Phytochemical evaluation of *Aglaomorpha coronans* from Nepal

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Abstract: The rhizomes of *Aglaomorpha coronans* is used to cure back pain by the local people of Lamjung district. The medicinal value depends on the presence of biologically active phytochemicals with drug like properties. Phytochemical evaluation of *A. coronans* of Nepalese origin has not been reported yet. Hence, the present study is focused on the phytochemical analysis, evaluation of antioxidant, antibacterial activities of various extracts and GC-MS profiling of ethyl acetate extract. The rhizomes were extracted with the hexane, chloroform, ethylacetate, methanol and 50% aqueous methanol. Phytochemical screening of ethyl acetate, methanol and 50% aqueous methanol extracts showed the presence of phenolics, flavonoids, tannins and sugar. Their total content as well as antioxidant activity were determined. The highest amounts of phenolics, flavonoids and tannins were detected in the ethyl acetate extract (292.14±1.82 mg GAE/g, 322.90±3.94 mg CE/g, 516.66±0.42 mg TAE/g). The highest amounts of sugars were detected in the methanol extract (205.64±0.16mg GE/g). In DPPH radical scavenging assay, ethyl acetate extract showed the highest activity (IC_{50} 37.54 µg/ml). The chloroform, ethyl acetate and methanol extracts showed antibacterial activity against *S. aureus.* GC-MS analysis of ethyl acetate extract showed the presence of more than 20 compounds. However, 9 compounds, mainly polyol, low molecular weight phenolic acids and terpenes/sterols were identified by comparing the mass fragmentation pattern of each compound with the standard NIST mass spectral database. The presence of 3,4-dihydroxy benzoic acid, Olean-13(18)-ene and β-sitosterol justifies the anti-inflammatory and analgesic properties of *A. coronans* in traditional medicine.

Keywords: Antibacterial; Antioxidant; GC-MS; Phytochemicals.

Introduction

The treatment of infections and health disorders with herbal medicines is usually not entirely a placebo medicine but really involves secondary metabolites[1]. Plants contain hundreds of active ingredients and their combined action enhances the synergetic effect that maybe potentially useful for the development of prophylactic as well as therapeutic agents for multiple targets. Drug discovery and development should not always be limited to the discovery of a single molecule and the current belief on one drug one disease approach. Preparation of standardized plant extracts and isolation and identification of phytochemicals from plant materials are crucial for drug discovery as they provide unlimited opportunities for new drug leads[2]. It is well known that plants collected on the basis of ethnopharmacology are more informative in the drug development process than the random collection. Some anticancer drugs and lead compounds as well as antimicrobial compounds were discovered based upon traditional medicine[3,4]. Traditional medicine and healing systems are widely practiced in every community of Nepal and it is therefore necessary for the scientific validation of such empirical knowledge.

Pteridophytes are not well investigated phytochemically in comparison to angiosperms. *Aglaomorpha coronans* (Wall. ex Mett.) Copel belongs to Polypodiaceae family [synonym: *Drynaria coronans* (Wall. ex Mett.) J. Sm. ex T, *Pseudodrynaria coronans* (Wall ex. Mett.) Ching,

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Polypodium coronans wall.] is an epiphytic fern, locally known as 'kamaru' and commonly known as Santa Rosa or basket fern as it forms a ring shaped-basket around tree-trunks. Rhizomes are thick, creeping, making thick bracket around host, covered by golden tomentose scales. It is distributed in Nepal at an altitude between 1000-1200 m above sea level[5]. The rhizomes are used by the local people of Lamjung to cure back pain and bone injuries. It is one of the components of traditional Chinese medicine 'GuSuiBu' which have been claimed to cure body ache, inflammation, cancer, ageing, blood stasis and bone injuries[6].

Some flavonol glycosides like Kaempferol-3-*O*-(6" -*O*-feruloyl-4" -*O*-acetyl)-*β*-D-glucopyranoside, kaempferol-3-*O*-(6" -*O*-feruloyl)-*β*-D-glucopyranoside, kaempferol-3-*O*-(6" -*O*-acetyl)-*β*-D-glucopyranoside, astragalin and isoquercitrin have been isolated from the whole plant of *Pseudodrynaria coronans* from China[7]. Total content of phenolics, flavonols, phenylpropanoids, anthocyanidin, epicatechin and naringni have been reported from *Pseudodrynaria coronans* from Taiwan[8]. Comprehensive literature review revealed that only antibacterial activity of ethanolic extract of *A. coronans* of Nepalese origin has been reported[9]. However, phytochemical investigation has not been reported elsewhere. The value of such plant can be explored only by phytochemical analysis. Hence, the purpose of the present study is to estimate the content of total phenolics, flavonoids, tannins and sugars, evaluation of antioxidant, antibacterial activities and chemical profiling of ethyl acetate extract by GC-MS technique.

Materials and Methods

Plant materials

The fresh rhizomes of *A. coronans* were collected from the Sundar bazzar municipality of Lamjung district in May 2018. The plant sample was authenticated by comparison with the herbarium species deposited at Central Department of Botany, Tribhuvan University. The voucher specimen (#AC-2018-AK) was deposited at Research Center for Applied Science and Technology, RECAST. The collected rhizomes were cleaned, washed with water, shade dried, powdered and stored in an air tight container.

General experimental procedure

All the solvents and chemical were of analytical grade and purchased from local vender. Gallic acid was purchased from Merk, Germany, DPPH and (±)-Catechin were purchased from Sigma Chemical Company, USA. Absorbance was measured using Chemito UV-VIS Spectrophotometer. GC-MS was recorded using GC-MS-JEOL AccuTOF GCX Time of Flight Mass spectrometer.

Extraction

The dried and powdered rhizomes (100 g) were extracted successively with hexane (400 ml), chloroform (300 ml), ethyl acetate (300 ml) and methanol (250 ml) in a Soxhlet extractor for 6-7 hours until the last extract became colorless. The remaining residue after extraction with methanol was refluxed with 50% aqueous methanol (200 ml) for 2 hours. The respective extracts were concentrated under rotary evaporator at reduced pressure to obtain solid or semi-solid extracts. The dried extracts were kept in fridge for further analysis.

Phytochemical screening

The presence different classes of natural product in the different extract were analyzed by reacting with different reagents following the standard protocol[10].

Estimation of total phenolic content (TPC)

The total phenolic content in the plant extracts were estimated by using phenol Folin-Ciocalteu reagent (FCR) based on colorimetric method[11]. The diluted extract (1 mL) was mixed with 5 mL of Folin-Ciocalteu reagent (10% v/v) and 4 mL of sodium carbonate (7% w/v). The mixture was shaken and incubated for 30 min at 40 °C in a water bath before absorption was measured at 760 nm. Total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram extract (mg GAE/g extract). All extracts were analyzed in triplicate.

Estimation of total flavonoid content

The total flavonoid content in plant extract was determined using Aluminium chloride colorimetric method[12]. The

diluted extract (1 mL) was mixed with 4 mL of double distilled water and 0.3 mL of $NaNO_2$ (5 % w/v). After 5 minutes, 0.3 mL of $AlCl_3$ (10% w/v) and after 1 minute 2 mL of 1 M NaOH was added. The total volume of the mixture was made up to 10 mL by the addition of 2.4 mL double distilled water and mixed thoroughly. Absorbance of the pink color mixture was determined at 510 nm. Total flavonoid content was expressed as catechin equivalents (CE) in milligrams per gram extract (mg CE/g extract). All extracts were analyzed in triplicate.

Estimation of total tannin content

The total tannin content in plant extracts was estimated by using Folin-Ciocalteu method[13]. The diluted extract (1 mL) was mixed with 8.4 mL of double distilled water, 0.5 mL of FC reagent (10% v/v) and 0.1 mL of sodium carbonate solution (7% w/v). The mixture was then shaken well and the allowed to stand for 30 minutes and then absorbance was taken at 700 nm. Total tannin content was expressed as tannic acid equivalents (TAE) in milligrams per gram extract (mg TAE/g extract). All extracts were analyzed in triplicate.

Estimation of total carbohydrate/sugar content

The total carbohydrate/sugar content in plant extracts was estimated by using anthrone reagent[14]. The extract (100 mg) was hydrolyse with dilute HCl and subjected to serial dilutions. The diluted extract (2 mL) was mixed with 8 mL of freshly prepared anthrone reagent (200 mg of anthrone in 100 mL ice-cold 95% conc. H_2SO_4). The mixture was shaken well and heated for 8 minutes at boiling water bath. Then, cooled rapidly and the absorbance was measured at 630 nm. Total sugar content was expressed as glucose equivalents (GE) in milligrams per gram extract (mg GAE/g extract). All extracts were analyzed in triplicate.

Determination of antioxidant activity using DPPH free radical

The antioxidant activity was determined using DPPH free radical[15]. A mixture of 2.5 ml of DPPH (0.10 mM in methanol) and 0.5 ml of extract of different concentrations were kept in the dark for 30 min. Absorbance was recorded at 517 nm. A control was prepared by mixing 0.5 ml methanol and 2.5 ml 0.1 mM methanolic DPPH solution. DPPH radical scavenging activity (%) was calculated as follows:

$$\% \text{ of radical scavenging } = \frac{Ac - As}{Ac} \times 100$$

Where, Ac=Absorbance of control and As= Absorbance of solution. The percentage scavenging was then plotted against concentration and IC_{50} value of the extracts determined graphically.

Determination of antibacterial activity

Agar well diffusion method was used for the evaluation of antibacterial activity of the crude extracts[16]. Gram-positive bacteria (*Staphylococcus aureus*, ATCC 25923) and Gram-negative bacteria (*Escherichia coli*, ATCC 25922) were selected to determine the antibacterial activity. An aliquot of 10 µL of sample of each concentration (500 mg/mL prepared in 50% DMSO) was introduced into each well of 4 mm diameter so that the exact amount of extracts in each well was 5 mg. Negative control experiments were performed using equivalent volume of 50% DMSO and positive control experiments were performed by use of a standard antibiotic, neomycin (10 µg/well). 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. Inhibition of the bacterial growth in the presence of extracts was measured in the form of zone of inhibition (ZOI).

GC-MS analysis of ethyl acetate extract

Analytical GC-MS of ethyl acetate extract was carried out on JEOL AccuTOF GCX Time of Flight Mass-spectrometer fitted with Agilent 7693A type GC injector and a ZB-5MSplus capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm). The initial oven temperature was maintained at 80 °C with a hold 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 250 °C while GC interface temperature and inlet temperature was kept at 300 °C. Diluted sample (0.5 µL) was injected at 300 °C 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 an 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 in the gas chromatogram were identified by processing the GC-MS data with MSAxel software (Version 1.1.6.17127 Copyright 2014-2015 JEOL Ltd.) and comparing it with the mass spectral database of the National Institute of Standard and Technology, NIST mass spectral library 2.2.

Results and discussion

Extractive values in different solvents

The hexane, chloroform, ethyl acetate, methanol and 50% aqueous methanol extracts were prepared from 100 g of powdered rhizomes of *A. coronans*. The high amounts of extracts were obtained with methanol and 50% aqueous methanol while low amounts of extracts were obtained with chloroform and ethyl acetate. These results indicated that *A. coronans* contain more polar phyto-constituents. The yields of various extracts are given in Table 1.

Phytochemical screening

The phytochemical screening of *A. coronans* extracts showed that alkaloids were absent in all the extracts. The hexane and chloroform extracts were devoid of all the tested phytochemicals except terpenoids. All the tested phytochemicals were present in ethylacetate, methanol and 50% aqueous methanol extracts except saponins in the ethyl acetate extract. The results are presented in Table 1.

Table 1: Yield of extracts and results of phytochemical screening.

Extracts	Hexane	$CHCl_3$	EtOAc	MeOH	50% aq. MeOH
Yield	2.73 g	1.05 g	1.47 g	8.90 g	7.46 g
Alkaloids	-	-	-	-	-
Terpenoids	+	+	+	+	+
Flavonoids	-	-	+	+	-
Phenolics	-	-	+	+	+
Glycosides	-	-	+	+	+
R. sugars	-	-	+	+	+
Saponines	-	-	-	+	+
Tannins	-	-	+	+	+

Total phenolic content

The total phenolic content in different extracts was calculated from regression equation of calibration curve (y = 0.0157x, R^2=0.9984) and was expressed as mg of GAE per gram of dry extract. The results showed that the ethyl acetate extract contains the highest amounts of phenolics (292.14±1.82 mg GAE/g extract) while methanol (37.48±5.41 mg GAE/g extract) and 50% aqueous methanol extracts (33.37±7.24 mg GAE/g extract) contain relatively low amounts of phenolics. It was reported that the aqueous extract of *Pseudodrynaria coronans* collected from Taiwan contain only 130.10 mg phenolics/g extract when catechin was used as a standard[7]. This could be due to the poor solubility of some phenolics in aqueous medium. The total phenolic contents calculated in ethyl acetate, methanol and 50% aqueous methanol extract of *A. coronans* are presented in Table 2.

Total flavonoid content

The total flavonoid content in different extracts was calculated from the regression equation of calibration curve (y = 0.0026x, R^2= 0.9978) and expressed as mg catechin equivalent (CE) per gram of dry extract. The results showed that the ethyl acetate extract contains the highest amounts of flavonoids (322.90±3.94 mg CE/g extract) while relatively low amounts of flavonoids were detected in methanol extract (9.87±1.00 mg CE/g extract) and it was

absent in 50% aqueous methanol. In contrast to our results, it was reported that the aqueous extract of *Pseudodrynaria coronans* collected from Taiwan contain only 7.42 mg flavonoids/g extract when quercetin was used as a standard[7]. The total flavonoid contents of different extracts are presented in the Table 2.

Table 2: Total phenolic, flavonoid, tannin, sugar and DPPH free radical scavenging activity of extracts.

Extracts	EtOAc	MeOH	50% MeOH
TPC (mg GAE/g dry extract (Mean ± S.D) (n=3)	292.14±1.82	37.48±5.41	33.37±7.24
TFC (mg CE/g dry extract) (Mean ± S.D) (n=3)	322.90±3.94	9.87±1.00	-
TTC (mg TAE/g dry extract) (Mean ± S.D) (n=3)	516.66±0.42	57.32±2.24	40.40±7.31
TSC (mg GE/g dry extract) (Mean ± S.D) (n=2)	110.01±1.01	205.64±0.16	149.09±0.06
IC_{50} µg/ml against DPPH (Mean ±S.D) (n=2)	37.54±1.25	80.25±1.37	143.05±1.72

Total hydrolysable tannin content

The total tannin content in different extracts was calculated from the regression equation of calibration curve (Y=0.0072x, R^2 = 0.9981) and expressed as mg tannic acid equivalent (TAE) per gram dry extract. The results showed that the ethyl acetate extract contain the highest amounts of tannin (516.66±0.42 mg TAE/g extract) while relatively low amounts of tannins were detected in methanol (57.32±2.24 mg TAE/g extract) and 50% aqueous methanol extracts (40.40±7.31 mg TAE/g extract). The total tannin contents of different extracts are presented in the Table 2.

Total sugar content

The total sugar content in different extracts was calculated from the regression equation of calibration curve (Y = 0.0104x, R^2 = 0.9923) and expressed as mg glucose equivalent (GE) per gram of dry extract.

The total sugar content in methanol extract was found to be high (205.64±0.16 mg GE/g extract) in comparison to 50% aqueous methanol (149.09±0.06 mg GE/g extract) and ethyl acetate extract (110.01±1.01 mg GE/g extract). The results are presented in Table 2.

Antioxidant activity

In DPPH radical scavenging assay, the lowest IC_{50} value was shown by the ethyl acetate extract (37.54±1.25 µg/ml) followed by the methanol extract (80.25±1.37) while the 50% aqueous methanol extract showed somewhat higher IC_{50} value (143.05±1.72). The stronger antioxidant activity of the ethyl acetate extract could be due to the presence of higher amounts of phenolics/flavonoids/tannins as evidenced by phytochemical analysis (Table 2).

Antibacterial activity

In antibacterial assay, only chloroform, ethyl acetate and methanol extracts of *A. coronans* showed antibacterial activity against gram positive bacteria, *S. aureus* at a concentration of 5 mg/well. However, all the tested extracts did not show activity against *E. coli*. The results are presented in Table 3. In contrast to our findings, it was reported that the ethanolic extract of *A. coronans* showed activity against *E. coli*[9]. It is well known that plant collected from different sites and seasons showed great chemical diversity which ultimately results in the diversity of their biological activities[17]. Generally, phytochemicals present in plant extracts show antibacterial activity by disrupting bacterial outer membrane integrity thereby affecting the membrane potential[18]. The phytochemicals present in our extracts could not able to disrupt the outer membrane of *E. coli* to cause leakage of cellular contents.

GC-MS analysis of ethyl acetate extract

The chemical composition of ethyl acetate extract of *A. coronans* was determined by GC-MS technique. GC-MS analysis of ethyl acetate extract showed the presence of more than 20 compounds. However, 9 compounds were identified by comparing the mass fragmentation pattern of each compound with the standard NIST mass spectral database. They include mainly polyol, low molecular weight phenolic acids and terpenes/sterols. The results are given in Table 4. The gas chromatogram is shown in Fig 1. The mass spectrum of one of the compound eluted at 9.55 min and its comparison with the mass spectrum of 3,4-dihydroxy benzoic acid available in NIST library is shown Fig 2a and Fig 2b respectively.

Phenolic acids have beneficial effects on human health such as reducing the risk of various diseases[14]. 3,4-dihydroxy benzoic acid, known as protocatechuic acid, is a naturally occurring phenolic acid. It has structural similarity with gallic acid, caffeic acid, vanillic acid and syringic acid. It is widely distributed in most edible plants, fruits, bran, brown rice, onion scales. Its diverse pharmacological activities has been reported such as antioxidant, antibacterial, anticancer, antiulcer, antidiabetic, antiageing, antiviral, anti-inflammatory, analgesic, antiatherosclerotic, cardiac, hepatoprotective, neurological and nephroprotective activities[19,20]. Similarly, phytosterols are widespread in plants like vegetable oils, nuts, cereal products, vegetables, fruit and berries. Among them β-sitosterol is the major component[21,22]. Diverse pharmacological activities of β-sitosterol have been reported such as anti-inflammatory, chemoprotective, analgesic, immunomodulatory, antioxidant, neuroprotective and antidiabetic[23]. It was reported that olean-13(18)-ene has significant anti-proliferative and anti-inflammatory activities[24].

The antioxidant and antibacterial activities of ethyl acetate extract of *A. coronans* could be due the presence of protocatechuic acid along with other unidentified phenolics and flavonoids as well as phytosterols like β-sitosterol. The presence of these bioactive compounds justifies the use of *A. coronans* in traditional medicine to treat pain and inflammation.

Table 3: Results of antibacterial assay.

Extracts	Bacteria	Inhibition zone mm
Hexane	*S. aureus*	-
Chloroform	*S. aureus*	18
Ethylacetate	*S. aureus*	17
Methanol	*S. aureus*	18
50% aq. methanol	*S. aureus*	-

Table 4: **Results of GC-MS of ethyl acetate extract.**

S. No	RT	Name of compounds
1	2.91	Glycerin
2	4.06	1,2,3-propanetriol, 1-acetate
3	4.41	Glycerol 1,2-diacetate
4	8.10	2-butyl phenol
5	8.10	4-hydroxy benzene acetic acid
6	9.55	3,4-dihydroxy benzoic acid
7	17.89	Olean-13(18)-ene
8	18.52	β-sitosterol
9	20.73	9,19-cyclolanostan-3-ol,24-methylene-,3β-

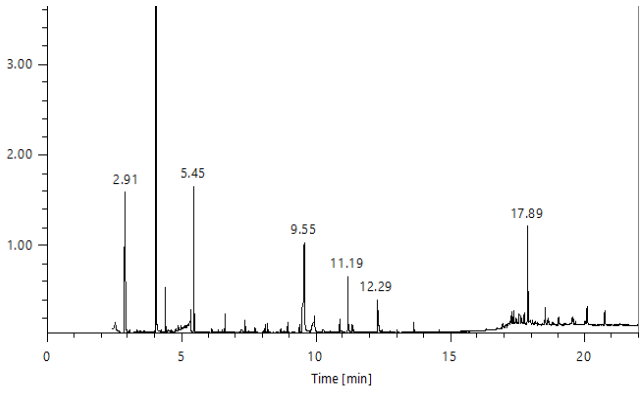

Fig 1. Gas chromatogram of ethyl acetate extract.

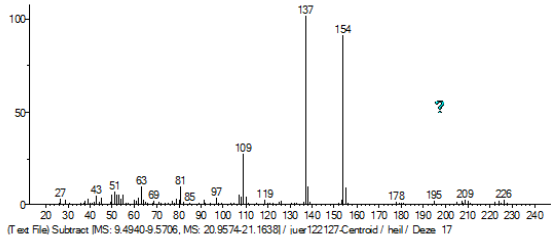

Fig 2a. Mass spectrum of the compound eluted at 9.55 min.

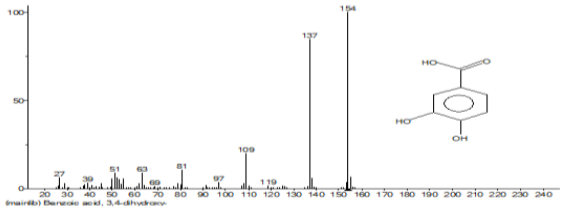

Fig 2b. Mass spectrum of 3,4-dihydroxy benzoic acid.

Conclusions

The present research highlighted the phenolic, flavonoid, tannin, sugar content of different extracts, their antioxidant, antibacterial activities and GC-MS profiling of ethyl acetate extract of *A. coronans.* The ethyl acetate extract is a good source of phenolics, flavonoids and tannins with high antioxidant activity. The methanol extract is a good source of carbohydrates. The chloroform, ethyl acetate and methanol extracts are the sources of antibacterial compounds. The presence of protocatechuic acid, β-sitosterol as analgesic and anti-inflammatory compounds justifies the traditional use of this plant in pain relief. Thus, this plant could be the source for obtaining many therapeutically valuable metabolites against pain and other ailments.

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