PHYTOCHEMICAL INVESTIGATION AND SCREENING OF SOME BIOACTIVITIES OF ROOTS OF *COCCULUS HIRSUTUS* (L.) DIELS (Kywet nabaung)

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Abstract

Nowadays, there has been increasing interest in medicinal plants having enormous health effects which include bioactivities and secondary metabolites. In this research, root of *Cocculus hirsutus* (L.) Diels (Kywet nabaung) was chosen to be studied. Some phytoconstituents, nutritional value, EDXRF value, and bioactivities such as antimicrobial, antioxidant and acute toxicity activities of roots of *C. hirsutus* were investigated. Antimicrobial activities of pet ether, ethyl acetate, 95 % ethanol, methanol and watery extracts of *C. hirsutus* were investigated against six species of microorganisms such as *Bacillus subtilis, Bacillus pumilus*, *Candida albicans, Escherichia coli*, *Staphylococcus aureus, and Pseudomonas areuginosa* by agar well diffusion method. According to DPPH free radical scavenging assay, ethanol extract of roots of *C. hirsutus* (IC_{50} 76.84 g / mL) was found to have higher antioxidant activity than that of watery extract. Acute toxicity of 95% ethanol extract of roots of *C. hirsutus* was also investigated by OECD guide line. The main aim of this study is to reveal some medicinal properties of selected plant.

Keywords: *Cocculus hirsutus* (L.) Diels, phytoconstituents, antimicrobial activity, antioxidant activity, acute toxicity test

Introduction

Cocculushirsutus(L.) Diels (Synonym – *Cocculusvillosus*) locally called Kywetnabaung, belonging to the family Menispermaceae is a climbing scandent shrub with hairy sepals (Kirtikar, and Basu, 1981). *C. hirsutus* is widely distributed to Sudan, Central Asia, China, India and Myanmar. The plant grows all over India, especially in dry regions. In Myanmar, it is known by various names in different regions. It grows on sandy and gravelly soil and can form a dense cover on top of other plants.In traditional medicine *C. hirsutus* is used in various state of Myanmar by most people especially in rural areas depending on herbal medicines to treat many diseases such as rheumatism, arthritis, muscle swelling, insect bites, pain, etc. The leaves and roots of *C. hirsutus* have great medicinal value. The juice of leaves contains a mucilage (Chadha, 1950). It mixed with water has the property of coagulation into a green jelly- like substance. The plant is a climber with

Figure 1 Images of *Cocculushirsutus* (L.) Diel (Kywetnabaung) rootsand leaves

green flowers bloom in February-March and fruits in May-June. Seeds are curved and fleshy with annular embryo. Roots are hairy and dark brown in colour (Chopra RN *et al.*, 1958).

Some Chemical Constituents and Medicinal Uses of *C. hirsutus*

The plant of *C. hirsutus* has been reported to contain essential oil, β-sitosterol, ginnol, glycosides, sterols and alkaloids. Preliminary phytochemical analysis of the leaves showed presence of alkaloids, phenolic compounds, glycosides, and carbohydrates. Roots are reported for the presence of D-trilobine and coclaurine, sterols and resins (Viqaruddin and Tahir 1986). The leaves and roots of *C.hirsutus* have great medicinal value and are used both internally and externally for medicinal purpose. Root is bitter and is used as alterative, laxative, demulcent, tonic, diuretic, antiperiodic in fever, in malaria, joint pains, in treatment of skin diseases constipation and kidney poblems (Chopra RN *et al.*, 1996). Decoctions of the root mixed with long pepper, is used in chronic rheumatism and syphilitic cachexia (Nandkarni KM 1976). Roots rubbed with bonduc nuts in water are given for stomach problems especially in children. Roots act as an aphrodisiac and tonic. This study focus on investigation of some phytoconstituents, nutritional values, elemental analysis, and screening of some bioactivities of roots of *C. hirsutus*.

Materials and Methods

Sampling of Plant Material and Identification

The roots of *Cocculus hirsutus* (L.) Diels (Kywet nabaung) belonging to the family Menispermaceae were collected from Thegon Township, Bago Region, Myanmar, during January and February , 2019. After collection, the scientific name of the plant was identified at the Botany Department, University of Yangon. The collected fresh samples were washed and air-dried at room temperature for two weeks and then ground into powder and stored in air-tight container.

Preliminary Phytochemical Investigation of the Roots of Kywet nabaung

In order to classify the types of organic constituents present in sample, preliminary phytochemical tests such as alkaloids, α- amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, phenolic compounds, reducing sugars, saponin, starch, steroids, tannins and terpenoids on roots samples were carried out according to the appropriate reported methods. (Trease and Evans, 1980, MariniBettolo*etal*., 1981; Shriner *et al*., 1980; Robinson, 1983; Finar, 1969; Tin, 1972).

Determination of Some Nutrient Values of the Samples

Determination of some nutritional values present in samples such as moisture, ash, fiber, protein, fat and carbohydrate contents were carried out by Association of Official Analytical Chemists (AOAC) method (AOAC, 1990).

Elemental Analysis of the Roots of Kywet nabaung by Energy Dispersive X-ray Fluoresence (EDXRF) Spectrometry

For this measurement, pellets of the sample were first made. X-ray spectrometer permits simultaneous analysis of light element to heavy element. Energy dispersive X-ray fluorescence spectrometer (Shimadzu EDX - 700) can analyze the elements from Na to U under vaccum condition. X-ray fluorescence uses X-rays to excite and unknown sample. The individual elements in the sample are detected by using semiconductor detector [Si - Li] that permits multi-elements, simultaneous analysis. In this way, EDX - 700 spectrometer determines elements that are presents in the sample.

Determination of Polar and Non polar Soluble Extracts

Accurately weighed 50 g of each of dried powdered samples was placed in five conical flasks and 200 mL each of PE (60 – 80 C), ethyl acetate, 95 % ethanol, water and methanol solvent was added to each flask. These flasks were closed with

aluminum foil, shaken for 6 h using shaker machine and then allowed to stand for 18 h. Each one was then separately filtered taking precautions against loss of solution. The filtrate was concentrated by removal of the solvent under reduced pressure to give the respective pet-ether crude extract, ethyl acetate, 95 % ethanol and watery extracts were also prepared by similar manner mentioned in above procedure. Each plant extract was dried at normal pressure on a water bath and stored under refrigerator for screening some bioactivities. The percentages of each solvent soluble extracts were calculated (British Pharmacopeia, 1973).

Screening of Some Biological Activities of the Samples

In this screening, antimicrobial, antioxidant and acute toxicity activities on KNBR were studied.

- **Screening of antimicrobial activity of the roots of Kywet nabaung**

The antimicrobial activity screening of different crude extracts *viz.*, pet-ether, ethyl acetate, methanol, 95% ethanol and watery extracts of KNBR were tested against six species of microorganisms *viz., Bacillus pumilus, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* by employing agar well diffusion method at Myanmar Pharmaceutical Industrial Enterprise Department (MPIED). (Panda, S. K., 2011; Panda, S. K., 2012).

- ***In Vitro* Screening of Antioxidant Activity of Some Crude Extracts from the Roots of Kywet nabaung by DPPH assay**

The antioxidant activities of 95 % EtOH, and watery extracts of the roots of Kywet nabaung were studied by DPPH (1, 1-diphenyl-2-pricryhydrazyl) free radical scavenging UV spectrophotometric assay method. This method is based on the reduction of coloured free radical DPPH in ethanolic solution by different concentrations of each sample. The antioxidant activity was expressed as 50 % oxidative inhibitory concentration (IC_{50}). The lower the IC_{50} value, the higher the antioxidant activity of the sample. In this experiment, ascorbic acid was used as a standard. Accurately weighed 2 mg of each test sample was separately dissolved in 20 mL of 95 % EtOH under vigorous shaking. The mixture solution was filtered and the stock solution was obtained. Desired concentrations (500g/mL, 250g/mL, 125 g/mL, 62.5 g/mL and 31.25 g/mL) of sample solutions were prepared from this stock solution by serial dilution with appropriate amount of 95 % EtOH. DPPH (4.7381 mg) was thoroughly dissolved in 100 mL of 95 % EtOH. This solution (120 M DPPH solution) was freshly prepared in the brown coloured flask and kept in refrigerator for no longer than 24 h. Two mg of ascorbic acid was dissolved in 20 mL of 95 % EtOH and used as a standard solution. Blank solution was prepared by mixing 1.5 mL of the test sample solution with 1.5 mL of 95 % EtOH (Musa, K. H., *et al.*, 2011). The control solution was prepared by mixing 1.5 mL of 120 M DPPH solution and 1.5 mL of 95 % EtOH. The sample solution was also prepared by mixing thoroughly 1.5 mL of 120 M DPPH solution and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature for 30 minutes. After 30 minutes, the absorbance of these solutions was measured at 517 nm by using UV spectrophotometer (Devasagayam, T.P.A., *et al.*, 2004).

$$\% \text{ Oxidative Inhibition} = \frac{A_{DPPH} - \left(A_{\text{test sample}} - A_{Blank}\right)}{A_{DPPH}} \times 100$$

A_{DPPH} = absorbance of DPPH in EtOH solution

$A_{\text{test sample}}$ = absorbance of (sample + DPPH) solution

A_{Blank} = absorbance of (sample + EtOH) solution

$$\text{Average}, \overline{X} = \frac{X_1 + X_2 + X_3 + \cdots + X_n}{n}$$

$$\text{Standard deviation (SD)} = \sqrt{\frac{\left(\overline{X} - X_1\right)^2 + \left(\overline{X} - X_2\right)^2 + \left(\overline{X} - X_3\right)^2 + \cdots + \left(\overline{X} - X_n\right)^2}{n-1}}$$

where, $\overline{X}$ = average % inhibition oxidation
$X_1, X_2, X_3, \ldots, X_n$ = % inhibition of test sample solution
n = number of times

Then, IC_{50} (50 % oxidative inhibitory concentration values) were also calculated by linear regressive excel programme.

- **Screening of Acute Toxicity of the Roots of *C. hirsutus***

In this study, acute toxicity effect of ethanol extract of KNBR (two doses) were determined on albino mice by the methods of OECD Guidelines for the Testing of Chemicals 423, at Laboratory Animal Services Division, Department of Medical Research (DMR),Yangon. According to the test description, total number of 9 adult female albino mice, weighing (25-30g) were selected and divided into three groups. Each group contained three animals. They were maintained in accordance with the recommendation of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication N. 85-23, revised 1996) for studies involving experimental animals. They were fasted for 18 h before giving the extracts. Group (A) mice were orally administrated with 95 % EtOH extract of *C. hirsutus* roots 2000 mg / kg dose. Group (B) mice were given orally with 95 % EtOH extract of KNBR 5000 mg / kg dose. Group (C) mice performed as a control group and they were treated with clean water and normal animal food. All groups of mice were kept in the three mouse cages in the separated room at the room temperature of 26 ± 1º C. After administration of extract on each group of animals, they were observed first 6 h continuously for mortality and behavior changes. Then check the animals each 24 h for fourteen days. The mortality during this period was noted (Nil or percent death). The results obtained from acute toxicity are described in discussion section (OECD, 1998; OECD, 2000).

Results and Discussion

The phytochemical results showed that KNBR contain alkaloids, -amino acids, carbohydrates, glycosides, phenolic compounds, reducing sugars, saponins, tannin, starch and terpenoids. But cyanogenic glycosides, steroids and flavonoids were found to be absent in KNBR.

Nutrient Values of Roots of Kywet nabaung by AOAC Method

From the nutrient values results, it was found that moisture (17.96), ash (2.35), proteins (6.14), crude fibre (28.21), crude fat (0.88) and carbohydrate contents (44.46) of KNBR were estimated by AOAC method. Energy values of KNBR is 233 kcal/100 g.

Elemental Analysis of the Roots of Kywet nabaung by Energy Dispersive X-ray Fluorescence (EDXRF) Method

In the present study, relative abundance of elements present in KNBR was determined by EDXRF spectrometer. It can be observed that KNBR contains K, Ca, S, Fe, Mn, Cu, P, Ba and Zn as constituent elements. According to the results, KNBR contained some elements necessary for human health such as Ca, Mn, and P.

Preparation of Various Crude Extracts by Direct Extraction Method from the Roots of Kywet nabaung (KNBR)

In Kywet nabaung roots, (1.67 g, 3.34 %) in yield of 95 % EtOH, (4.30 g, 8.6 %) in yield of H_2O, (1.00 g, 2 %) in yield of EtOAc, (1.10 g, 2.2 %) in yield of PE, (2.00 g, 4 %) in yield of MeOH extracts from roots were obtained and these extracts were kept for bioactivity studies.

Some biological activities such as antimicrobial activity, antioxidant and acute toxicity of various extracts of KNBR were investigated.

Results of antimicrobial activity of various crude extracts from the Roots of Kywet nabaung

In this investigation, crude extracts were tested on six species of microorganisms *viz., Bacillus subtilis, Bacillus pumilus, Candida albicans, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The observed microbial inhibition zone diameters of different crude extracts of KNBR are summarized in Table 1. Among the tested crude extracts of KNBR, EtOAc extract showed the most potent antimicrobial activity against all the tested microorganisms (inhibition zone diameter, ID**:** 2830 mm) and it showed the most prominent activity against *B. pumilus, B. subtilis, C. albicans* and *E. coli.* And MeOH extract of KNBR showed the antimicrobial activity against all the tested microorganisms (inhibition zone diameter, ID**:** 18 24 mm). But, EtOH extract of KNBR showed activity against all the tested microorganisms (ID**:** 1517 mm) except *B. subtilis* and *E. coil.* Watery extract and PE extract of KNBR did not show the antimicrobial activity tested. Among the tested crude extracts of KNBR, EtOAc and MeOH extracts of KNBR were found to possess the highest antimicrobial activity.

Table 1 Microbial Inhibition Zone Diameters of Different Crude Extracts from the Roots of Kywet nabaung

Microorganisms	Types of Microorganisms	Sample	Inhibition Zone Diameters (mm)				
			H_2O	PE	EtOAc	95% EtOH	MeOH
Bacillus pumilus	Gram (+) ve	KNBR	-	-	30	15	23
Bacillus subtilis	Gram (+) ve	KNBR	-	-	30	-	23
Candida albicans	Fungus	KNBR	-	-	30	17	23
Escherichia coli	Gram (-) ve	KNBR	-	-	30	-	23
Pseudomonas aeruginosa	Gram (-) ve	KNBR	-	-	28	15	18
Staphylococcus aureus	Gram (+) ve	KNBR	-	-	29	15	24

Agar well diameter = 10 mm

10 mm 16 mm = (+) KNBR = Kywet nabaung Root

17.20 mm 20 mm = (++)

21 mm and above = (+++) () = no zone of inhibition

***In vitro* antioxidant activity of some crude extracts from the roots of Kywet nabaung**

The antioxidant activity was expressed as 50 % oxidative inhibitory concentration (IC_{50}). The lower the IC_{50} value, the higher the antioxidant activity of the sample. In this experiment, ascorbic acid was used as a standard. The antioxidant activity of the crude extracts of KNBR were determined for five different concentrations; 31.25 g / mL, 62.5 g / mL, 125 g / mL, 250 g / mL and 500 g / mL of each samples in EtOH solvent. Antioxidant activity of 95 % ethanol and watery crude extracts from KNBR was expressed in terms of percent inhibition. From these experimental results, it was found that as the concentrations of sample increased, the absorbance values were found to decrease and the antioxidant activity increased. Among the tested two crude extracts of KNBR, the antioxidant activity

of 95 % EtOH extract (IC_{50} 76.84 g / mL) showed higher antioxidant activities than watery extract (IC_{50} 94.57 g / mL). Figure 4 and Table 2 show bar graph of percent oxidative inhibition of crude extracts of KNBR at various concentration in comparison with standard ascorbic acid. In the present study, crude extracts of KNBR were found to have higher antioxidant activity. From the results, it can be inferred that due to the high antioxidant potential, KNBR may be used in prevention of diseases related to oxidative stress such as coronary heart disease, neurodegenerative diseases, Parkinsons disease and even in various types of cancer.

Table 2 Percent Oxidative Inhibition and IC_{50} Values of Crude Extracts from the Roots of Kywet nabaung and Standard Ascorbic Acid

Extracts	Percent Oxidative Inhibition (%) (mean ± SD) in different concentration (µg / ml)					IC_{50} (µg/mL)
	31.25	62.5	125	250	500	
(EtOH)	17.81±0.05	45.56±0.06	63.93±0.09	91.20±0.03	106.29±0.02	76.84
(watery)	21.61±0.05	37.68±0.01	61.34±0.02	95.32±0.05	100.91±0.01	94.57
Ascorbic acid	30.20±0.09	51.28±0.08	80.45±0.08	83.28±0.07	90.00±0.07	60.59

KNBR = Kywet nabaung Roots

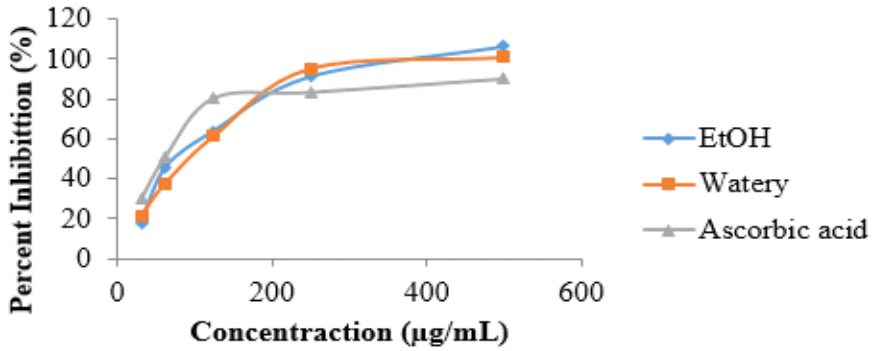

Figure 4 Percent oxidative inhibition Vs concentration (g / mL) of crude extracts from the Roots of Kywet nabaung and standard ascorbic acid

Acute toxicity test of the roots of *C. hirsutus*

For acute toxicity screening of roots of *C. hirsutus*, EtOH extract was done with the dosage of 2000 mg / kg and 5000 mg / kg body weight in each group of albino mice. The condition of mice groups was recorded after fourteen days administration. The results showed no lethality of the mice was observed up to fourteen days administration. Each group of animals was also observed still alive and did not show any visible symptoms of toxicity like restlessness, respiratory disorders, convulsion, aggressive activities, coma and death.

Table 3 Acute Toxicity Effect of Ethanol Extract of *C. hirsutus* Roots on Albino Mice Model after Two Weeks Administration

No	Groups	Extract Administration	Dosage (mg / kg)	No. of death	% of death after 14 days
1	Group A	EtOH Extract	2000	Nil	0
2	Group B	EtOH Extract	5000	Nil	0
3	Group C	No administration	Nil	Nil	0

Figure 5 Acute toxicity test for ethanolic extract of Kywet nabaung roots on albino mice model

Conclusion

The present studies on roots of *Cocculus hirsutus* (L.) Diel (Kywet nabaung) provide the following information. The preliminary phytochemical investigation revealed the presence of alkaloids, -amino acids, glycosides, reducing sugars, terpenoids and saponins in roots of *C. hirsutus*. From the results of some physico-chemical analyses, in KNBR it was found that 17.96 of moisture, 2.35 of ash, 0.88 of fat, 28.21 of crude fiber, 6.14 of protein and 44.46 of carbohydrates. Elemental analysis by EDXRF method revealed that roots of *C. hirsutus* contained K, Ca, S, Fe, Cu, Sr and Rb elements. From the screening of antimicrobial activities of roots of *C. hirsutus*, MeOH and EtOAc extracts showed the most potent antimicrobial activity against all the tested microorganisms (inhibition zone diameter, ID**:** 1830 mm) and EtOH extract showed the activity against *B. pumilus*, *C. albicans*, *S.aureus* and *P.aeruginosa* (ID**:** 1517 mm). In the *in vitro* antioxidant activity screening EtOH extract of KNBR was found to have higher antioxidant activity than that of watery extracts. From study on the acute toxicity activity of the 95 % EtOH extract of roots of *C. hirsutus*, the results showed no lethality of the mice was observed up to fourteen days after administration. According to the study, the presence of phytoconstituents, essential nutrient for good health, antimicrobial and antioxidant activities, and absence of acute toxicity activity confirm that *C. hirsutus* may be used as antimicrobial, preventive infection and enhances healing.

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