PHYTOCHEMICAL ANALYSIS AND DETERMINATION OF ANTIMICROBIAL, ANTIOXIDANT AND ANTICANCER ACTIVITY OF THE LEAF ETHANOLIC EXTRACTS OF *PIPER SARMENTOSUM* ROXB. IN LAPUYAN ZAMBOANGA DEL SUR, PHILIPPINES

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ABSTRACT: *Piper sarmentosum* Roxb. belongs to the genus Piper in which traditional application described as remedy for several diseases. Considering its vast potential as effective pharmacological agent, a study was conducted to assess the phytochemical and biological activities of *P. sarmentosum* leaf extracts, with particular focus on their anti-microbial, anti-oxidant and anticancer properties. Results for antimicrobial test revealed that it has positive activity against the tested microorganisms namely, *Bacillus subtilis* and *Klebsiella pneumonia*. In terms of the antioxidant activity of the extracts, using DPPH scavenging assay, it can be observed that *P. sarmentosum* has an IC_{50} of 55.25 ppm, suggesting a potentially significant source of antioxidants. The extracts also exhibited a strong cytotoxic activities against three human cancer cell lines; cervical carcinoma (HeLa), breast cancer (MCF-7), and colon adenocarcinoma (HT-29). The antimicrobial and antioxidant activities of the leaf extract could be attributed to the tannins and flavonoids identified using phytochemical analysis together with steroids and alkaloids. Further analysis of the bioactive compounds using GC-MS analysis revealed the presence of phytochemicals that have anticancer potentials of which the abundant contents were Asarone (73.72%), 3 – (4- methoxyphenyl) propionic acid (7.58%), and Phytol (3.67%). The results of the study show that the ethnomedicinal application of *Piper* species has a pharmacological basis and that phytochemical search of active compounds inspired by the knowledge from ethnomedicinal application could be vital in drug discovery.

Keywords:

Asarone, Ethnopharmacology, GC-MS, Medicinal plants, Piper

INTRODUCTION: Medicinal plants have presented a lot of potential for the development of new pharmacologically significant compounds in treating different diseases.

It is a natural product that occurs naturally in plants and contains complex chemical structures, which can lead to the discovery of novel drugs, benefitting humanity [1-2]. The most significant of these bioactive chemicals are alkaloids, tannins, flavonoids, and phenolic compounds [3].

The Philippines has a diverse species of plants of which most of it was used for medicinal purposes. Among these are the *Piperaceae* or the pepper family. It is one of the largest families of basal dicots, widely distributed in tropical and subtropical regions of both hemisphere [4]. In Southeast Asia, the genus is distributed in the Indo-Malayan region, South India and Sri Lanka with ~600 species [5]. The abundant *Piperaceae* in the Philippines is the genus *Peperomia* Ruiz & Pavon and *Piper* Linnaeus [6-7] in

which genus Piper is composed of a diverse specialized metabolism that includes alkaloids, terpenoids, and flavonoids of which these properties of plant volatile compounds make them potential source for antibacterial, antifungal and antiviral agents in medical, cosmetic and technological applications [8].

Piper sarmentosum Roxb. belongs to the genus *Piper* locally known as “kaduk” in Malaysia, “sirih duduk” or “mengkadak” in Indonesia. As described by Rahman *et al*., 2016 and Chan and Wong, 2014, is an herb with slender branchlets, green stem, rounded flowers that have a unisexual ovary, fruits are obovoid berry, pungent and aromatic in taste [9-11]. Recent studies suggested that the plant phytochemical contents involved alkaloids, phenols, and polyphenols, flavonoids, and tannins. The plant also demonstrated a high antioxidant, antibacterial, cytotoxic, anti-inflammatory, antifungal, anti-dengue, and atherosclerosis activity [12-17].

The traditional application, described *P. sarmentosum* Roxb. as a remedy for several diseases that includes treatment of fever and indigestion, [18] toothache, headaches, and for the treatment of coughs and rheumatism. In the Philippines, the ethnomedicinal application of the plant has not yet been recorded. The Subanen tribe in Lapuyan, Zamboanga del Sur identified the plant as medicinal, that was utilized by them in treating kidney problems. The locals referred to the plant as thalon-thalon.

The lack of scientific data on traditional knowledge, demand for plant-based drug discovery to fill the gap and identify new ways to counteract multifactorial diseases. Therefore, there is a need to screen medicinal plants for relevant active compounds to be a basis for further biomedical researches and considering the vast potential of *Piper sarmentosum* Roxb. as effective pharmacological agent, a study was conducted to assess the phytochemical and biological activities of *P. sarmentosum* leaf ethanolic extracts in Lapuyan Zamboanga del Sur, Philippines, with particular focus on their anti-microbial, anti-oxidant and anticancer properties.

MATERIALS AND METHODS:

Collection of Plant Sample: The plants were collected in the municipality of Lapuyan Zamboanga del Sur, Philippines. Before the collection, permits and consent were obtained from the Mayor and the local Subanen Tribal leader. The collection of plant samples were not possible without the help of our guide and also one of the Tribal leader (“Thimuay”) in the area. Pictures were taken for the confirmation and identification of the plant by a botanist and systematist. Also the latitude, longitude, and elevation at the different sampling areas were noted using a handheld Garmin GPSMAP 76S system. The leaves of the plant were stored in an airtight container with silica gel.

Extract Preparation: The leaves of *P. sarmentosum* Roxb. were cut into pieces and air-dried at room temperature. It was then powdered using dry mechanical grinder. One hundred fifty (150) grams of powdered leaves were soaked in 500 ml of absolute ethanol for a week with regular stirring. The extract was filtered with Whatman Number 1 and concentrated in rotary evaporator at 40-50 °C under reduced pressure to obtain a semisolid material and was stored in storage vials for further analysis.

***In-vitro* Antimicrobial Assay:** The test for antimicrobial inhibition capacity of the leaf ethanolic extracts was the Agar well diffusion method against selected test microorganisms from the Microbiological Research and Services Institute, University of the Philippines Diliman. The following bacterial strains were used as test organisms: Gram-negative bacteria- *Klebsiella pneumonia* (UPCC 1360), and gram-positive bacteria- *Bacillus subtilis* (UPCC 1295) was prepared in 0.1% peptone water.

The growth inhibition positive control was ofloxacin (5.0 µg), and the negative control was absolute ethanol. A 3.0 mm thick pre-poured nutrient agar (NA) plates were inoculated with the respective microbial suspension by swabbing the agar surface. After that the procedure was repeated for two more times,

constantly rotating the plate 60°C each time, ensuring even distribution of the inocula. There were three (3) equidistant wells made on the agar plate with the help of a cork borer (10.0 mm), and about 200 µl of the plant extract was placed in each of the wells. The plates then were incubated at 35 °C and observed after 24 h.

The inhibition zone was measured in millimeters and the mean diameter of the inhibition zone was calculated. The antimicrobial index (AI) is done by the following formula:

AI = (Diameter of the inhibition zone – Diameter of the well) / (Diameter of the well)

DPPH – Free Radical Scavenging Assay: The free radical scavenging activity of the extracts was evaluated based on the scavenging activity of DPPH (1, 1-diphenyl-2-picryl hydrazyl) by measuring the reduction of the absorbance measured at 517 nm. The protocol was adapted from Jacinto *et al.,* 2009, and Hou *et al.*, 2004 with modifications [19-20].

DPPH is reduced from a stable free radical that is purple in color into diphenylpicryl hydrazine that is yellow with the presence of antioxidants. In each experiment, the tested sample alone in methanol was used as blank while the DPPH solution in methanol was used as the control. The standard utilized is L-ascorbic acid. The percent of DPPH discoloration of the samples was calculated and the result was expressed as percentage inhibition using the following formula:

% Inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$

In which $A_{control}$ is the absorbance values of the control and A_{sample} for the sample absorbance values.

***In-vitro* MTT Assay:** Powdered plant extracts were weighed and dissolved in a 1% DMSO to make a stock solution of extracts (1000 µg/mL). The solution was divided into aliquots for future use. Anti-cancer activity of the plants was determined using MTT (3-(4, 5-dimethylthiazolyl- 2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay against three cancer cell lines *viz*, Cervical carcinoma (HeLa), Breast Cancer (MCF-7), and Colon adenocarcinoma (HT-29). From the prepared monolayers, medium was removed and cells were treated with the extracts at different concentrations (100, 80, 60, 40, and 20) µg/mL before it was incubated for 24 h. After incubation, the treated cells and medium at each well was removed and washed with PBS before it was replaced with 25 µL MTT (2.5mg/mL) and was then further incubated for 4 h. The supernatant was then removed and 100 µL of DMSO was added and the absorbance was measured at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01, and A153601, Packard BioScience, CT, USA). Percent cell viability and inhibition were calculated and compared with the untreated cells as negative control.

Phytochemical Screening: Evaluation of the different photo components of the leaf ethanolic extracts were performed following the standard phytochemical methods described by Aguinaldo *et al*., 2005 [21]. The method has been modified according to the Laboratory of the Department of Chemistry Mindanao State University – Iligan Institute of Technology. A Three (3) point scale (+ turbid, ++ moderate, and +++ heavy) in scoring was based on the Handbook of the Philippine Medicinal Plants.

GC-MS Analysis: For the GC-MS analysis of the leaves, it was performed following the protocol of Chipiti *et al.,* 2015 [22]. A 1.0 mg of the leaves crude extract was diluted with a mixture of 0.5 mL absolute methanol and 0.5 mL dichloromethane to separate chlorophyll. The upper layer with no chlorophyll (10.0 µL) was be taken and further diluted with 1.0 mL hexane.

The GC-MS analysis was carried out using Agilent Technologies 7890A GC system coupled to a mass detector, 5975C Mass Selective detector, and driven by Agilent Chemstation software and with HP-5MS 30m × 0.25mm × 0.25 µm df capillary column. The carrier gas was ultra-pure helium at a flow rate of 1.0 mL/ min. and a linear velocity of 37 cm/s. The temperature of the injector was set at 320 °C. The instrument

was set to an initial temperature of 70 °C, which was programmed to increase to 280 °C at the rate of 10 °C/ min with a hold time of 4 min. at each increment injection. An aliquot of 1.0 µL sample was injected in a split mode 100: 1.

The mass spectrometer was operated in the electron ionization mode at 70eV and electron multiplier voltage at 1859V.

Other MS operating parameters were: ion source temperature 230 °C, quadruple temperature 150 °C, solvent delay 3 min and scan range 22-550 amu (automatic mass unit).

The compounds were identified by direct comparison of the mass spectrum of the analyte at a retention time to that of a reference standard found in the National Institute of Standards and Technology (NIST) library. Total GC running time was 25-30 min [20]. Similarity index more than 80% was considered [23].

Statistical analysis: All experiments were carried out in three replicates. Data were expressed in Mean ± Standard Deviation. Statistical analysis was carried out with SPSS software (version 20.0).

RESULTS AND DISCUSSION: Ethnobotany and traditional medicine have been utilized for centuries, particularly the application of medicinal plants for therapeutic purposes. In view of these details, this study describes the biological activities of the leaf ethanolic extracts of *P. sarmentosum* collected from the Municipality of Lapuyan Zamboanga del Sur, the Philippines with a particular focus on its antimicrobial, antioxidant and anticancer properties as well as its phytochemical components and analysis.

TABLE 1: COLLECTED *PIPER* SPECIES IN LAPUYAN, ZAMBOANGA DEL SUR, PHILIPPINES

Scientific name	*Piper sarmentosum* Roxb.
Common name	Wild pepper/betel (English)
Local name	Thalon-thalon
Sampling location	Lower Salambuyan, Lapuyan Zamboanga del Sur

Antimicrobial Assay: The agar well diffusion assay for the antimicrobial test was performed to determine the antimicrobial activities of the leaf ethanolic extracts against gram-positive and gram-negative bacteria

namely *B. subtilis* and *K. pneumoniae* respectively. The results exhibited inhibition zones ranging from 20 mm to 25 mm diameter.

The positive control, Ofloxacin 5 µg displayed a resistant result. Extracts from *P. sarmentosum* demonstrated maximum antibacterial activity against gram-negative bacteria *K. pneumoniae* with a 1.5 antimicrobial index **Table 2**. The results of the antimicrobial test revealed that the ethanolic leaf extract has positive activity against the tested microorganisms.

FIG. 1: LEAF ETHANOLIC EXTRACT FROM *PIPER* SPECIES PLATES FOR AGAR WELL ASSAY SHOWING ZONES OF INHIBITION AND THEIR ANTIMICROBIAL INDICES AGAINST GRAM-POSITIVE BACTERIA A. *BACILLUS SUBTILLIS* (UPCC 1295), B. *KLEBSIELLA PNEUMONIAE* (UPCC 1360) AND C. OFLOXACIN

TABLE 2: ANTIMICROBIAL ANALYSIS OF THE *PIPER* LEAF ETHANOLIC EXTRACTS AND POSITIVE CONTROL OFLOXACIN

DPPH - Free Radical Scavenging Assay: Antioxidant values in the DPPH free radical scavenging assay of the leaf extracts were calculated by its ability to reduce DPPH, which is a stable free radical. The results were recorded in terms of percent (%) inhibition, and the amount of extract required scavenging half the DPPH free radical (IC_{50}) as shown in **Table 3**.

TABLE 3: ANTIOXIDANT ACTIVITY OF *PIPER* SPECIES BY DPPH SCAVENGING METHOD

Samples	DPPH scavenging activity (%) Mean of triplicate analysis								IC_{50} ppm
	5 ppm	**10 ppm**	**20 ppm**	**30 ppm**	**50 ppm**	**100 ppm**	**200 ppm**	**300 ppm**	
P. sarmentosum	33.93	37.04	41.93	41.48	46.22	55.11	62.22	64.00	55.25

Anticancer Analysis: The ethanolic leaf extracts of the studied *Piper* species were evaluated for their anticancer activities against three (3) human cancer cell lines namely, Cervical carcinoma (HeLa), Breast Cancer (MCF-7), and Colon adenocarcinoma (HT-29) using MTT assay. The IC_{50} values of the plant's extract were obtained against the three cancer cell lines shown in **Table 4**. Also their percent (%) inhibition on the different cell lines at different concentrations (100, 80, 60, 40, and 20) µg/mL were illustrated in the figure below **Fig. 2**.

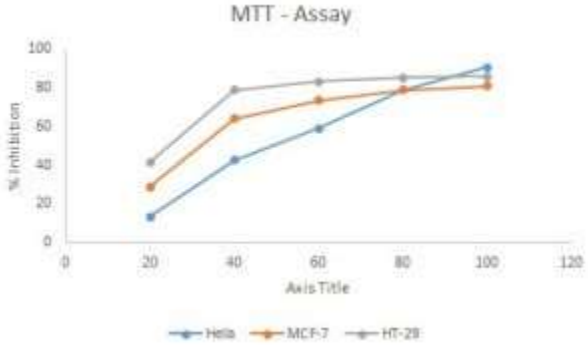

FIG. 2: PERCENT INHIBITION OF THE LEAF ETHANOLIC EXTRACTS OF *PIPER* SPECIES ON CANCER CELL LINES

TABLE 4: CYTOTOXICITY OF THE LEAF ETHANOLIC EXTRACTS OF PIPER SPECIES ON CANCER CELL LINES

Species	IC_{50} (µg/mL) Mean ± SD		
	Cervical carcinoma (HeLa)	**Breast Cancer (MCF-7)**	**Colon adenocarcinoma (HT-29)**
P. sarmentosum	51.61 ± 23.03	30.02 ± 6.84	24.97 ± 5.52

Phytochemical Screening and GC-MS Analysis: The initial assessment of the phytochemicals on the *P. sarmentosum* Roxb leaf ethanolic extracts revealed that it can be classified as a good source of natural products **Table 5**. Results of the screening concluded that the plants extract has a varying amount of phytochemicals present. It was also observed that high amount of steroids, tannins, flavonoids, and alkaloids commonly occurred among them, however absence or lack of anthraquinones and cyanogenic glycosides.

Further analysis of the bioactive compounds of the ethanolic leaf extracts was done using GC-MS that showed different numbers of peaks indicating differences in its phytochemical constituents **Fig. 3**. The list of the compounds that were characterized and quantified by comparing the mass spectra with the Wiley, NIST and Pest library with over 80% similarity were listed and identified in **Table 6**. Biological activities of the listed phytocompounds were also presented and are based on the following references [24-25].

TABLE 5: PHYTOCHEMICAL SCREENING OF THE LEAF ETHANOLIC EXTRACTS OF PIPER SPECIES

Sample	Alkaloids	Anthraqui-nones	Cyanogenic glycosides	Flavonoids	Saponins	Steroids	Tannins	Reducing sugar
P. sarmentosum	**(+++)**	**(-)**	**(-)**	**(+++)**	**(+)**	**(+++)**	**(+++)**	**(+)**

Note: (+) indicates present: +turbid, ++moderate, +++heavy; (-) indicates absent

TABLE 6: PHYTOCHEMICALS IDENTIFIED IN THE LEAF ETHANOLIC EXTRACT OF *PIPER SARMENTOSUM* USING GC-MS

S. no.	Reference Compounds with the Wiley, NIST, and Pest Library	Molecular Formula	Retention Time	Similarity Index	Area %	Activity
1	Alloocimene	$C_{10}H_{16}$	7.72	80	0.15	Insecticides, Larvicides, Pesticides
2	Benzene propanoic acid	$C_9H_{12}O_2$	7.85	97	1.10	Antibacterial, Antifungal

3	α- cubebene	$C_{15}H_{24}$	8.25	98	0.98	Antioxidant, Antimicrobial
4	Trans-caryophyllene	$C_{15}H_{24}$	8.92	97	0.96	Anti-inflammatory, Anti-allergic, Antioxidant
5	α-caryophyllene	$C_{15}H_{24}$	9.47	89	0.26	Anti-inflammatory, Anti-allergic, Antioxidant
6	Germacrene D	$C_{15}H_{24}$	9.95	98	2.82	Antimicrobial, Insecticides
7	1H – indene, 2,3,3a,4 - tetrahydro	$C_{15}H_{24}$	10.06	96	0.59	No activity Reported
8	Cis-methyl isoeugenol	$C_{11}H_{14}O_2$	10.16	98	2.27	Insecticides, Antiseptic, Anti-inflammatory
9	Naphthalene	$C_{10}H_8$	10.22	95	1.02	Anti-tumor, Analgesic, Anti- bacterial, Anti-inflammatory Sedative, Fungicide
10	Delta-cadinene	$C_{15}H_{24}$	10.67	97	0.69	Antibacterial, Antifungal
11	3 – (4-methoxyphenyl) propionic acid	$C_{10}H_{12}O_3$	11.94	97	7.58	Anti-inflammatory
12	Trans – isoelemicin	$C_{12}H_{16}O_3$	12.71	98	0.86	Antibacterial, Antifungal
13	Asarone	$C_{12}H_{16}O_3$	13.16	99	73.72	Anti-bacterial, Antifungal, Anticancer
14	neophytadiene	$C_{20}H_{38}$	15.13	97	0.45	Fungicide
15	Hexadecanoic acid	$C_{16}H_{32}O_2$	16.85	87	0.46	Anti-oxidant, Hypocholesterolemic Nematicide, Pesticide, Lubricant, Antiandrogenic, Flavour
16	phytol	$C_{20}H_{40}O$	18.07	93	3.67	Anticancer, Antimicrobial, Anti-inflammatory
17	Tributyl acetyl citrate	$C_{20}H_{34}O_8$	19.50	90	2.41	Pharmaceutical excipient

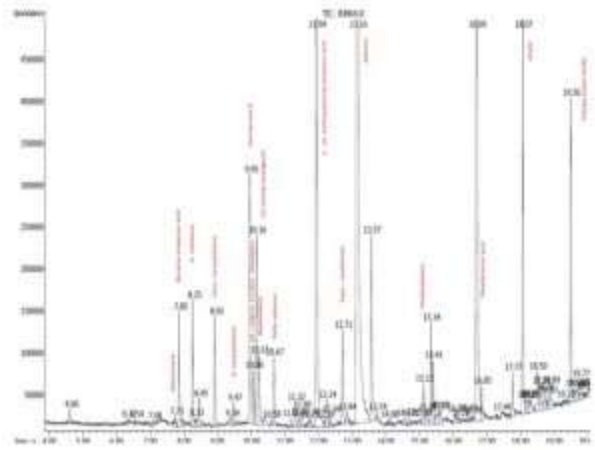

FIG. 3: GC-MS CHROMATOGRAM OF THE LEAF ETHANOLIC EXTRACTS OF *PIPER SARMENTOSUM* ROXB.

DISCUSSION: On the recent review study conducted with a particular focus in the leaf ethanolic extracts of *P. sarmentosum* Roxb, it revealed that the constituents of the extracts include alkaloids, lignans, flavones, unsaturated amides, and other phytocomponents, of which the major contents are amide alkaloids and their different types on the basis of their chemical structure and skeletons that caused the differences on their pharmacological activities [26].

The results above also can be comparable with the initial phytochemical review on the genus *Piper* with alkaloids/amides as the major phytochemicals present [27-28]. The antimicrobial and antioxidant activities present in the study can be attributed due to the presence of secondary metabolites of different chemical types in the plant material. Presence of the phytochemicals like flavonoids, tannins, and steroids in the extracts indicated that *P. sarmentosum* is phenol rich compounds. The existence of flavonoids that have been shown to exhibit effects on the cell wall degradation of cell components leading to death of bacteria [29-30].

The study also shows that the studied *Piper* species leaf ethanolic could be utilized as a cytotoxic and anti-cancer agent against Cervical carcinoma (HeLa), Breast Cancer (MCF-7) and Colon adenocarcinoma (HT-29). GC-MS analysis revealed the presence of seventeen (17) compounds for *P. sarmentosum*, that have a similarity index of more than or equal to 80%. The identified compounds possess many biological activities. The major phytochemicals identified in *P. sarmentosum* include Asarone (73.72%), phytol (3.67%) and 3 – (4- methoxyphenyl) propionic acid (7.58%), of which demonstrated to inhibit cancer cells [31, 24].

A number of other compounds were also detected through GC-MS that have a noteworthy medicinal value. Also, other compounds listed in **Table 6** that does not have anticancer activity were potentially studied and used for pharmacological work as a good agent for anti-inflammatory, antimicrobial, antifungal, insecticidal, nematicide, larvicide and antioxidant.

The GC-MS analysis for the leaf ethanolic extracts of *Piper* species is the first stage towards understanding the nature of the active phytochemicals in the studied medicinal *Piper*. Though, further studies like isolation of individual compounds and analyzing its bioactivity as well as understanding its toxicity profile will produce significant results.

CONCLUSION: Results for antimicrobial test presented that it has positive activity against the tested microorganisms namely, *Bacillus subtilis* and *Klebsiella pneumoniae*. In terms of the antioxidant activity of

the extracts, using the DPPH scavenging assay, it can be observed that *P. sarmentosum* has an IC_{50} of 55.25 ppm, suggesting a potentially significant source of antioxidants. The extracts also exhibited a strong cytotoxic activities against three human cancer cell lines; cervical carcinoma (HeLa), breast cancer (MCF-7), and colon adenocarcinoma (HT-29). It was observed that *P. Sarmentosum* is more effective against HT-29 followed by MCF-7 and HeLa respectively.

The antimicrobial and antioxidant activities of the leaf extract could be attributed to the tannins and flavonoids identified using phytochemical analysis together with steroids and alkaloids. Further analysis of the bioactive compounds using GC-MS analysis revealed the presence of phytochemicals Phytol, Asarone, and Naphthalene that have anticancer potentials. Major phytochemicals were also detected through GC-MS belonging to seven chemical classes (monoterpenes, phenylpropanoids, sesquiterpene, hydrocarbons, fatty acid, diterpenes, and ester) of which the abundant contents were Asarone (73.72%), 3 – (4-methoxyphenyl) propionic acid (7.58%) and Phytol (3.67%).

It is suggested that further studies like isolation of individual compounds and analyzing its bioactivity as well as understanding its toxicity profile will produce significant results. Moreover, clusters of genes for the biosynthesis regulating the metabolite profiling of the plant species need to further investigated. Finally, the results of the study showed that the ethnomedicinal application of *Piper* species has a pharmacological basis and that phytochemical search of active compounds inspired by the knowledge from ethnomedicinal application could be vital in drug discovery.

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