Evaluation of Acute Toxicity of Ethanolic Plant Extract and Isolation of Compounds from the Root of *Polypodiumvulgare*L.(Hsay-poe-ti)

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Abstract

In this research work, one of Myanmar indigenous medicinal plants, *Polypodiumvulgare*L., which belongs toPolypodiaceae family, Myanmar named Hsay-poe-ti (root) was selected for chemical investigations. Firstly, preliminary phytochemical screenings of selected sample were carried out by test tube method. Furthermore, mineral contents of Hsay-poe-ti (root) were determined by EDXRF technique. Moreover, antimicrobial activities of selected sample were measured by Agar well diffusion method on six tested organisms. And then, the acute toxicity studied of 95% ethanol extract of roots of Hsay-poe-tiwas evaluated by OECD guideline 425 by using animal models with various doses. As a result of acute toxicity test, LD_{50} value of ethanol extract of Hsay-poe-tiroot was found to be more than 3000 mg/kg/day as the lethal effect was not detected in all mice.Finally, three pure compounds were isolated from the roots of Hsay-poe-tiby using Thin Layer and Column Chromatographic separation methods. FT IR spectra of these isolated compounds were measured and the functional groups containing in these compounds were assigned. The aim of present studies is to investigate the root of *Polypodiumvulgare*L. (Hsay-poe-ti) by chemically and pharmaceutically.

Keywords:EDXRF, *Polypodiumvulgare*L., OECD guideline 425,LD_{50}, FT IR

Introduction

*Polypodiumvulgare*L.,(family; Polypodiaceae) is commonly knownas the polypody is indigenous to Europe,Africa and Eastern Asia (Ollgaard B. and Tind K.,1993).*Polypodiumvulgare*L.is a small, winter green perennial fern attaining a height up to 30cm, extensively dispersed throughout theWorld.The surface of the root is hard, hairy, ragouts and longitudinally fissured. The upper surface presents several hairs like tubercles or scaly projections, each projection is curved, 0.5inch long and fissured.The rhizome which is used as drug is fibrous, knotty mud colored with black or red tinged.The drug is characterized by an astringent, sweet and nauseous test and brittleness in fracture.Plant has been used medicinally as anti-epileptic, cardio tonic, anti-spasmodic, and digestive (Khory R.N. &Katrak N.N., 1981).

It is also effective in piles, leprosy, bronchospasm, melancholia and rheumatic disorders (Dar P. A., *et al*, 2012). Tea made from roots is used for pleurisy, sore throat, stomach ache and poultice of root for inflammation.In European herbal medicine, it is traditionally used as a treatment for hepatitis, jaundice and as a remedy for indigestion and loss of appetite(Foster. S. and Duke. J. A., 1990). The root is alterative, anthelmintic, cholagogue, demulcent, diuretic, expectorant, pectoral, purgative and tonic.It was also considered of value for lung ailments and liver diseases. The herb is constipation in children and was also previously used to treat intestinal parasites, especially tapeworms.Polypody has also the reputation to have blood cleaning and anti-rheumatic action(Chevallier, A., 1996).

Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use (CDER). Before the medicinal plant could be used as medicines, it must be ensured to be safe(P. Lalitha, *et al*, 2012). The Organization for Economic Cooperation and Development (OECD) defines acute toxicity as "the adverse effectsare occurring within a short time of (oral) administration of a single

dose of a substance or multiple doses given within 24 hours (OECD guideline, 2008). The LD_{50} test is used to determine the acute toxicity of a substance. This is the dose at which the test substance is lethal to 50% of the test animals(Gallagher, M. E., 2003). A key stage in ensuring the safety of drugs is to conduct toxicity tests in appropriate animal models.

In the present study, the phytochemical screenings, mineral contents, antimicrobial activity, acute toxicity and isolation of pure compounds from the roots of *Polypodiumvulgare*L. were carried out. The result of recent investigations could be supported that the root of *Polypodiumvulgare*L.is medicinally used as safely drugs.

Botanical Description

Figure 1.Plant and root of *Polypodiumvulgare*L.

Botanical name - *Polypodiumvulgare*L.

Family name - Polypodiaceae

Common name - Polypody

Local name - Hsay-poe-ti

Genus - *Polypodium*

Species - *P. vulgare*

Part of Used - Roots

Medicinal use - hepatitis and jaundice,bile secretion, gentle laxative, the common cold and sore throat, and dry cough

Material and Method

Sample Collection

The roots of *Polypodiumvulgae* L. were collected from Pin-da-ya Township, Shan State, Myanmar. It was wash with water and cut into small pieces. Dried in air with roomtemperature. It was stored at well stopper bottle and which was used for experiment.

Preliminary Phytochemical Tests for the Roots of *Polypodiumvulgare* L.

Phytochemical tests were done on the various extracts of sample by test tube method (Harbone J.B., 1984).

Determination of Some Physicochemical Properties of Roots of *Polypodiumvulgare* L.

The Some Physicochemical Propertiessuch asmoisture, ash and pH of the roots of *Polypodiumvulgare* L. were determined by AOAC method (AOAC, 2000).

Mineral Content in Roots or *Polypodiumvulgare* L.

The mineral contents of roots of *Polypodiumvulgare* L. were measured by EDXRF Spectroscopy Method. EDXRF spectrum was measured at the Department of Chemistry, Monywa University. It was shown in Figure 3 and the results were described inTable1.

Antimicrobial Activities of Roots of *Polypodiumvulgare* L.

The antimicrobial activities of the roots of *Polypodiumvulgare* L. were determined by Agar well diffusion method on six tested organisms at Central Research and Development Center (CRDC), Insein, Yangon. The sixorganisms are *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans*and *Escherichia coli.* The results of antimicrobial activities were shown in figure 4 and table 2.

Study on Acute Toxicity of 95% EthanolicExtract of the Roots *Polypodiumvulgare* L.

Site of Study

Study on acute toxicity of 95% ethanolic extract of the roots of *Polypodiumvulgare*L. was carried out at the Department of Biotechnology, Mandalay Technological University, Patheingyi Township, Mandalay Region.

Preparation of Roots Extract for Acute Toxicity Test

About 580 g of air dried sample of the roots of *Polypodiumvulgare* L. were percolated with 2.5L of 95% ethanol stored in a well stopper bottle. After one month, it was evaporated at room temperature for about one week. Totally (30.95g) of ethanol extract was obtained. The dried extract obtained was stored in desiccators. For testing the effect of the sample on experimental animals, the required amount of extract was taken and dissolved with distilled water to get the required dosage of extract.

Method for Acute Toxicity Test

Acute toxicity test for animal models (*Mus. Muscles mice*) were used in this study. The mice with 30g of body weight and age of 4-6 week were selected to use and divided into seven groups (control group and six treatments groups).One group included 3 mice each. The extracts were given at the dosage of 500, 1000, 1500, 2000, 2500 and 3000 mg/kg/day respectively. A total of 18 mice were used to give orally the sample extract. The mice were administered 2 times per day of 12 hours interval for 6 days regularly. After administering the extract orally, the mice were watched for 14 days for their health and behavior.

Figure 2. Administration of ethanolicplant extract to experimental mice

Extraction and Isolation of Compounds from the Roots of *Polypodiumvulgare* L.

Extraction Procedure

The sample 100 g was extracted with 95%ethanol 750 mL for one month and then filtered and the filtrate was concentrated. The residue was re-extracted with 100 mL of ethyl acetate and ethylacetate extract (4.32g) was obtained.

Isolation of Pure Compound from the Roots of *Polypodiumvulgare* L

The ethyl acetate crude extract (4.32g) was separated by Column Chromatography method. The silica gel 70-230 mesh as used as adsorbent and eluent used as n-hexane and ethyl acetate with various ratio from non- polar to polar. Totally, (178) fractions were collected. Each fraction was checked by TLC and the same R_f value fractions were combined. Eight combined fractions were obtained. Combined fraction (III), (IV), and (VII) gave only one spot on TLC. Pure solid compound was purified by crystallization and checked by Thin Layer Chromatography method.

The pure compound I, yellow oily compound (0.0240 g), pure compound II, yellow crystal compound (0.0317 g) and pure compound III, brownish yellow amorphous compound (0.0338 g) were collected from the column separation. The yield percent of pure compound I, II and III were found to be (0.56%), (0.73 %) and (0.82 %)based upon the ethyl acetate crude extract. Isolated pure compounds were rechecked by phytochemical tests. Pure compound I gave positive for terpene test, pure compound II responded positive for phenol and polyphenol test and compound III gave positive for glycoside and steroid tests.

Determination of Functional Groups in the Isolated Compounds

Isolated compounds I, II and III from the roots of *Polypodiumvulgare* L. were sent to Monywa University for the measurement ofFTIR spectra. The functional groups containing in these isolated compounds were assignedand identified with structure of chemical constituents in root of *Polypodiumvulgare*L..The FT IR spectra of isolated compounds were shown in figure 5, 6, and 7.

Results and Discussion

Phytochemical Test for the Roots *of Polypodiumvulgare* L.

Phytochemical tests were done by test tube method which gave theinformation of secondary metabolite compounds present in roots of *Polypodiumvulgare* L.

According to the results of phytochemical tests, the root of *polypodiumvulagre* L. consist of alkoloid, carbohydrate, flavonoid, glycoside, phenolic compound, polyphenol, reducing sugar, saponin, steroid, tannin and terpene compounds respectively.

Determination of Some Physicochemical Properties of root of*Polypodiumvulgare* L.

The some physicochemical properties of the root of *polypodiumvulgare* L. such as moisture and ash were performed by AOAC method. As the results of Physicochemicaltests, the selected sample contains moisture 0.137% and ash 1.667% respectively.

Mineral Content in Roots of*Polypodiumvulgare* L.

Mineral contents in the roots *of Polypodiumvulgare* L. were also analyzed by EDXRF spectroscopy. The results are shown in figure 3 and Table 1.

Table 1 shows that tenessential elements (K, Si, Ca, S, P, Mn, Fe, Ti, Cu, and Zn) were found in the roots of *Polypodiumvulgare*L.. Among them, potassium in the highest amount in the sample. Decreasing order of mineral content is K>Si>Ca>S>P>Mn>Fe>Ti>Ca>Zn. These results indicate that roots of *Polypodiumvulgare* L. is a rich source of minerals for health benefit. There was non-toxic metal containing in this selected sample.

Table 1. Mineral Content in Roots of *Polypodiumvulgare*L.

No.	Elements	Relative Abundance (%)
1.	Potassium (K)	0.353
2.	Silicon (Si)	0.257
3.	Calcium (Ca)	0.130
4.	Sulfur (S)	0.088
5.	Phosphorus (P)	0.075
6.	Manganese (Mn)	0.017
7.	Iron (Fe)	0.012
8.	Titanium (Ti)	0.002
9.	Copper (Cu)	0.002
10.	Zinc (Zn)	0.001

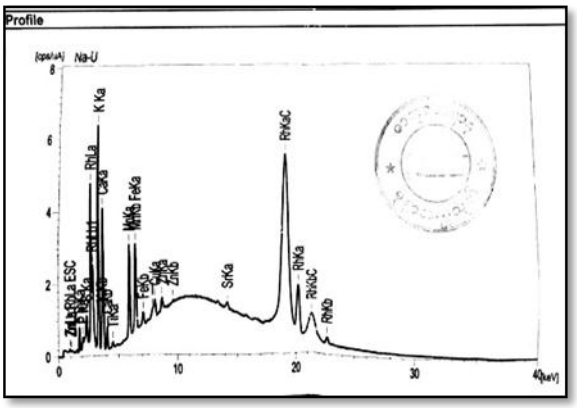

Figure 3.EDXRF spectrum of roots of *Polypodiumvulgare* L.

Antimicrobial Activities of Roots of *Polypodiumvulgare* L.

The antimicrobial activities of roots of *Polypodiumvulgare* L. were performed by Agarwell diffusion method on six tested microorganisms. These results are tabulated in Table 2 and figure 4.

Table2.Antimicrobial Activities of Roots of *Polypodiumvulgare*L.

Sample	**Extracted Solvents**	**Inhibition zone diameter**					
		I	**II**	**III**	**IV**	**V**	**VI**
Polypodium vulgare L. (Root)	n-hexane	-	-	-	-	-	-
	EtOAc	14mm (+)	14 mm (+)	15mm (+)	12 mm (+)	12 mm (+)	13 mm (+)
	EtOH	17 mm (++)	18 mm (++)	18 mm (++)	16 mm (++)	17 mm (++)	16 mm (++)

Agar-well -10mm
10mm-14mm (+)
15mm-19mm (++)
21mmabove (+++)

Organisms
I = *Bacillus subtilis* IV = *Bacillus pumilus*
II = *Staphylococcus aureus* V = *Candida albicans*
III = *Pseudomonas aerugunosa* VI = *Escherichia coli*

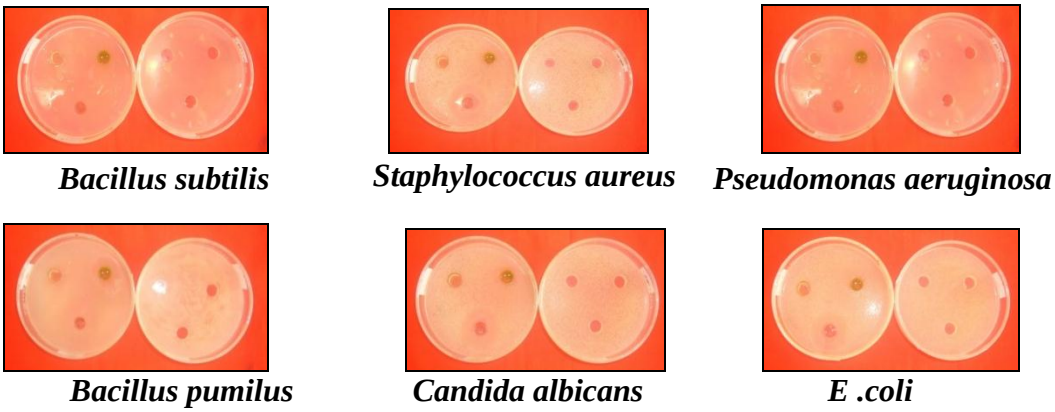

Figure 4.Antimicrobial activities of roots of *Polypodiumvulgare*L.

According to the results of antimicrobial activities tests, ethanol extract of selected sample responds medium activity on all tested organisms. Ethyl acetate extract has also low activities on six tested organisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans*and *Escherichia coli*. n-Hexane extract has no activity on all tested organisms.

Study on Acute Toxicity of 95% EthanolicExtract of Roots of*Polypodiumvulgare* L.

Acute Toxicity of 95% ethanolic extract of the Roots of *Polypodiumvulgare* L. was investigated. Acute toxicity studies were done by mices with various doses (500mg/kg, 1000 mg/kg, 1500 mg/kg, 2000 mg/kg, 2500mg/kg, and 3000 mg/kg) for six groups. Each group contains 3 mice and oral administrated. After (14)days period, all mice were no dead and alive with any toxic symptoms. The results are described in table 3.

Table 3.LD_{50}Value of Sample Extract of *Polypodiumvulgare* L. After Two Weeks Oral Administration of Doses for Acute Toxicity Test

Extract	Groups	No. of Mice/ Group	Dose of Extract (mg/kg)	Observed Period	Ratio of Dead and Tested	Death%
Ethanolic Extract of Roots of *Polypodiumvulgare*L.	I	3	500	Two weeks	0/3	0
	II	3	1000	Two weeks	0/3	0
	III	3	1500	Two weeks	0/3	0
	IV	3	2000	Two weeks	0/3	0
	V	3	2500	Two weeks	0/3	0
	VI	3	3000	Two weeks	0/3	0
	Control	3	D/W 10 mL/kg	Two weeks	0/3	0

As a resultthis experiment, LD_{50} value of ethanolextract *Polypodiumvulgare* L. was found to be more than 3000 mg/kg/day of the lethal effect was not detected in all mice.Hence, the selected plant sample was safely used as drugs more than 3000 mg/kg/day.

Isolation of Pure Compounds from the Roots of *Polypodiumvulgare* L.

Thin Layer chromatograms of isolated Compound I, II and III

The isolated compound I, II and III were checked by TLC, using n-hexane :ethyl acetate with various ratio, iodine developerand UV detector. The R_f values of these three isolated compounds were determined as follows.

Isolated compound-I

Solvent system = n Hexane: Ethylacetate(3 : 2, v/v)

Developer = Iodine vapor, UV lamp

Adsorbent = Aluminium pre-coated silica gel plate

R_f = 0.36

Isolated compound-II

Solvent system = n Hexane: Ethylacetate(1 : 1, v/v)

Developer = Iodine vapor, UV lamp

Adsorbent = Aluminium pre-coated silica gel plate

R_f = 0.34

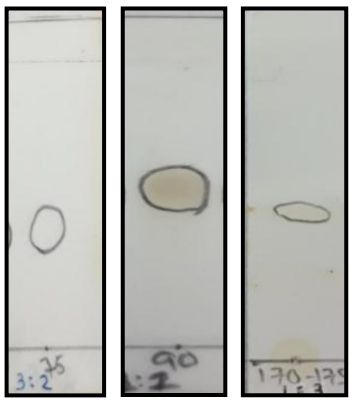

Figure 5. Thin layer chromatograms of isolated compounds I, II, and III

Isolated compound-III

Solvent system = n Hexane: Ethylacetate(1 : 3, v/v)

Developer = Iodine vapor, UV lamp

Adsorbent = Aluminium pre-coated silica gel plate

R_f = 0.33

Determination of Functional Groups in the Isolated Compounds

FT IR assignments of isolated compounds

FT IR spectrum of isolated compound I was measured at the Department of Chemistry, Monywa University. FT IR spectra of isolated compound – I, II and III are shown in figure-6, 7 and 8.

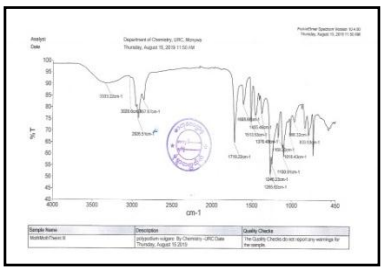

Figure 6. FT IR spectrum of isolated compound – I

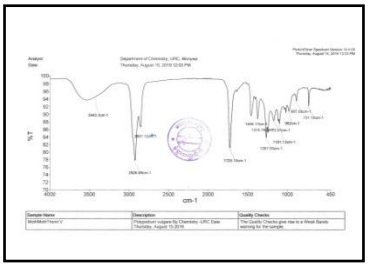

Figure7. FT IR spectrum of isolated compound – II

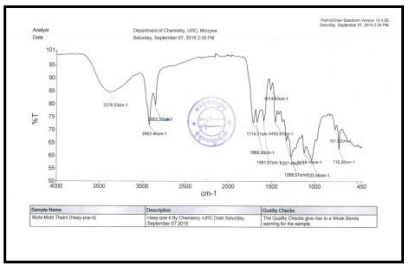

Figure 8. FT IR spectrum of isolated compound – III

According to FTIR spectrum, figure-6,isolated compound I should consists of O-H group, sp^3 hydrocarbon, carbonyl group, C-H in plane bending vibration of sp^3 hydrocarbons, C-C-O stretching vibration of alcohol group, C-O-C stretching vibration of ether group, C-H out of plane bending vibration of sp^3 hydrocarbon respectively. In accordance with phytochemical test and FT IR assignment, isolated compound I may be terpene compound.

According to FT IR spectrum, figure-7, isolated compound II consists of O-H group, sp^2 hydrocarbon, sp^3 hydrocarbon, (C=O) carbonyl group, aromatic benzene ring, allylic hydrocarbon, C-C-O stretching vibration of alcohol group, C-O-C stretching vibration of ether group, =C-H out of plane bending vibration of trans or E and cis or Z alkenic groups respectively. As a result of phytochemical test and FT IR assignment, isolated compound II may be phenoliccompound.

According toFT IR spectrum, figure-8,isolated compound III containsof O-H group, sp^3 hydrocarbon, carbonyl group, C-H in plane bending vibration of sp^3 hydrocarbons, C-C-O stretching vibration of alcohol group, ether functional group, C-H out of plane bending vibration of sp^3 hydrocarbon respectively.These functional groups were identified with the functional groups of Osladin compound which is a major chemical constituent in selected sample. As a result of phytochemical test and FT IR assignment, isolated compound III may be steroidal glycoside Osladin compound.

Conclusion

Polypodiumvulgare L. is locally used as drugs for gentle laxative medicine, whichcontainseffective chemical constituents and good minerals. This plant should not be used for serious antimicrobial drugs because it has medium and low antimicrobial activities in various solvent extracts. In accordance with the acute toxicity test,*Polypodiumvulgare*L. extract could be observed the any toxic symptoms and death in test animals.As the results of present research work, *Polypodiumvulgare*L. could be used as safety drugs for orally administrated up to dose 3000mg/ kg/day.

Acknowledgements

Weare extremely grateful to DrThidaWin, Rector, University of Mandalay and Professor DrYi YiMyint, Head of Department of Chemistry, University of Mandalay, Myanmar, for their permitting, understanding, kindly helps and good guidance on our research studies.

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