Original Research Article

Anti-inflammatory, Antioxidant and Cytotoxicity Studies on *Lycopodiella cernua* (L.) J. Sm. in Bukidnon, Philippines

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ABSTRACT

Introduction: *Lycopodiella cernua* is a widely distributed species recognized for several uses in traditional medicine. Although relatively common in the wild, relevant pharmacological data on Philippine *L. cernua* population seems scanty despite its use in traditional medicine among some of the country's tribal groups. **Objectives:** The study aims to validate the medicinal potential as anti-inflammatory and antioxidant to strengthen traditional claims of *L. cernua*. **Materials and Methods:** Crude methanolic extraction was done for aerial and ground parts of *Lycopodiella cernua*. The extracts were measured for total phenolic content (TPC) and total flavonoid content (TFC) with Folin-Ciocalteu and aluminum chloride methods, respectively. Antioxidant activity was also determined based on DPPH radical scavenging activity relative to ascorbic acid. Anti-inflammatory activity was detected by enzyme cyclooxygenase (COX-2 and COX-1) inhibition assays. Moreover, cytotoxicity tests of extracts against neonatal human epidermal keratinocytes (HEKn) and lung adenocarcinoma (A549) cell line were done using MTT assays. **Results and Discussion:** Phenolics (aerial: 33.04 ± 2.82 mg GAE/g; ground: 4.91 ± 0.1 mg GAE/g) and flavonoids contents (aerial: 11.46 ± 7.19 µg QE/g; ground: 5.82 ± 5.09 µg QE/g) were relatively low. DPPH radical scavenging activity was also relatively low (aerial: 19.13 ± 0.88 %; ground: 12.21 ± 0.9 %). However, COX-2 inhibition activity of the ground parts was 74.78 ± 18.61%. Moreover, the COX-2: COX-1 ratio of 1.29 of the aerial parts suggests selectivity for COX-2. Both extracts were found nontoxic against HEKn normal cell lines and A549 cancer cell lines. **Conclusion:** *L. cernua* is nontoxic and has anti-inflammatory and antioxidant activities.

Key words: Antioxidant, Anti-inflammatory, Phytochemicals, *Lycopodiella cernua.*

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INTRODUCTION

Lycopodiella cernua is a widely distributed species recognized for several uses in traditional medicine. There are also reports that validate its medicinal potential. Inhibitory activities of certain isolated compounds from the said species on B-secretase 1 and cholinesterase had been reported.[1,2] Ho *et al.*[3] observing similar cholinesterase inhibitory activity on alkaloid extracts earlier suggests the possibility that this bioactivity can be useful in addressing Alzheimer's disease. But the need for more empirical data is still evident because the species holds potential to address other health issues such rheumatism, hepatitis and dysentery, bruises, burns and scalds.[1,2,4] Although relatively common in the wild, relevant pharmacological data on Philippine *L. cernua* population seems scanty despite its use in traditional medicine among some of the country's tribal groups.[5] Thus, whatever empirical data that can validate its medicinal potential will also strengthen traditional claims on its medicinal value. Moreover, further studies are also useful to address conservation issues of this species which like other wildlife species are facing imminent negative

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population pressure due to problems on climate change and various anthropogenic activities. The data in this study probably represent a small part of what needs to be discovered for this species.

MATERIALS AND METHODS

Collection, identification and preparation of plant material

Lycopodiella cernua (L.) J. Sm (Figure 1) of the family Lycopodiaceae was collected from Mt. Musuan in Central Mindanao University, Musuan, Bukidnon, Philippines. Pertinent field data were recorded. Specimens were identified based on diagnostic morphological characters and were compared to the voucher specimens deposited at the Herbarium of the University Museum of Central Mindanao University (CMUH). Vouchers for the herbarium were also prepared. Identification was verified also through DNA barcoding using the rbcL gene.

Molecular identification using the chloroplast rbcL gene was employed to further confirm the identity. The modified CTAB method[6] was used to extract the total genomic DNA from silica-dried frond. The rbcL region was amplified through PCR using the primer pair rbcLaF (ATGTCACCACAAACAGAGACTAAAGC) and rbcLaR (GTAAAATCAAGTCCACCRCG). The amplification was carried out using a Veriti® thermal cycler (P/N 4375786, Life Technologies) with the following condition: heated lid (98°C); initial denaturation at 95°C for 5 min; 25 cycles of denaturation (94°C for 30 s), annealing (55°C for 1 min), and extension (72°C for 1 min); and final extension (72°C for 10 min). At the end of the thermal cycling, the amplicons were resolved through an agarose electrophoresis (1% agarose, 0.5× TBE), stained with Gel Red® and visualized with GelDoc™ EZ documentation (Bio-Rad Technologies, Inc). Amplicons were sequenced by Macrogen, Inc. (South Korea) for two-directional sequencing. Sequences were edited using BioEdit™, analyzed using Basic Local Alignment System Tool (BLAST) and Barcode of Life Database (BOLD) Identification System and deposited in Genbank.

Figure 1: Habit of *Lycopodiella cernua* (L.) J. Sm. (N 07°52.72 E 125°04.06) in Mt. Musuan, Bukidnon, Philippines.

Fresh and dry weights of the collected samples were recorded. Percent moisture loss was also calculated:

$$M_n = ((W_w - W_d)/W_w) \times 100$$

Wherein, M_n = moisture content (%) of material, W_W = wet weight of the sample, and W_d = weight of the sample after drying.

Aerial and ground parts were milled separately using a heavy duty miller then sieved to produce finer and homogenous product.

Methanolic Extraction

Powdered samples were exhaustively soaked in methanol (100 g sample /500 mL solvent). Soaked samples were then filtered and the filtrate dried *in vacuo* at 40°C using rotary evaporator. The concentrates were stored at -4°C.

Qualitative Phytochemical Analysis

The crude methanolic extracts were subjected to preliminary phytochemical screening for alkaloids, anthraquinones, phenolics, saponins, tannins and terpenoids using bioautographic assay via thin-layer chromatography (TLC) as described by Gracelin *et al.*[7]

Determination of Total Phenolic Content (TPC)

The Folin-Ciocalteu method using a 96-well microtiter configuration was used for the total phenolic content determination of the aerial and ground part extracts of *L. cernua*. Extracts were dissolved in DMSO: methanol: water (15:5:2) solution at 2 mg/mL.[8] Twenty (20) µL of the extract was added with 80 µL (1:10) Folin-Ciocalteu reagent (Sigma). After 30 min, 80 µL of 5% Na_2CO_3 was added as modification of the procedure in Bayili *et al.*[9] The microplate was incubated at room temperature for 2 h then read at 750 nm using MultiSkan Go (ThermoScientific). A standard calibration curve using gallic acid with $R^2 = 0.9998$ was used to determine concentration.

Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the aerial and ground part extracts of *L. cernua* was determined using Aluminum Chloride method in a 96-well microtiter configuration.[10] Thirty (30) µL of fern extract at 2 mg/mL in DMSO:methanol: water (15:5:2) was added with 30 µL of 10% $AlCl_3$. Thirty (30) µL of 1M sodium acetate was then added to the mixture followed by 110 µL of water. The plate

was incubated at room temperature for 30 min and absorbance was read at 415 nm in the MultiSkan Go (ThermoScientific). Quercetin was used as the standard.

Determination of Antioxidant Activity

Aerial and ground part extracts of *L. cernua* (50 µl) in 15 DMSO: 5 methanol: 2 water at a final concentration of 0.33 mg/ml were added with 150 µl DPPH in a 96-well plate. Ascorbic acid at 0.4 mg/ml was used as positive control. Solvent (DPPH + solvent) and extract (extract + solvent) blanks were included. After 30 min of incubation, absorbance was read at 517 nm. DPPH Scavenging activities were computed in percentage relative to ascorbic acid.[11]

$$\% \text{ DPPH radical scavenging activity} = [(A_0-(A_1-A_2)/A_0] \times 100$$

Where: A_0, A_1 and A_2 are the absorbance respectively of solvent blank, sample and extract blanks. The values of the extracts were then divided by the values of ascorbic acid.

Anti-inflammatory Assay (COX-Inhibition Assay)

The ability of the extracts to inhibit the enzyme cyclooxygenase 2 (COX-2) and cyclooxygenase-1 (COX-1) was determined using a COX inhibition assay kit following the manufacturer's instructions (Cayman Chemicals, Inc., USA). The extracts were assayed at a final concentration of 100 ppm. Two trials (4 replicate wells/trial) were done for each extract. Celecoxib (Celebrex) was used as positive control. Selectivity index was computed by getting the ratio of % COX-2 inhibition and % COX-1 inhibition.

MTT Cytotoxicity Assay using Normal Cells

All extracts were assayed at two concentrations: 200 ppm and 20 ppm. The proliferative activity on HEKn was determined using the MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) kit (Vybrant, Life Technologies). Cells at passage 3 were plated in a 96-well plate at 5,000 cells/well concentration. The cells were incubated for 24 h. The media was changed before adding with plant extract at a final concentration of 20 ppm and incubated again at 37°C for 48 h. Then, cells were labeled by adding 10 µL of 12 mM MTT stock solution and incubated at 37°C for 4 h. After labeling the cells with MTT, all but 25 µL of medium were removed from the wells. Then, cells were incubated at 37°C for 10 min with 50 µL of dimethyl sulfoxide (DMSO). Absorbance per well was determined by optical density at 540 nm using a microplate reader. Two trials at three replicates per extract were carried out.

MTT Cytotoxicity Assay using lung cancer cells

MTT cytotoxicity assay adapted from Mosmann[12] was employed in this study. Cells (human lung adenocarcinoma A549) were seeded into sterile 96-well microtiter plates using a seeding density of 6 x 10^4 cells/well. The plates were then incubated overnight at 37°C and 5% CO^2. Samples at 4 mg/mL DMSO were serially diluted to four different concentrations: 1000 µg/mL, 500 µg/mL, 250 µg/mL and 125 µg/mL in a master dilution plate (MDP). From the MDP, 10 µL of each concentration were dispensed onto the plated cells to obtain the final screening concentrations, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL. Doxorubicin was used as the positive control while DMSO served as the negative control. Three replicate wells were used per concentration. The treated cells were incubated for 72 hrs in 5% CO^2 at 37°C. Removal of media from the microtiter plate was done after incubation. This was followed by addition of 20 µL 3-(4, 5- dimethylethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) at 5 mg/mL PBS. After incubation, the media were removed from the microtiter plate and 20 µL 3-(4, 5- dimethylethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) at 5 mg/mL PBS was added. The cells were then incubated again at 37°C and 5% CO_2 for four hours. After which, DMSO was added to each well to terminate the reaction. Absorbance was read at 570 nm using a microplate reader. The concentration required to kill 50% of the cell population or the Inhibition Concentration 50 (IC_{50}) was determined using the software "icpin", which uses linear interpolation method. Three trials were done for each sample.

RESULTS

Morphological and Molecular Identification

Based on plant habit and morphological characteristics such as sori shape and distribution, frond type, rhizome characteristics, and presence/absence of scales or hair, the collected plant specimens were identified as *Lycopodiella cernua* (L.) J. Sm. Furthermore, the rbcL region with an average length of 561 base pairs was successfully sequenced and confirmed the initial identification at 100 % identity when compared to GenBank and BOLD databases.

Qualitative and Quantitative Phytochemical Analysis of Crude Methanolic Extracts

The results in this study showed that the *L. cernua* samples had detectable levels of saponins and terpenoids only (Table 1). These observations were seen on the crude extracts from both aerial and ground parts of the species which were subjected to seven phytochemical screening

tests. It was also found that the aerial parts of this species had higher levels of TPC (33.04 ± 2.82 mg GAE/g) and TFC (11.46 ± 7.19 µg QE/g) compared to extracts from the ground parts at values of 4.19 ± 0.10 mg GAE/g and 5.82 ± 5.09 µg QE/g. respectively (Table 2).

DPPH Radical Scavenging Assay

The free radical DPPH [1,1-Diphenyl-2-picrylhydrazyl radical, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl] scavenging activity in *L. cernua* for methanolic extracts from the aerial parts and ground parts were 19.13 ± 0.88 % and 12.21 ± 0.9 %, respectively, relative to the 0.4 mg/ml ascorbic acid standard (Table 3).

Cyclooxygenase Inhibition Assay

The methanolic extracts from the aerial parts of *L. cernua* exhibited high anti-inflammatory activity against COX-2 (74.78 ± 18.61 %) compared to its activity against COX-1 (57.80 ± 3.46 %) (Table 3). These values yield a ratio of 1.29. The ground parts of the plant had low activity for COX-2 inhibition so its COX-1 inhibition was no longer determined.

MTT Cytotoxicity Assay using HEKn and A549 cells

Findings on the cytotoxic activities using the MTT cell proliferation assay showed that the crude methanolic aerial and ground extracts were nontoxic to HEKn cell line (Table 4). This is based on the 20 ppm LC_{50} standard of the US National Cancer Institute Plant Screening Program.[12] There was also no demonstration of toxicity against lung adenocarcinoma A549 using the MTT assay, as shown by the absence of linear interpretation of the data (Table 5).

DISCUSSION

Plant identification is an essential step in natural products research. Traditionally, plants are identified based on morphological features. However, morphology as a sole criterion for identification may pose ambiguity due to

Table 1: Phytochemical tests in crude methanolic extracts of *Lycopodiella cernua* using thin-layer chromatography (TLC).

Phytochemicals	Plant Part	
	Aerial	Ground
Alkaloids	-	-
Anthraquinones	-	-
Saponins	+	+
Tannins	-	-
Terpenoids	+	+

Table 2: Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of *Lycopodiella cernua* plant parts.

Plant part	TPC (mg GAE/ g sample)	TFC (µg QE/ g sample)
aerial	33.04 ± 2.82	11.46 ± 7.19
ground	4.91 ± 0.1	5.82 ± 5.09

Table 3: DPPH radical scavenging activity (%) and Cyclooxygenase Inhibition Activity (%) of *Lycopodiella cernua.*

Plant part	% DPPH Radical Scavenging Activity	% Cyclooxygenase Inhibition		
		COX-2	COX-1	Selectivity Index (COX-2/ COX-1)
aerial	19.13 ± 0.88	74.78 ± 18.61	57.80 ± 3.46	1.29
ground	12.21 ± 0.9	35.83 ± 8.24	*	*

Values are mean ± SE (n=3). (*) - not carried out

Table 4: Cytotoxicity assay of *Lycopodiella cernua* using human epidermal keratinocytes (HEKn).

Plant part	Concentration (ppm)	Mean ± SD
aerial	200	54.75 ± 0.82
	20	95.43 ± 7.50
ground	200	84.33 ± 0.83
	20	93.74 ± 32.18

Table 5: Cytototoxicity assay of *Lycopodiella cernua* using lung adenocarcinoma A549 cell lines.

Sample	Dose (µg/mL)	Trial 1	Trial 2	Trial 3
Aerial Parts	0	0.66 ± 0.25	0.69 ± 0.11	0.76 ± 0.04
	50	0.69 ± 0.03	0.76 ± 0.15	1.07 ± 0.12
	25	0.83 ± 0.03	0.57 ± 0.17	0.78 ± 0.17
	12.5	0.88 ± 0.03	0.63 ± 0.23	0.79 ± 0.08
	6.25	0.85 ± 0.01	0.64 ± 0.15	0.72 ± 0.01
	IC_{50}	No LI	No LI	No LI
Ground Parts	0	0.78 ± 0.02	0.94 ± 0.04	0.85 ± 0.02
	50	0.38 ± 0.04	0.45 ± 0.02	0.44 ± 0.02
	25	0.50 ± 0.01	0.70 ± 0.07	0.54 ± 0.03
	12.5	0.56 ± 0.01	0.77 ± 0.01	0.61 ± 0.03
	6.25	0.61 ± 0.02	0.65 ± 0.00	0.60 ± 0.01
	IC_{50}	49.05	48.20	No LI
Doxorubicin	0	0.50	0.53	0.53
	25	0.11	0.14	0.21
	12.5	0.11	0.17	0.22
	6.25	0.11	0.14	0.21
	3.125	0.10	0.15	0.22
	IC_{50}	1.99	1.95	2.63

No LI = no linear interpretation.

the very diverse plant forms and this is aggravated further in ferns and its allies since these plants lack flowers and fruits. Hence, traditional method is reinforced with molecular methods.[13] In this study both morphological and molecular data confirmed the identity of the plant as *Lycopodiella cernua* (L.) J. Sm.

The qualitative phytochemical screening of plant extracts is an important initial step leading to the discovery of novel drugs. In this study, only two phytochemicals out of seven tests done on both aerial and ground parts were positive. These were saponins and terpenoids. This study confirms what was earlier reported regarding the presence of terpenoids[14] in this species but the findings on saponins appears to have no previous comparison. Moreover, Choung *et al.*[1] also reported alkaloids in *L. cernua*. The present study however, does not rule out the possibility that the Philippine *L. cernua* samples used had undetectable amounts of alkaloids. Another possibility is that the environment could influence gene expression in a species. In relation to this, it is necessary to refer to the report of Liu *et al.*[15] who found that climatic and edaphic factors greatly influence the production of active substances in the anti-cancer plant *Sinopodophyllum hexandrum* (Royle) T.S. Ying. In particular, they found that ecological conditions in Jingyuan, Ningxia Province, and Yongdeng, Gansu Province favor production of podophyllotoxin and lignans in the said species but in Shangri-La, Yunnan Province, and Nyingchi, Tibet, conditions were favorable for quercetin and kaempferol production in the same species.

As to bioactivities, serratene triterpenes in *L. cernua* was reported by Zhang *et al.*[14] for their inhibitory effects against *Candida albicans* secreted aspartic proteases (SAP). Choung *et al.*[1] also showed cognitive-enhancing effect of alkaloid fractions from *L. cernua* on amnesic mice. Moreover, according to Cambie and Ashe in 1994, as cited by Baltrushes,[4] there are alkaloids from this species which have been patented for Hay fever treatment. It also appears that the distribution of TPC and TFC differs between *L. cernua* and two related Pteridophyte taxa reported earlier. In the 2017 report of dela Cruz *et al.*[10] TPC and TFC levels of *Drynaria quercifolia* and *Microsorum punctatum* were higher in the ground parts instead**.** The undetectable levels or probable absence of tannins (Table 1) indicates that there could be other types of phenolic compounds. The TPC and *TFC* values for *L. cernua,* however, appear modest when viewed against those reported on other species particularly those obtained from many of the popular Philippine fruits.[16]

The evaluation of the antioxidant activity in methanolic extracts of *L. cernua* using the free radical DPPH (1,1-Diphenyl-2-picrylhydrazyl) scavenging activity indicates the presence of antioxidant molecules that donate electrons resulting to a reduced DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate). By comparison, this is relatively lower than the DPPH radical scavenging activity reported for some vegetables like *Ipomoea batatas* (89.53 ± 0.2 %) and *Corchorus olitorius* (63.76 ± 4.1 %) using 1 mg/mL ascorbic acid as standard.[17]

There is higher anti-inflammatory activity of the crude methanolic extracts of aerial parts of *L. cernua* using COX-2 compared to its activity against COX-1. This indicates selectivity. When comparing this to commercial products, this selectivity as indicated by the COX-2: COX-1 ratio of 1.29 is lesser than the value for Aspirin (2) but greater than that for Ibuprofen (0.67).[18] It has been suggested that the anti-inflammatory activities of drugs could result from its ability to inhibit cyclooxgenases which catalyze the bioconversion of arachidonic acid to prostaglandins.[19] Of the two currently established COX isozymes, COX-1 which is produced by a variety of tissues is needed in many physiological functions such as gastro protection and vascular homeostasis. On the other hand, COX-2 being induced by mitogenic and proinflammatory stimuli, is believed to be involved in inflammatory response. This indicates that selectivity for COX-2 inhibition could be the more useful direction to follow in addressing inflammatory issues. At least this has been considered for disorders like Alzheimer's disease, rheumatoid arthritis, osteoarthritis, and some solid tumors.

The crude methanolic extracts of *L. cernua* did not show cytotoxic activity on the proliferative activity of HEKn cell lines using the MTT assay. The species further showed nontoxic activity against lung adenocarcinoma A549. The above observations are based on the 20 ppm LC_{50} standard of the US National Cancer Institute Plant Screening Program [12]. This observation is important to note in the light of the anti-inflammatory activity demonstrated by this species using COX-1 and COX-2. This means that further development using this species in addressing inflammation issues has the assurance of its nontoxic activity against normal cells, at least on HEKn cells.

CONCLUSION

Lycopodiella cernua (L.) J. Sm. was evaluated for its phytochemical properties, antioxidant and anti-inflammatory activities as well as cytotoxicity. The presence of saponins and terpenoids were observed at detectable levels. The TPC and TFC were higher in the aerial parts than

in the ground parts. Free radical DPPH scavenging activity was observed but this was lesser than the ascorbic acid standard suggesting low level of antioxidant activity for most samples. Anti-inflammatory activity was also observed with apparent selectivity for COX-2 inhibition. Although there was no observed toxicity on lung adenocarcinoma A549 cell line, its apparent absence of toxic activity on the proliferation of normal neonatal human epidermal keratinocytes (HEKn) makes it potentially useful for addressing inflammatory issues requiring COX-2-selective drugs. These observed bioactivities appear to be the highlight of this study.

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CONFLICT OF INTEREST

The author declare that there is no conflict of interest.

ABBREVIATIONS

COX: cyclooxygenase; **TPC:** Total Phenolic Content; **TFC:** Total Flavonoid Content; **HEKn:** Human Epidermal Keratinocytes, neonatal; **DPPH:**1,1-Diphenyl-1-(2,4,6-trinitrophenyl) Hydrazyl; **GAE:** Gallic Acid Equivalent; **QE:** quercetin equivalent.

SUMMARY

The study reports on the anti-inflammatory, antioxidant and cytotoxic activity of crude methanolic extracts from the aerial and ground parts of Lycopodiella cernua, underscoring the following observations: (1) That, the DNA barcode of the samples used had 100% initial identity to the NCBI and BOLD databases for Lycopodiella cernua; (2) That, saponins and terpenoids were at detectable levels in both extracts; (3) That, TPC, TFC and %DPPH radical scavenging activity were higher in the aerial extracts; (4) That, the aerial part extract was COX2-selective at selectivity index of 1.29 and; (5) That, both extracts were nontoxic to human epidermal keratinocytes and lung A549 adenocarcinoma cell line.

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