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RESEARCH ARTICLE

Chemical and biological analysis of extracts of *Acorus calamus* L.

Aaradhana Pokharel[1], Arjun Thapa[1], Homa Karki[1], Rakesh Kumar Yadav[1], Samjhana Bharati[1], Timila Shrestha[1], Puspa Lal Homagai[1], Binita Maharjan[1]*, Ram Lal Shrestha[1,2,*]

[1] *Department of Chemistry, Amrit Campus, Institute of Science and Technology, Tribhuvan University, Lainchaur, Kathmandu, Nepal*

[2] *Institute of Natural Resources Innovation, Kalimati, Kathmandu, Nepal*

*Email: binitamhrjan@gmail.com, swagatstha@gmail.com

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Abstract:

The powdered plant materials of *Acorus calamus* were subjected to successive extraction using the cold percolation method with methanol, hexane, and chloroform solvent. Qualitative phytochemical analysis of methanol, hexane, and chloroform extracts showed the presence of alkaloids, saponins, glycosides, sterols, triterpenoids, and carbohydrates. Five different major compounds were identified by GC-MS analysis of the chloroform extract, with isoprothiolane (83.11%) being the most prevalent. The total phenolic content in the chloroform extract was calculated 17.39 mg Gallic acid equivalent/g and the total flavonoid content was 3.37 mg quercetin equivalent/g of dry extract. The IC_{50} value of chloroform extract was found to be 576.19 µg/mL, and the LC_{50} value was found to be 66.21 µg/mL. Antibacterial activity was shown in *Staphylococcus aureus* in chloroform extract with a ZOI of 7 mm.

Keywords: *Acorus calamus*, GC-MS, TPC, TFC, Antibacterial, Antioxidant, Cytotoxicity

Introduction:

Acorus calamus L. is a tall perennial wetland monocot plant with a cylindrical, up to 2.5 cm thick, creeping and widely branched, aromatic rhizome, purplish-brown to light brown externally and white internally [1,2]. It is a member of the Acoraceae family and is usually referred to as a sweet flag. The native name for it is "Bojho" in Nepal. It grows in subtropical and temperate locations all over the world. It comes from Asia and is also found in America, Africa, and Europe. It is primarily found in the terai and hilly parts of Nepal and in wet and marshy areas [3,4].

A. calamus has been known as a stimulant, a bitter herb for the appetite, and used as a digestive aid [5,6]. It has a long history of usage and many conventional and ethno-medicinal applications. The aromatic rhizomes and leaves have traditionally been used for medicinal purposes. The rhizome is used as a substitute for ginger, cinnamon, and nutmeg because of its odour and spicy flavor. Since ancient times, it has been used in a number of medical systems, including Ayurveda, Unani, Siddha, Chinese medicine, etc., to treat a wide range of ailments, including nervous disorders, loss of appetite, chest pain, cough, bronchitis, colic, fever, inflammation, depression,cramps, diarrhoea, digestive disorders, flatulence, gas, indigestion, tumours, skin disorders, etc.[7]. Different pharmacologic behaviours of rhizomes of *A. calamus* such as anti-convulsant, cardiovascular, hypolipidemic, immunosuppressive, anti-inflammatory, antioxidant, anti-diarrheal, anti-microbial, anti-cancer, and anti-diabetic have been recorded. β and α-asarone genotoxicity and mutagenicity have been identified, restricting their use at high doses[5].

Materials and Methods:

Collection of the Plant materials

About 8 kg of the plant of *A. calamus* was collected from Gorkha District, Nepal, at about 1135 m altitude in April 2019. The collected leaves and roots were washed and shade dried. About 3 kg of powder were taken for extraction after grinding into powder.

Extraction

The powdered plant was exhaustively extracted with methanol three times by cold percolation process for 20 days. The content was filtered and concentrated over the Rota evaporator. The crude methanol extract so obtained was proceeded for successive extraction with n-hexane in a separating funnel with vigorous shaking and continuous release of air. The crude hexane extract was collected from the upper layer, and the lower aqueous layer was again mixed with chloroform for further extraction in the separating funnel with vigorous shaking and continuous release of air, taking great care. The lower chloroform layer was concentrated over Rota evaporator. The obtained methanol, hexane and chloroform extracts were then subjected to various phytochemical screening, GC-MS tests and different bioactivities.

Phytochemical Screening

The chemicals which occur naturally in plants are phytochemicals. They play a crucial role in many diseases, such as asthma, arthritis, cancer, etc.[8]. For methanol, hexane, and chloroform extracts, phytochemical screening was done using the standard procedures. The phytochemical tests include tests for alkaloids, flavonoids, glycosides, amino acids, proteins, triterpenoids, carbohydrates and saponins [7,9].

Gas Chromatography-Mass Spectroscopy

The GC-MS analysis of chloroform extract was performed at the Department of Food and Technology and Quality Control, Babarmahal, Kathmandu, Nepal. The following conditions were met for the GC-MS analysis on the GCMS-QP 2010 gas chromatography-mass spectrometer. The ion source temperature and interface temperature were both sustained at 200°C and 250°C, respectively, while helium was utilized as the carrier gas in a Rtx-5MS column with sizes of 60 m by 0.32 mm by 0.25 m (micro meter). The column's temperature was set at 50°C and 300°C with, hold periods of 1.0 and 5.0 min, respectively. The injection volume was 1 L, with a 1:90 split ratio. For identification, a comparison to the Mass Library was included.

Antibacterial Activity

The agar well diffusion method was employed to test antibacterial test in Muller-Hinton Agar. The wells in the incubated media plates were made with a sterile cork borer (4 mm), and each well was labelled suitably. The working solution of the plant extracts was then added to each well using a micropipette. In a separate well, the solvent (DMSO) was tested for activity alongside a control. After that, the extracts were distributed all over the medium by leaving the plates closed for 30 minutes. The plates were incubated for 6 hours at 37°C. After the requisite amount of incubation, the plates were inspected for the zone of inhibition around the well, which is designated by a clear zone without growth, and this region was noted [10,11].

Antioxidant Assay

The antioxidant assay was carried out on DPPH. Initially, a stock solution was developed. A 1 mg/mL solution is generated when a 1 mg sample is dissolved in 1 mL of methanol. Similarly, employing a two-fold dilution procedure, numerous concentrations of the 1000 µL (1 mL) extracts were prepared, including 1500, 1000, 500, 250, and 125 µg/mL. 500 L (0.5 mL) of these solutions were mixed to 1500 µL (1.5 mL) of 0.1 mM DPPH (4 mg DPPH in 100 mL methanol). Aluminum foil was used to shield the solutions from light, and they were vigorously shaken for two minutes.In a darkened room, the solutions were held at room temperature for 30 minutes. After 30 minutes, their absorbance was measured at 517 nm against methanol as a blank. As a control, the absorbance at 517 nm of a combination of 1.5 mL of DPPH solution and 0.5 mL of methanol was measured. A calibration curve was constructed.

The percentage of radical scavenging activity was calculated using the formula below.

$$\text{Percentage scavenging} = \frac{(A_0 - A_T)}{A_0} \times 100\%$$

Where, A_0 = Absorbance of the DPPH

A_T = Absorbance of the DPPH free radical solution containing the sample extract.

The effective sample concentration required to scavenge 50% of the DPPH free radicals is described as the 50% inhibitory concentration (IC_{50}). Plotting the extract concentration against the matching scavenging action allowed us to calculate the IC_{50} values using the dose inhibition curve in the logarithmic range [9,12–17].

Total Phenolic Content Assay

The total amount of phenolic compounds in chloroform extracts was calculated using the Folin-Ciocalteu method. 1 ml of Folin-Ciocalteu reagent was placed in a beaker, and distilled water was added 10 times to dilute it. Gallic acid was diluted in 1 mL of distilled water at a 1 mg to 1 mL ratio, or 1000 µg to 1 mL. The following ingredients were combined: 0.1 mL of sample (1 mg/mL in methanol), 1 mL of Folin-Ciocalteu phenol reagent (1:10 water dilution), and 0.8 mL of an aqueous 1 M Na_2CO_3 solution. Before measuring the absorbance of the reactants at 765 nm in contrast to the control (methanol), the reaction mixture was allowed to stand for approximately 15 minutes in complete darkness. As a standard, gallic acid was used. Gallic acid equivalents per grams of dried extract are used to assess the total phenolic content [18–21].

Using the following equation, the amount of total phenolic content in the sample was calculated as milligrams of gallic acid equivalent:

$$TPC = \frac{C * V}{m}$$

Where, C= concentration of gallic acid from curve (mg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

Total Flavonoid Content Assay

After an hour of mixing with 1 mL of 2% $AlCl_3$ (dissolved in methanol), the sample was analysed for absorbance at 415 nm in comparison to a blank (methanol). As a standard, quercetin was used. In mg of quercetin equivalents per gram of sample material, the total flavonoid concentration is provided [22].The following formula was used to determine the sample's total flavonoid content in mg of quercetin equivalent:

$$TFC = \frac{C * V}{m}$$

Where, C= concentration of quercetin from curve (mg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

Brine Shrimp Lethality Assay

To assess the cytotoxic potential of bioactive substances, lethality experiments on *Artemia salina* (brine shrimp) are frequently used. Because aseptic procedures are not employed, it is a quick (24-hour), low-cost, and straightforward test. For 24 hours, brine shrimp nauplii were exposed to solutions containing varying percentages of chloroform extracts of *A. calamus* [11,23]. The figure of motile nauplii was used to determine the efficacy of the chloroform extracts. Extracts that have LC_{50} values less than 1 mg/mL are regarded bioactive when utilizing the Brine Shrimp Lethality Assay to assess toxicity.

Results and Discussion:

Phytochemical analysis of *A. calamus* extract

Phytochemical analysis of *A. calamus* extract was done in different solvents extracts such as methanol, hexane, and chloroform. The results showed that in all extracts, alkaloids were present, in methanol extract, flavonoids were present. Sterols, triterpenoids, glycosides, carbohydrates and saponins were present in all extracts.

GC-MS Analysis

The composition of chloroform extract of *A. calamus* was analyzed by GC-MS. It revealed the presence of five major compounds, which are listed below:

Table 1: *Phytochemical screening results of different extracts*

S.N.	Phytochemical Constitutions	Tests	Qualitative Analysis		
			Methanol	Hexane	Chloroform
1.	Alkaloids	Mayer's Test	+	+	+
		Dragendroff's Test	+	+	+
		Wagnor's Test	+	+	+
2.	Flavonoids	Shinoda test	+	-	-
		Alkaline reagent	+	-	-
		Ferric Chloride test	+	+	+
3.	Glycosides	Keller Killiani Test	+	+	+
4.	Amino Acids	Ninhydrin Test	-	-	-
5.	Protein	Birut Test	+	-	-
		Millan's Test	-	-	-
6.	Sterols and Triterpenoids	Salkowski's Test	+	+	+
7.	Carbohydrate	Molisch's Test	+	+	+
		Fehling's Test	+	+	+
8.	Saponins	Froth Test	+	+	+

"+" denotes presence, whereas "-" denotes absence.

Table 2: *List of components revealed by GC-MS in the chloroform extract of A. calamus*

S.N.	Name of Compound	Retention Time	Molecular Formula	Molecular Weight	Area %
1.	Benfuresate	14.246	$C_{12}H_{16}O_4S$	256	4.03
2.	Cinerin	15.474	$C_{20}H_{28}O_3$	316	7.52
3.	Isoprothiolane	17.754	$C_{12}H_{18}O_4S_2$	290	83.11
4.	Allethrin	17.916	$C_{19}H_{26}O_3$	302	2.39
5.	Cypermethrin	18.639	$C_{22}H_{19}Cl_2NO_3$	415	2.95

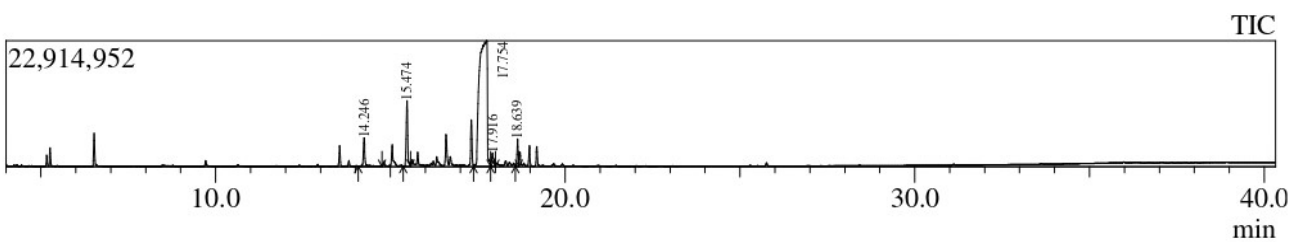

Figure 1: *Chromatogram of GC-MS analysis of chloroform extract of A. calamus*

Antibacterial screening analysis

The chloroform extract of *A. calamus* was examined against six bacteria samples; *Klebsiella pneumoniae, Bacillus subtilis, Micrococcus luteus, Enterobacterosp., Pseudomonas aeruginosa,* and *Staphylococcus aureus* for their antibacterial potential. Chloroform extract of *A. calamus* showed activity against *Staphylococcus aureus* KCTC 1916 with ZOI of 7 mm.

Table 3: *Zone of inhibition in diameter (mm) of Chloroform Extract of A. calamus*

Test Organism	Chloroform Extract of *A. calamus (15µl)*
Bacillus subtilis KACC 10114	No activity
Klebsiella pneumoniae KCTC 2242	No activity
Staphylococcus aureus KCTC 1916	7mm
Micrococcus luteus KACC 13377	No activity
Enterobacter cloacae subsp. dissolvens KACC 13002	No activity
Pseudomonas aeruginosa KACC 10232	No activity

Antioxidant Screening Analysis

The antioxidant potential is inversely proportional to the IC_{50} value, which may be estimated using linear regression of % inhibition against the antioxidant activity. High antioxidant activity is indicated by a lower IC_{50} value. The standard method is used for all calculations. Absorbance was measured at the wavelength of 517nm.

Table 4: *Result of DPPH assay of Chloroform extract of A. calamus*

Concentration (µg/mL)	% Scavenged
1500	91.95
1000	66.18
500	37.20
250	16.26
125	3.38

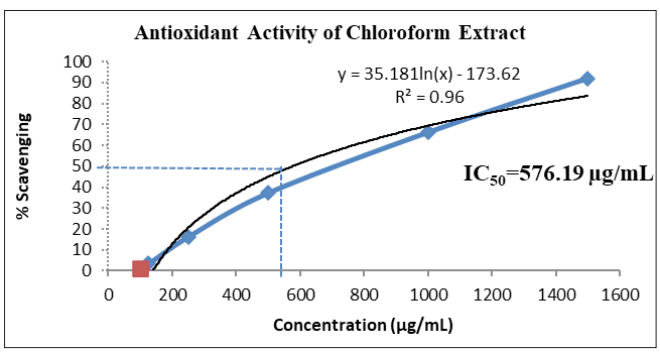

Figure 2: *Graph showing the results of the DPPH test on the chloroform extract*

This study showed that the IC_{50} value of chloroform extract of *A. calamus* was 576.19 µg/mL.

Total Phenolic ContentAnalysis:

The total phenolic content was estimated as mg of gallic acid equivalent by means of the calibration curve for gallic acid. The following data was logged after measuring the absorbance of solution:

Table 5: *Absorbance of Gallic acid*

Concentration(mg/mL)	Average Absorbance (nm)
10	0.03
20	0.12
40	0.27
60	0.44
80	0.60
100	0.79

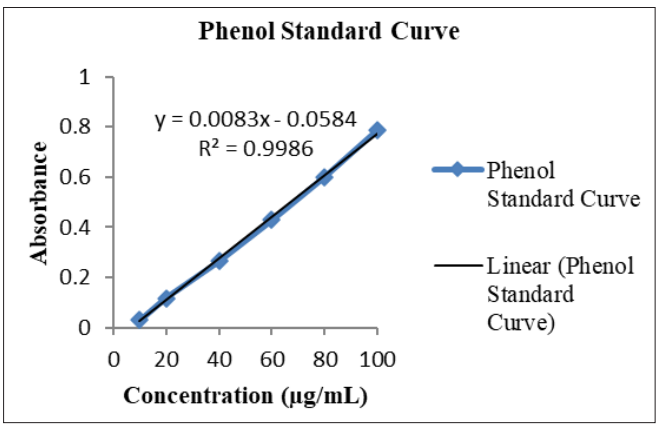

Figure 3: *Gallic acid calibration curve for determining total phenolic content*

Below is a table with the relevant information and the overall phenolic content:

Table 6: *Total phenolic content in chloroform extract of A.calamus*

Concentration (mg/mL)	Average Abs	Gallic acid concentration (mg/mL)	Gallic acid equivalent (mg/g)
1	0.086	0.001739	17.39

Chloroform extract of *A. calamus* was discovered to have a total phenolic content of 17.39 mg of gallic acid equivalent per gram, as indicated in Table 6.

Total Flavonoid Content Analysis

The amount of total flavonoid per grams of dried material is presented as mg of quercetin equivalents.

Table 7: Absorbance of quercetin

Concentration (mg/mL)	Average Absorbance
5	0.06
10	0.12
20	0.43
40	0.96
80	1.90

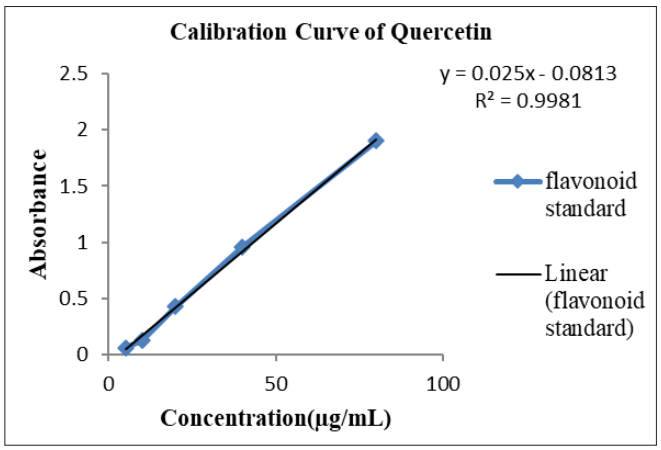

***Figure 4:** Calibration curve of quercetin for total flavonoid content determination*

The following table displays the total flavonoid content along with the necessary information.

Table 8: Total flavonoid content in chloroform extract of A. calamus

Concentration (mg/mL)	Average Abs	Quercetin concentration (mg/mL)	Quercetin equivalent (mg/g)
1	0.003	0.00337	3.37

From the analysis, the total flavonoid content in the plant of *A. calamus* was 3.37 mg quercetin equivalent /g of dry extract.

Brine Shrimp Lethality Analysis

From the lowest concentration to the highest concentration, it was observed that the level of lethality displayed by the extractives was directly proportional to those concentrations. This concentration-dependent increase in the percentage mortality of Brine Shrimp nauplii caused by *A. calamus* implies that these extracts contain some harmful constituents.

The table below contains the results of the Brine Shrimp Lethality assay:

Table 9: Effect of chloroform extract of A. calamus on brine shrimp

Concentration (µg/ml)	% Mortality
500	100
250	80
125	60
62.5	50
31.25	30
15.625	20

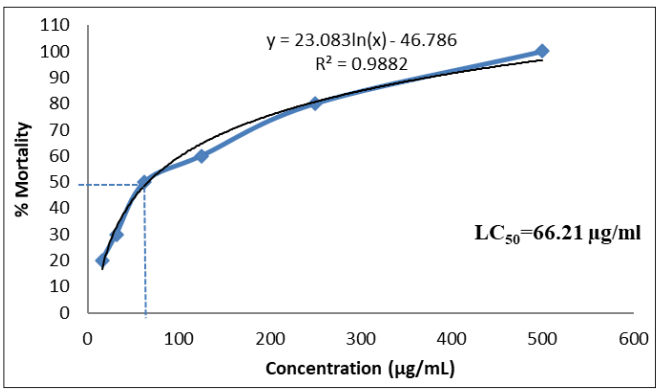

***Figure 5:** Graph of A. calamus chloroform extract concentration vs shrimp mortality rate*

This study showed that the LC_{50} value of chloroform extract of *A. calamus* was 66.21 µg/mL.

Conclusion

Phytochemical screening of methanol, hexane and chloroform extract showed the presence of alkaloids, flavonoids, carbohydrates, triterpenoids, and saponins. GC-MS analysis showed the presence of five major components with isoprothiolane (83.11%) being the abundant one. The chloroform extract showed activity against *Staphylococcus aureus* with ZOI 7mm and other bacteria didn't show any activity.The total phenolic content was found to be 17.39 mg/g gallic acid equivalent and total flavonoid content was 3.37mg/g quercetin equivalent. Through DPPH scavenging assay, the IC_{50} value of chloroform extract was determined to be 576.19 µg/mL. The chloroform extracts of *A. calamus* exhibited toxic activity against the brine shrimp with LC_{50} values of 66.21 µg/mL and were considered as containing active components.

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