PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT, ANTIMICROBIAL ACTIVITIES AND GC-MS PROFILING OF DRYMARIA DIANDRA BLUME

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Abstract: *Drymaria diandra* is used in Nepalese traditional medicine to treat various ailments. The hexane, dichloromethane, ethyl acetate, methanol and 50% aqueous methanol extracts of *D. diandra* were prepared and screened for the presence of different classes of phytochemicals. Methanol and 50% aqueous methanol extracts showed the presence of phenolics, flavonoids and carbohydrates so their total contents were estimated. The highest amount of phenolics and flavonoids were found in methanol extract (190.58±2.21 mg GAE/g extract and 69.25±7.91 mg CE/g extract respectively). The highest amount of sugar was found in 50% methanol extract (185.60±2.53 mg GE/g extract). Similarly, the extracts were tested for the antibacterial activity against one gram negative bacteria, *Escherichia coli* and one gram positive bacteria *Staphylococcus aureus.* All the tested extracts showed good antibacterial activity against both bacteria with the inhibition zone ranging from 10 -22 mm. The free radical scavenging activity was determined using DPPH free radical. Both methanol and 50% aqueous methanol extracts showed strong antioxidant activity (IC_{50} 13.61and 16.94 µg/ml respectively). The GC-MS analysis of hexane extract revealed the presence of more than 22 compounds. However, 9 compounds were identified by comparing the mass fragmentation pattern of each compound with the standard NIST mass spectral database.

Keywords: Antioxidant; Antimicrobial; GC-MS; Phytochemical.

INTRODUCTION

Medicinal plants are still a significant source of drugs and leads. They play a vital role in preventing, healing and curing of human diseases because of the presence of different classes of phytochemicals[1]. The search for new pharmacologically active agents obtained by screening plant extracts has led to the discovery of many clinically useful drugs that play a major role in the treatment of human diseases[2].

The search for antibacterial from medicinal plants has received much attention because of the emergence of drug-resistant pathogenic bacteria to the existing drugs[3]. Again the search for natural antioxidants has received much attention which can help to prevent oxidative damage occurring in the body[4]. It is known that onset and progression of many chronic and degenerative diseases are caused by disparate physiological, pathological, environmental and lifestyle factors[5].

In these days, the concept of multi-target drugs or multi-component therapy is gaining increased attention with the discovery that many diseases like hypertension are best treated by multi-drug or multi-target therapies[6]. Thus, natural antioxidants are gaining popularity among consumers and scientific community because

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epidemiological studies have indicated that their frequent consumption is associated with a lower risk of cardiovascular disease and cancer[7]. It is well known that many valuable drugs derived from plants were discovered through their application in traditional medicine. The acceptability, convenience and accessibility of traditional medicine make them important resources for the discovery of bioactive molecules with therapeutic effects. However, plants synthesize diversity of phytochemical which results in the diversity in their biological activities and drug like properties[8].

Drymaria diandra, (Synonyme: *D. cordata*) commonly known as Chickweed and locally known as 'Abijalo'belongs to Caryophyllaceae family, is a creeping herb grows in dense patches in moist and shady places throughout Nepal to about 2000 m.The plant has been traditionally used to treat diverse ailments. The juice of the root is inhaled to treat sinusitis, the juice of the plant is useful to treat gastric, ulcer, fever, indigestion and conjunctivitis. The plant has laxative and cooling effect, the aerial parts are eaten as a vegetable and paste of the plant is useful for peptic ulcer, fever, cough, cold and headaches[9]. Literature review revealed that many bioactive constituents have been isolated from *D. diandra* . A novel flavonoid glycoside[10], cyclic peptides[11], drymaritin, a new anti- HIV alkaloid and diandraflavone, a new C- glycoside flavonoid[12], new flavone glycosides[13] have been isolated. In our previous investigation, *D. diandra* did not show wound healing activities[14].Quantification of phenolics, flavonoids, antioxidant and antimicrobial activity of methanol extract have been carried out[15,16]. In contrast, scientific investigation has not been made on different extracts like hexane, dichloromethane, ethyl acetate, methanol and 50% aqueous methanol. Thus, the present study was carried out to determine the antimicrobial activity of all the five extracts, total phenolic, flavonoid and sugar content in polar extracts, their antioxidant activity and metabolite profiling of hexane extract by GC-MS technique to detect the presence of various bioactive compounds.

MATERIALS AND METHOD

Plant Materials

The plant material was collected from Bhaktapur district in February 2016 and authenticated by comparing with the voucher specimens deposited at Central Department of Botany Tribhuvan University, Kathmandu, Nepal. The voucher specimen (DD-16-SK) was deposited at Research Centre for Applied Science and Technology, RECAST, Tribhuvan University.

General experimental procedure

DPPH and authentic (±)- catechin were purchased from Sigma Chemical Company, USA. Gallic acid was purchased from Merck, Darmstadt, Germany. Aluminium chloride (SD fine-chemicals), Folin- Ciocalteu (SD fine-chemicals) and anthrone (Thomas baker) were purchased from a local vendor. All other chemicals and solvents were of analytical grade and purchased from local vendors. Absorbance was measured using Chemito UV- VIS Spectrophotometer. Analytical GC-MS was carried out on JEOL AccuTOF GCX Time of Flight Mass-spectrometer fitted with Agilent 7693A type GC injector and a HP5 capillary column.

Extraction

The dried and powdered aerial parts of *D. diandra*(100 g) were extracted successively with hexane (250 ml, 6 times, 18 h), dichloromethane (300 ml, 6 times, 18 h), ethyl acetate (200 ml, 4 times, 18 h) and methanol (200 ml, 5 times, 18 h) by cold percolation. The residue after extraction with methanol was refluxed with 50% aqueous methanol (400 ml, 4 h). The extracts were filtered separately and the solvent was evaporated under reduced pressure in a Rotavapor to get respective crude extracts. The dried extracts were stored at -20 ºC for further use.

Phytochemical screening

The freshly prepared crude extracts were tested for the presence of various classes of phytochemical using standard procedures[17].

Determination of total phenolic content

Total phenolic content in plant extracts was determined by using Folin-Ciocalteucolouri metric method based on oxidation-reduction reaction[18].Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram dry extract (mg/g).

Determination of total flavonoid content

Total flavonoid content was determined by aluminum chloride colorimetric assay[19]. Total flavonoid content of the extracts was expressed as mg catechin equivalents (CE) per gram of dry extract (mg/g).

Determination of total sugar content

Total sugar content in plant extracts was estimated by using anthrone reagents based colorimetric method[20]. Total sugar content of the extracts was expressed as mg glucose equivalents (GE) per gram of dry extract (mg/g).

Calculation

The total phenolic, flavonoid, and sugar content were calculated in all the extracts separately using the formula, C=cV/mwhere, C, Total content of phenol/flavonoid/sugar compounds in mg/g, c, concentration of gallic acid/(±)-catechin/D-glucose established from the calibration curve in mg/mL, V, volume of extract in mL, m, weight of plant extract. Data were recorded as a mean (±) standard deviation of three determinations of absorbance for each concentration, from which linear correlation coefficient (R^2) value was calculated using MS Office Excel 2007. The linear regression equation for a straight line is, [y = mx +c] where, y, absorbance of extract, m, slope of the calibration curve, x, concentration of extract, c, intercept. Using this regression equation, concentrations of extracts were calculated. From the calculated values of concentration of each extract, the total phenolics, flavonoid and sugar content were calculated.

Determination of antioxidant activity using DPPH free radical

Antioxidant activity of the selected extracts was assessed using DPPH free radical[21].

Determination of antibacterial activity

One Gram positive bacteria, *S. aureus* (ATCC 25923) and one Gram negative bacteria, *E. coli* (ATCC 25922) were used to determine the antibacterial activity of the extracts. Agar well diffusion method was usedto determine the antimicrobial activity[21].

GC-MS analysis of hexane extract

Analytical GC-MS of hexane extract was carried out on JEOL Accu TOF GCX Time of Flight Mass-spectrometer fitted with Agilent 7693A type GC injector and a HP5 capillary column (29.2 m x 0.25 mm i.d., film thickness0.25 µm). The initial oven temperature was maintained at 80 °C withhold time of 1 minute. The temperature was gradually increased to 320 °C at a rate of 15 °C/minute. The maximum oven temperature was maintained at 325 °C and kept at final temperature for 5 minutes. The ion chamber temperature was maintained to 250 °C while GC interface temperature and inlet temperature was maintained to 300 °C. Diluted sample (0.5 µL) was injected in the splitless mode. Helium was used as a carrier gas with a flow rate of 1.2 mL/min. MS was operated in electron impact mode with ionization energy of 70 eV. Full scan mass spectra were acquired from 25-600 amu. The total run time was 22 minutes. The detected compounds were identified by processing the raw GC-MS data with msAxel software (Version 1.1.6.17127 Copyright 2014-2015JEOL Ltd.) and comparing with NIST mass spectral library (Mass spectral Program for the National Institute of Standards and Technology/Environment Protection Agency/National Institute of Health Mass Spectral Library, Version 2.2).

RESULTS AND DISCUSSION

The aerial parts of dried and crushed *D. diandra* (100 g) was successively extracted with hexane, dichloromethane, ethylacetate and methanol by percolation. The residue was refluxed with 50% aqueous methanol to get the respective extracts. The highest amount of extract was obtained with methanol and the lowest amount was obtained with 50%methanol.The results of the yield of the extracts are shown in Table 1. The results of the phytochemical screening of different extracts revealed that only terpenoids and quinones are present in the hexane, dichloromethane andethyl acetate extracts. Phenolics, flavonoids, tannins, glycosides and reducing sugars are present in the

methanoland 50% aqueous methanol extracts. The results are shown in Table 1.

Table 1. Yield and phytochemical screening of different extracts

Extracts	Hexane	CH_2Cl_2	EtOAc	MeOH	50% MeOH
Yield in g	2.66	3.22	2.09	5.04	1.54
Phenolics	-	-	-	+	+
Flavonoids	-	-	-	+	+
Alkaloids	-	-	-	-	-
Terpenoids	+	+	+	-	-
Glycosides	-	-	-	+	+
Tannins	-	-	-	+	+
R. sugars	-	-	-	+	+
Quinones	+	+	+	-	-

(+) indicates present and (–) indicates absent

Total phenolic content

Plant derived polyphenols including flavonoids and tannins possess several biological properties and thusnecessitate evaluating their presence in different extracts prepared in different organic solvents. A simple andreproducible Folin-Ciocalteu (FC) method was applied for the determination of total phenolic content using gallic acid as a standard although there is possible interference from other readily oxidizable compounds present in plant extracts. The absorbance values obtained at different concentrations of gallic acid was used for the construction of calibration curve. FC method is based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybedic/phosphotungstic acid complexes to form blue colored complexes, $(PMoW^{11}O_{40})^{-4}$, that are determined spectrophotometrically at 760 nm. The total phenolic content in different extracts were calculated from the calibration curve using regression equation Y = 0.013x, R^2 = 0.999 followed by the formula C = cV/m and expressed as mg gallic acid equivalent (GAE) per g of extract in dry weight (mg/g). The results indicated that the total phenolic content in the methanol extracts (190.58±0.2.21 mg GAE/g extract) was found to be higher than in the 50% aqueous methanol extract (82.42±8.41 mg GAE/g extract). The total phenolic content in our sample is relatively high in comparison to the literature data (122.45±0.96 GAE/g)[15]. This may be due to the difference in extraction method as well as the season of collection and collection site of the plant materials. The results are shown in Table 2. Further, the highest yield and total phenolic content obtained in the methanol extract of could be attributed to its high polarity. Another possible reason might be due to the establishment of complexes by phenolic constituents with other biomolecules such as proteins, carbohydrates[22].

Total flavonoid content

The total flavonoid content in different extracts was estimated by aluminium chloride colorimetric assay using (±)-catechin as a standard. The absorbance values obtained at different concentrations of catechin was used for the construction of calibration curve. The principle involved in aluminium chloride colorimetric method is that it forms acid stable complexes with the either keto groups and/or group of flavones and flavonols. In addition it also forms acid labile complexes with the ortho-dihydroxyl groups of the flavonoids which give pink colour in alkaline medium whose absorbance was measured spectrophotometerically. The total flavonoid content in different extracts were calculated from the calibration curve using regression equation Y = 0.004x, R^2 = 0.991 followed by the formula C = cV/m and expressed as mg (±)-catechin equivalent (CE) per g of extract in dry weight (mg/g). The results of this investigation indicated that the total flavonoid content inthe methanol extract (69.25±7.91 CE/g extract) was found to be slightly higher than that of the 50% aqueous methanol extract (61.86±4.91 CE/g extract). The total flavonoid content in our sample is relatively high in comparison to the literature data (11.51±0.30 QE/g extract)[15]. This could be due to the selection of different standards for the construction of calibration curve. Quercetin was used as a standard in the literature while catechin was used in our investigation. The results are shown in Table 2.

The ratios of total flavonoid to total phenolic content were found to be different in methanol and 50% aqueous

methanol extracts. In the case of methanol extract, the ratio (0.363) was observed which indicated that 36.3% of totalphenolics are flavonoids. Similarly in the case of 50% methanol extract, the ratio (0.750) was observed whichindicated that 75.0% of total phenolics are flavonoids. The results are shown in Table 2.

Total sugar content

Plant polysaccharides have significant therapeutic potential and could be the source of novel immunomodulatory medicine. Many immunomodulatory plant polysaccharides have been isolated and characterized from medicinal plants[23,24]. This has initiated to evaluate their presence in different plant extracts. Anthrone reagent based colorimetric assay using D-glucose as a standard was used to determine the total sugar content in plant extracts. Plant extracts containing sugars were first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural that with anthronale, an enol form of anthrone forms a green coloured product with an absorption maximum at 630 nm which was measured against blank consisting of anthrone solution and distilled water.The absorbance values obtained at different concentrations of glucose was used for the construction of calibration curve. The total sugar content in different extracts were calculated from the calibration curve using regression equation $Y = 0.013x, R^2 = 0.994$ followed by the formula C= cV/m and expressed as mg glucoseequivalents (GE) per g of extract in dry weight (mg/g). Our finding revealed that the 50% aqueous methanol extract was found to contain higher amount of carbohydrate (185.60 ±2.33 GE/g) than the methanol extract (61.48±3.16 GE/g). The results are shown in Table 2.

Antioxidant activity using DPPH free radical

The DPPH assay was carried out for methanol and 50% aqueous methanol extracts. The absorbance values were measured at wavelength 517 nm for different concentrations of extracts and the control. These values were used to calculate the percentage inhibitions of DPPH radical against the samples. The IC_{50} values of various extracts were calculated from the percentage inhibitions at various concentrations are given in Table 2. The results showed that the IC_{50} value of the methanol extract was found to be lower (13.61 µg/ml) than the IC_{50} value of 50% aqueous methanol extract (16.94 µg/ml). Our sample showed relatively strong antioxidant activity in comparison to the reported date (IC_{50} 26.27±0.19)[15]. The earlier reports have not compared the effect of different solvents during extraction. The stronger antioxidant activity may be correlated with the presence of higher amounts of phenolics/flavonoids in our sample as evidenced by our phytochemical analysis (Table 2). Antioxidants have the ability to prevent various oxidative. stress related cell damages mediated by free radicals. Many antioxidant molecules are recorded in several medicinal plants and thus will be beneficial in the treatment of several human diseases[25]. In the human body, cell damage may induce the generation of increased levels of free radicals. Various disorders including myocardial infarction, cancer, atherosclerosis and neurogenerative disorders are mainly correlated to these free radicals[26].

Antibacterial activity

Plants used in the traditional medicine could be an alternative source of antimicrobial drugs. In this regard, we evaluated the antimicrobial properties of different extracts of *D. diandra* and the results were very conclusive. The hexane, dichloromethane, ethylacetate, methanol and 50% aqueous methanol extracts were screened for antimicrobial activity against *S. aureus*and *E. coli* by agar well diffusion method. An aliquot of 5 and 10 mg each extract prepared in DMSO was introduced into each well. Negative control experiment was performed using equivalent volume of DMSO and positive control experiment was performed by the use of a standard antibiotic, amoxicillin disc (10 µg). At the end of the incubation period, the clear inhibition zones of bacterial growth around the wells were observed in the presence of

different extracts. The results are shown in Table 2. The results of antibacterial assay revealed that all the extracts showed activity against both bacteria. Microbial inhibition was found to vary slightly with the type of extracts evaluated. The greater activity was shown by dichloromethane extract with an inhibition zone of 22 mm against *E. coli* and 20 mm against *S. aureus*at a concentration of 10 mg/well. It could be due to more soluble bioactive compounds extracted with dichloromethane such as terpenoids and quinones (Table 1). A lesser activity was shown by 50% methanol extract with an inhibition zone of 10 mm against *E. coli* and 11 mm against *S. aureus* at a concentration of 10 mg/well. It could be due to the presence of mainly phenolics, flavonoids and tannins (Table1). Mostly gram negative bacteria exhibit more resistant properties against a wide range of antibiotics or chemical drugs when compared to gram positive bacteria due to the differences in their cell wall and outer membrane structures[27-29]. However, both gram positive and gram negative bacterial were effectively inhibited by all the extracts. Thus, the extracts of *D. diandra* could be beneficial in treating the diseases caused by *S. aureus* and *E. coli*. In contrast to our findings, it was reported that the methanol extract of *D. diandra* did not show antibacterial activity against *E. coli ,Klebsiella sp*. And *Serratia sp*[16].

Table 2. Antimicrobial activity, total phenolic, total flavonoid, total sugar and DPPH free radical scavenging activity.

Plant extracts	Bacteria	Inhibition zone (mm)			TPC mg GAE/g	TFC mg CE/g	Ratio TF/TP	TSC mg GE/g	DPPH IC_{50} (µg/ml)
		5 mg/well	10 mg/well	Control					
Hexane	*E. coli*	15	15	-	-	-	-	-	-
	S. aureus	12	12	-					
DCM	*E. coli*	17	22	-	-	-	-	-	-
	S. aureus	18	20	-					
EtOAc	*E. coli*	16	17	-	-	-	-	-	-
	S. aureus	12	15	-					
MeOH	*E. coli*	15	15	-	190.58±2.21	69.25±7.91	0.363	61.48±3.16	13.61
	S. aureus	12	13	-					
50% MeOH	*E. coli*	10	10	-	82.42±8.41	61.86±4.91	0.750	185.60±2.53	16.94
	S. aureus	11	11	-					
Amoxicillin	*E. coli*	-	-	22	-	-	-	-	-
	S. aureus	-	-	36					

Note: Inhibition zone was measured including well and disc

GC-MS of hexane extract

The chemical composition of hexane extract of *D. diandra*was determined by GC-MS technique. The chromatogram is shown in Fig 1. The GC-MS analysis of the hexane extract revealed the presence of more than 22compounds. However, 9 compounds were identified on the basis of the fragmentation pattern of each compound in mass spectrum and comparison with the standard NIST mass spectral database. The identified compounds were one diterpene, one phthalate ester, two hydrocarbons and four saturated aldehydes. The results are shown in Table 3. The Gas chromatogram is shown in Fig 1. The mass spectrum of one of the compound eluted at 9.861 min and its comparison with the mass spectrum of neophytadieneavailable in NIST library is shown Fig 2.

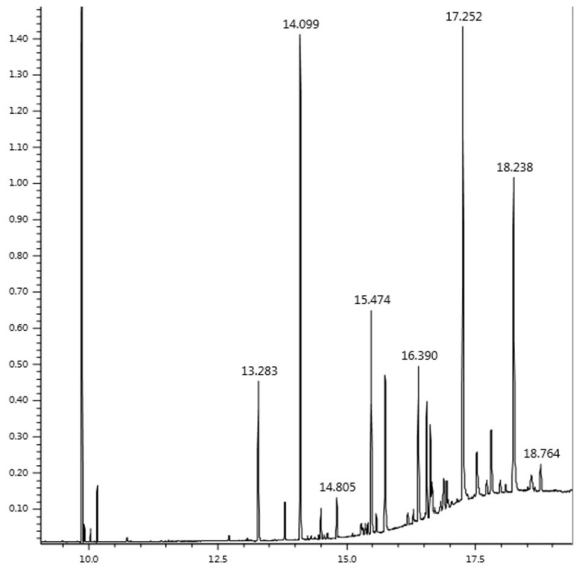
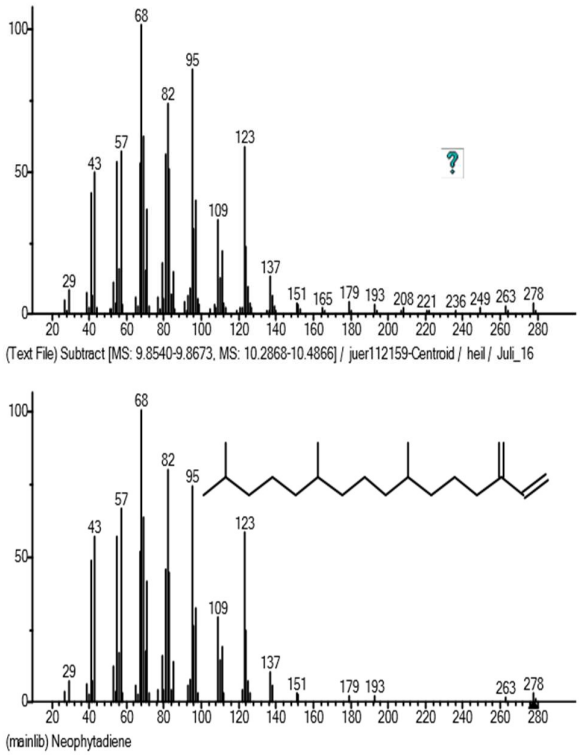


Figure 1: Gas chromatogram of hexane extract.

Figure 2: Mass spectrum of the compound eluted at 9.861 min (above) and the mass spectrum of neophytadiene available in NIST library (below).



Plants synthesize various chemicals to protect them against predators and microbial attacks. They differ from plant to the plant families. Neophytadiene, which is present in several plants and the green alga showed strongantibacterial activity[30]. Several aliphatic saturated and unsaturated aldehydes are produced by enzymatic cleavage of fatty acids when plants are attacked by microbes[31]. The long-chain aldehydes from olive oil have shown antibacterial activity against both gram-positive and gram-negative microorganisms that caused human intestinal and respiratory tract infections[32].The antibacterial activity of the hexane extract could be due to the presence of neophytadiene together with aldehydes like triacontanal and henicosanal and other compounds. Their synergistic/addative effect could be more pronounced than that of the individual component.

Table 3. Results of GC-MS of hexane extract.

Name of compounds	Chemical class	Retention time
Neophytadiene	Diterpene	9.861
Di-isooctyl phthalate	Phthalate ester	14.009
Tetracosanal	Saturated aldehyde	15.474
Hentriacontane	Hydrocarbon	15.745
Octacosanal	Saturated aldehyde	16.390
Stigmastan-3,5,22-trien	Sterol	16.559
2-Methylhexacosane	Hydrocarbon	16.624
Triacontanal	Saturated aldehyde	17.252
Henicosanal	Saturated aldehyde	18.238

CONCLUSION

The present research highlighted the phenolic, flavonoid, sugar content of different extracts, their antioxidant, antibacterial activity and GC-MS profiling of hexane extract of *D. diandra.* The polar extracts are the good

source of phenolics, flavonoids and carbohydrates with high antioxidant activity. Non polar and medium polar extracts are the source of antibacterial compounds. When comparing our results with the reported results, our plant looked different in terms of phytochemical contents and biological activity. Thus, this plant can serve as a new natural source for obtaining many therapeutically valued metabolites against various diseases. The results are in agreement with the traditional belief for which they use as medicine for the treatment of various ailment.

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