show that the plant *Desmodium velutinum* possess antioxidant, antihyperglycemic and hypolipidemic properties (Ozougwu *et al*., 2021). Hence the interest in the discovery of the phytochemicals

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contained in this plant. Investigative research on phytochemicals has become imperative owing to widespread use of traditional herbs for treatment not only in Nigeria and Africa but also, to a large extent, in all countries of the world. Due to the increased awareness of the significance of traditional medicine in human and animal healthcare, intensive investigation of the phytochemicals of some of the herbs used the conditions they are used to manage/treat is considered worthwhile (Seriki *et al*., 2019). Hence, this study was designed to ascertain the bioactive compounds of *Desmodium velutinum* root and to validate its use as a possible option as a therapeutic agent for drug formulation which could aid in the fight and alleviation of cancer, ulcer and other health complications.

Materials and Methods

Plant material. The fresh root of *Desmodium velutinum* collected from Orba in Udenu Local Government of Enugu State was identified by Mr. Alfred Ozioko, the botanist at Bio-resources Development and Conservation Programme, Nsukka, Enugu State, Nigeria.

Reagents and chemicals. The chemicals used for this study were of analytical grade and products of Sigma Aldrich (Saint Louis, Missouri, USA), British Drug House (BDH, England) and Andalucia (Spain). They include methanol, hydrochloric acid, chloroform, dragendorff's reagent, mayers's reagent, ammonium sulphate, ferric chloride, fehling solutions (A and B), ethanol, lead sub-acetate, bromine water, tetra-oxosulphate (VI) acid, ethylacetate, olive oil, picric acid, ammonium solution, wagners' reagent, acetic acid, concentrated ammonium hydroxide, diethyl ether, *n*-butanol, sodium chloride, petroleum ether, chloroform acetic anhydride, TCA-chloroform, potassium dichromate, thiamin, 2,6 dichlorophenol indophenol and α,α dipyridine.

Instruments and equipment. The types of equipment used were obtained from the Department of Biochemistry, The University of Nigeria Nsukka and Central Research Laboratory. They include conical flasks (Pyrex, England), water baths (Gallenkamp, England), beakers (Pyrex, England), Metler Weighing Balance (Toledo WB, China), filter papers (Whatman), test tubes (Pyrex, England), measuring cylinder (Pyrex, England), glass funnel (Pyrex, England), spectrophotometer (Spectronic 20D, Germany), water bath (Gallenkamp, England), refrigerator (Thermocool, England), centrifuge (Vickas Ltd, England) and rotary evapourator.

Method of extraction of plant materials. The fresh root of *D. velutinum* was washed in running water to remove unwanted materials and chopped into small pieces. The pieces were air-dried for two weeks and ground into a coarse powder. A quantity, of 1 Kg of ground coarse sample was weighed and soaked in 4.5 L of methanol solution, stirred and allowed to stand for 48 h. The suspension was filtered using a muslin bag followed by Whatman no. 42 filter paper. The filtrate was evapourated under reduced pressure and dried using a rotary evapourator at 55 °C. The concentrated extract was stored in a labeled sterile capped bottle at 1-8 °C. The % yield of the extract was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of extract (g)}}{\text{Weight of pulverized sample (g)}} \times 100$$

Reaction mechanism. The extraction of the plant was done *via* cold maceration. Maceration ruptures the cell structure and exposes the chemical constituents to react with the solvent and helps in removal of different plant component. At the end of the extraction, the micelle is separated from the marc by filtration or decantation. Subsequently, the micelle is then separated from the menstrum by evapouration in an oven or on top a water bath. This method is extensively used for the extraction of different types of bioactive compounds at laboratory scale.

Quantitative profiling of phytochemicals. This was carried out using the method described by Zhishen *et al*. (1999).

Total phenolic content. The total phenolics of the extract were determined using the Folin and Ciocalteu reagent. Sample and standard readings were made using a spectrophotometer at 765 nm against the reagent blank.

The test sample (0.2 mL) was mixed with 0.6 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent (1:1). After 5 min, 1 mL of saturated sodium carbonate solution (8% w/v in water) was added to the mixture and the volume was made up to 3 mL with distilled water. The reaction was kept in the dark for 30 min and after centrifuging the absorbance of blue colour from different samples was measured at 765 nm. The phenolic content was calculated as gallic acid equivalents GAE/g of dry plant material based on a standard curve of gallic acid.

Flavonoid content. Spectrophotometric method was used to determine the total content of flavonoid using aluminium chloride. The HPLC chromatogram of flavonoids as shown in Fig. 1. The plant extract of 1 mL and distilled water of 4 mL was taken in a 10 mL flask. 0.30 mL of 5% sodium nitrite and after 5 min, 0.3 mL of 10% aluminium chloride was mixed in the flask. 5 min later, 2 mL of 1 M sodium hydroxide (NaOH) was treated and diluted using 10 mL distilled water. The absorbance was measured for test and standard solutions using a reagent blank at 510 nm wavelength by ultraviolet (UV)-visible spectrophotometer. The total content of flavonoid was denoted as mg of Q E/g of extract.

Tannin content. About 0.1 mL of plant extract was added in 10 mL of volumetric flask containing the distilled water of 7.5 mL and Folin-Ciocalteu phenol reagent of 0.5 mL, 35% Na_2CO_3 solution of 1 mL and diluted to 10 mL using distilled water. The reagent mixture was well shaken and kept at 30 °C temperature for 30 min. The absorbance of standard and test solutions was analysed with blank at 725 nm wavelength by using UV-visible spectrophotometer. The tannin total content of tannin was expressed as mg of GAE/g of extract.

Identification of the polyphenolic compounds in the ethylacetate fraction. The identification of the polyphenolic compounds (flavonoids, phenolic acid and tannins) in the ethylacetate fraction were identified using HPLC according to the method described by Ortan *et al*. (2009) which is shown in Fig. 2.

HPLC Apparatus and chromatographic conditions. HPLC separation was achieved on an Agilent 1260 Series (Agilent Technologies, Santa Clara, California, USA) equipped with a 1260 Quat pump VL quaternary pump, 1260 ALS autosampler, 1260 TCC column thermostate and 1260 DAD VL diode array detector. The separation was done in a Hypersil BDS C_{18} column (4.6 × 100 mm i.d., 3.5 μm) with a C_{18} guard column (4 × 10 mm i.d., 3 μm). The mobile phases were (A) 0.5% acetic acid in water and (B) methanol using gradient elution: 10% B in A to 50% B in A for 40 min; 100% B for 10 min. This column was re-equilibrated with 10% B in A for 10 min before each analysis and the flow rate was set at 1.0 mL/min with the controlled temperature at 25 °C. DAD detector was set at the wavelength of 326 nm and injection volume was 5 μL for every sample and reference standard.

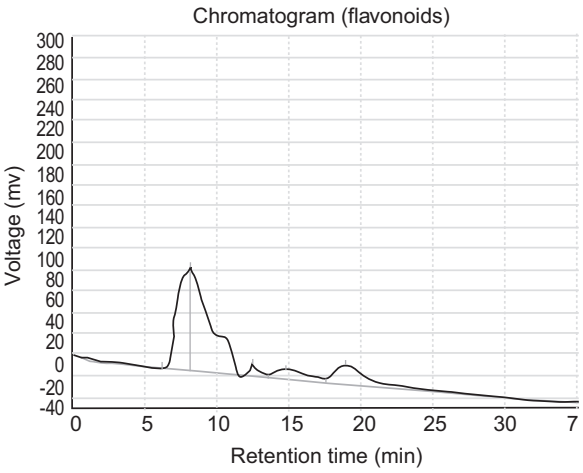

Fig. 1. HPLC Chromatogram of flavonoids.

Statistical analysis. The statistical analysis was conducted using statistical product and service solution version 20 (SPSS 20.0). The results are presented in mean of triplicates.

Results and Discussion

Percentage yield of methanol extract of desmodium *velutinum* root. The extraction of 1000 g of *Desmodium velutinum* root with methanol (80%) produced an extract with yield of 3.45%.

Percentage yield of ethylacetate fraction of *desmodium velutinum* methanol extract. The fractionation of 40 g of *Desmodium velutinum* root methanol extract using ethylacetate gave a percentage yield of 15%.

Phytochemical constituents of *D. velutinum* root. The result of phytochemical analyses of *D. velutinum* root as seen in Table 1 below shows alkaloids (6.82 ± 2.31 mg/g), phenolics (5.12 ± 0.06 mg/g), tannins (3.82 ± 0.03 mg/g) and flavonoids (1.03 ± 0.01 mg/g) saponins and steroids were not detected in the sample.

Table 1. Phytochemical constituents of *D. velutinum* root

Phytochemicals	Amount (mg/g)
Tannins	3.82±0.03
Flavonoids	1.03±0.01
Phenolics	5.12±0.06
Alkaloids	6.82±2.31
Saponins	-
Steroids	-

Result is mean ± SD; n = 3.

Polyphenolic compound composition of *Desmodium velutinum* methanol extract. The high performance liquid chromatography use for analysis of the ethylacetate fraction of *Desmodium velutinum* root methanol extract which shows the presence of flavonoids, phenols and tannins. The flavonoids were chlorogenic acid, catechin, rutin hydrate, caffeic acid, quercetin and un-identified flavonoid compound with retention time, 0.065 as seen in Table 2 and Fig. 2. The phenols identified were ascorbic acid, *p*-coumaric acid, rutin, ferulic acid, apigenin and an unidentified phenolic compound with retention time, 15.907 as seen in Table 3 where the tannins identified were ellagitannin and gallotannin as well as eight un-identified tannic compounds with different retention time as seen in Table 4 and Fig. 3.

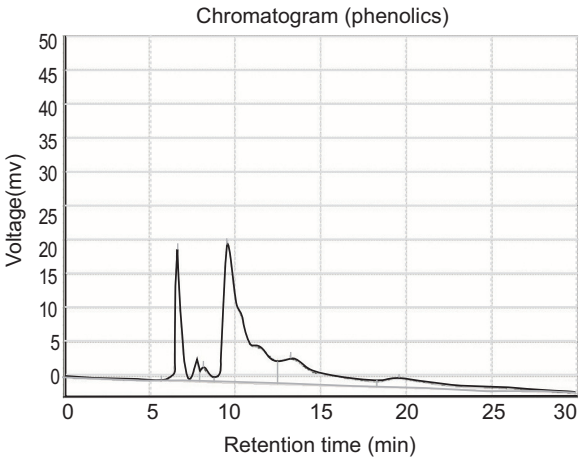

Fig. 2. HPLC Chromatogram of phenolics.

The present study evaluated the phytochemical and polyphenol constituents of *Desmodium velutinum* root using HPLC. The phytochemical constituents detected in this work which include alkaloids, tannins, phenols and flavonoids could contribute to the antimicrobial and physiological effects associated with the plant extract. Phytochemicals are naturally occurring compounds in plant such as fruits, vegetables, whole grains, beans, nuts and seeds. Many phytochemicals act as antioxidants, neutralizing free radicals and ameliorating the possible damage in to human system (Geetha and Geetha, 2014). It has become very fundamental to do investigative research on phytochemicals considering the extensive use of traditional herbs for treatment not only in Nigeria and Africa. Many phenolics and flavonoids have been reported to possess potent antioxidant activity and anti-cancer, anti-carcinogenic, anti-bacterial, anti-viral or anti-inflammatory activities to a greater extent (Dhalaria *et al*., 2020). Flavonoids which are found commonly in the leaves, flowering tissues and pollens which are important part of the diet because of their effects on human nutrition (Kumar *et al*., 2021). They act by the regulation of inflammatory mediators. The most important function of these phytochemicals is their antioxidant activity, as they are highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Makhaik *et al*., 2021). These secondary metabolites are natural

Table 2. Flavonoid compounds present in *D. velutinum* root ethylacetate fraction

Peak I.D.	Retention time (mins)	Height (cm)	Area (m^2)	Concentration (µg/100 g)
Chlorogenic acid	7.540	87519.945	822502.813	60.5252
Catechin	8.632	95571.477	2173638.500	114.2422
Rutin hydrate	10.498	8064.095	101499.523	12.5329
Caffeic acid	14.998	8422.607	290748.656	17.2555
Quercetin	18.815	17574.744	596835.938	36.8938

Table 3. Phenolic compounds present in *D. velutinum* root ethylacetate fraction

Peak I.D.	Retention time (mins)	Height (cm)	Area (m^2)	Concentration (µg/100 g)
Ascorbic acid	5.323	18731.861	84268.352	14.8288
p-Coumaric acid	7.557	2924.231	11461.243	1.7448
Rutin	8.632	2086.993	13481.462	2.0524
Ferrulic acid	9.915	20464.871	340399.219	57.8214
Apigenin	13.640	3804.233	134297.531	23.4451

Table 4. Tannins present in *D. velutinum* root ethylacetate fraction

Peak I.D.	Retention time (mins)	Height (cm)	Area (m^2)	Concentration (µg/100 g)
Ellagitannin	1.265	51838.543	432673.938	22.1371
Gallotanin	2.590	5906.009	272908.250	13.9629

antioxidants that have multiple biological effects and play an important role in the defense against cardiovascular disease, aging and cancer. The total phenolics, alkaloids, tannins and flavonoids contents of *Desmodium velutinum* methanolic root extract are presented in Table 1. The results indicate that the ethylacetate fraction of *D. velutinum* root had low total flavonoids content (1.03 ± 0.01 mg/g) and tannin content (3.82 ± 0.03 mg/g), this finding disagrees with that of Nkwocha *et al*. (2018) who reported moderate total flavonoids and tannin content in *D. velutinum* stem. Phenolic compounds were in moderate concentration (5.12 ± 0.06 mg/g) but in this study also differ from that reported study by Nkwocha *et al*. (2018) on *D. velutinum* stem. Saponins and steroids were not detected in *D. velutinum* root. The alkaloid content was moderate in concentration (6.82 ±2.31 mg/g) and is in line with the result obtained by Nkwocha *et al*. (2018) on *D. velutinum* stem. The results of phytochemical screening of *D. velutinum* showing the presence of tannins, flavonoids, alkaloids and phenols which are in line with the investigation made by Anowi *et al*. (2012). Alkaloids are a wide range of phytochemicals with varied structures and functions. They have numerous pharmacological functions as well as harmful effects depending on the amount taken and the overall quantity accumulating in the body at a time (Anowi *et al*., 2012).

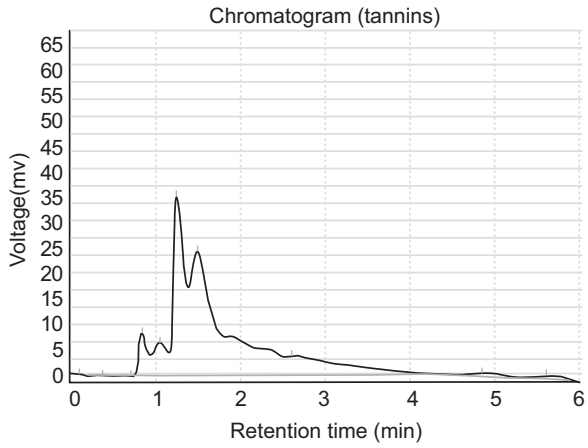

Fig. 3. HPLC Chromatogram of tannins.

The HPLC analysis results also indicated that the ethyl acetate fraction of *D. velutinum* methanol extract contains flavonoids such as chlorogenic acid, catechin, rutin hydrate, caffeic acid and quercetin. Various phenols were identified such as ascorbic acid, *p*-coumaric acid, rutin, ferulic acid and apigenin, tannins identified were ellagitannin and gallotannin as well as eight unidentified tannic compounds. The presence of several phytochemicals and bioactive substances suggest that ethylacetate fraction of *D. velutinum* methanol root extract could have various medicinal values notably anti-diarrhoeic, analgesic, antioxidative, hypolipidemic and antidiabetic activities.

Conclusion

This study shows that ethyl acetate fraction of *Desmodium velutinum* methanol root extract contains bioactive compounds such as tannins (3.82± .03 mg/g), flavonoids (1.03±0.01 mg/g) and phenolics (5.12±0.06 mg/g) which validates its possible option as therapeutic agent or for drug formulation, as it could aid in the prevention and alleviation of cancer, ulcer and other health complications.

Conflict of Interest. The authors declare that they have no conflict of interest.

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