STUDIES ON THE STRUCTURE AND BIOLOGICAL ACTIVITY OF THE CHEMICAL CONSTITUENTS OF THE LEAVES OF *IPIL, HANDALAMAY* AND *LIPANG ASO*

Mylene M. Uy and Anita P. Rivera
Chemistry Department, Mindanao State Univeristy-Iligan Institute of Technology

Abstract

Purification of the respective crude chloroform fractions of *Ipil*, *Handalamay* and *Lipang aso* through silica gravity column chromatography employing appropriate solvent systems have yielded a number of sub-fractions.

Brine shrimp lethality tests of selected sub-fractions revealed some of them to have very remarkable cytotoxicities against the brine shrimp *Artemia salina*. The highest cytotoxicity was exhibited by the *Ipil* sub-fraction **IBC4.4** which has acute LC_{50} range value of 59.43-61.93 ppm followed by the *Handalamay* sub-fraction **PAC4.3** (acute LC_{50} range=277.58-280.02 ppm) indicating that it only took six hours for these samples to kill 50% of the test animals. The next highest activities were showed by the *Ipil* sub-fraction **IBC4.2** (chronic LC_{50} range value=146.94-149.42 ppm) followed by the *Handalamay* sub-fractions **PAC 4.3**, **PAC4.4** and **PAC4.8** (chronic LC_{50} range values=277.58.42-280.02, 412.77-414.83 and 415.17-417.23 ppm, respectively) signifying that the samples took twenty-four hours to kill 50% of the test animals.

The HPLC purification of a *Lipang aso* sub-fraction has lead to the isolation of **24-ethylcholesterol** whose structure was determined from the NMR spectral data.

Almost all of the sub-fractions obtained exhibited radical-scavenging activity against the free-radical 2,2-diphenyl-1-picrylhydrazine (DPPH) indicating that the samples possess antioxidant potentials.

Infrared spectroscopic measurements revealed the presence of vinyl, carboxyl, hydroxyl, nitro and ether functionalities in the samples.

Phytochemical tests showed that saponins, flavonoids and alkaloids are found in all three medicinal plants. However, tannins are found only in *Ipil* and *Handalamay* while terpenoids are present only in *Ipil* and *Lipang aso*.

Introduction

This research work is focused on the Philippine plants - *Ipil* (*Intsia bijuga*), *Handalamay* (*Pipturus arborescens*) and *Lipang aso* (*Laportea meyeniana*).

These medicinal plants are endemic and utilized as traditional herbal medicines all throughout the country. The main objectives of this research are: (1) to isolate and purify the chemical constituents from the chloroform extracts of the three medicinal plants; (2) to subject the purified chemical constituents to cytotoxicity and antimicrobial assays; and (3) to elucidate the structures of the purified constituents.

Earlier, the initial cytotoxicity and antimicrobial activities as well as the fractionationation of the chloroform extracts of the plants' leaves were reported.

For the second year of research, the objectives are: (1) to further purify the chloroform fractions of the leaf extracts; (2) to determine the cytotoxicity of the constituents against the brine shrimp (*Artemia salina*); (3) to partially characterize the plant constituents by Infrared spectroscopy, (4) to measure the free-radical scavenging potentials of the constituents against 2,2-diphenyl-1-picrylhydrazine (DPPH); and (5) to identify the chemical constituents from the leaves of the plants. The completion of this research work has established a scientific validation of some of the folkloric medicinal uses of the three plants by having provided baseline data regarding their chemical compositions and biological activities. Eventually, this will contribute to the rational and sustained exploration and conservation of the gene pool of the particular medicinal plants for the benefit of the people. Once this is realized, the people with the support of their local government units, will be given the impetus to commercially market these medicinal plants as herbal medicines for their economic benefit and efficacy.

Review of Related Literature

Ipil (*Instia bijuga*), belongs to the Luguminosae family. It is a spreading tree up to 40 m tall, with prominent buttresses when mature. *Ipil* is widely distributed in Babuyan Islands, Northern Luzon and Mindanao. In the Philippines, the bark and leaves are used in traditional medicine for treating rheumatism, dysentery and urinary tract infection (Esperanza and Kitche, 2005) while the fruit is considered a laxative (Quisumbing, 1978).

Handalamay (*Pipturus arborescens*) is a small tree belonging to the Urticaceae family. It is common and widely distributed in the Philippines, found chiefly in thickets and second-growth forests at low and medium altitudes from Batan Island to Mindanao and Palawan. The scrapings of the bark are used externally as cataplasm for boils, while the leaves are used to cure herpes (Esperanza and Kitche, 2005). From the hexane extract of the leaves, triterpenes such as glutinone, friedelin and glutinol with a mixture of common sterols- campersterol, stigmasterol and sitosterol, were isolated (Gabona, 2000). A pure isolate active against *Bacillus subtilis* was obtained from the crude ethyl acetate extract of the leaves after chromatography (Rosal, 1995).

Lipang aso (*Laportea meyeniana*) is a small tree belonging to the Urticaceae family characterized by the presence of stinging hairs known for causing contact dermatitis. In Butuan City, Philippines, the extract of the plant's roots is used as a cure for ringworm. Dried powdered leaves are used to stop bleeding while a decoction of the leaves is used for nosebleeds (Esperanza and Kitche, 2005).

The antioxidant and cytotoxic activities as well as the phytochemical screening of the methanol extracts of the leaves of these three plants have been recently reported (Peteros and Uy, 2010).

Experimental

Selected fractions obtained from the chromatographic separation of the respective choloform extracts of the plants were further purified by (1) silica gravity column chromatography and (2) reversed-phase High-performance Liquid Chromatography (HPLC) employing appropriate gradient solvent systems.

The resulting sub-fractions/isolates were then subjected to brine shrimp (*Artemia salina*) lethality tests to determine their cytotoxicities. Lethal concentration (LC_{50}) values were established by the Reed-Muench method (Miya, et al., 1973).

Infrared spectroscopic profiling was performed using the Perken-Elmer FT-IR instrument of the Mindanao University of Science and Technology Chemical Laboratory Service Center, Cagayan de Oro City. NMR spectroscopic measurements were done in Nagahama Institute of Bio-science and Technology through Dr. Shinji Ohta.

The antioxidant potentials of the samples were measured using the 2,2-diphenyl-1-picrylhydrazine (DPPH) radical-scavenging activity (Moon and Shibamato,2009).

The phytochemical screening of the plants was conducted using standard methods. (Edeoga et.al., 2005)

Discussion of Results

A. Purification, Cytotoxicity Evaluation, Antioxidant Screening and IR profiling of the Chloroform Fraction of *Ipil*

The purification of the *Ipil* chloroform fraction **IBC4** (212.5g) on silica column eluted with chloroform-hexane and methanol-chloroform gradient solvent systems yielded the four sub-fractions **IBC4.1-IBC4.4**. Only two of the sub-fractions had considerable weights namely, **IBC4.2** (72.8 mg) and **IBC4.4** (90.5 mg). Purity check of these two samples by HPLC indicated that each of them have two major components.

Results of the brine shrimp lethality tests conducted on the two sub-fractions are presented in Table 1.

Table 1. Brine Shrimp Larvae Mortality after Exposure* to *Ipil* Sub-fractions

Sub-fraction	Dose (ppm)	Mortality (%)
IBC4.2	1000	93.5
	500	75.9
	300	62.7
	100	43.1
IBC4.4	1000	99.05
	500	94.94
	300	90.57
	100	54.29

*24-hrs for **IBC4.2** and 6-hrs for **IBC4.4**

The chronic and acute LC_{50} range value for **IBC4.2** and **IBC4.4** were estimated to be at 146.94-149.42 ppm and 59.43-61.93 ppm, respectively.

These results indicate that the more polar sub-fraction **IBC4.4** is more toxic than **IBC4.2** since after 6-hour exposure, 50% of the brine shrimp larvae were killed at a very low concentration of the test sample.

All the four sub-fractions except **IBC4.2** exhibited radical-scavenging activity against the free radical DPPH indicating the three sub-fractions to possess antioxidant potentials.

The IR spectroscopic measurements on the four sub-fractions (Table 2) revealed that most of them contain common structural features indicating similarities in the compound class to which they may belong.

Table 2. IR Spectroscopic Profiles of the **IBC** Sub-fractions

Sub-fraction	Funtionalities Indicated
IBC4.1	OH (hydroxyl), C=O (carbonyl), aldehyde CH, NO_2 (nitro)
IBC4.2	C=O (carbonyl), aldehyde CH, NO_2 (nitro)
IBC4.3	OH (hydroxyl), C=O (carbonyl), aldehyde CH, NO_2 (nitro)
IBC4.4	OH (hydroxyl), C=O (carbonyl), aldehyde CH, NO_2 (nitro), C=C (vinylic)

B. Purification, Cytotoxicity Evaluation, Antioxidant Screening and IR profiling of the Chloroform Fractions of *Handalamay*

Two fractions (**PAC2** and **PAC4**) obtained from the previous chromatographic separation of the *Handalamay* chloroform extract PAC were purified.

The purification of fraction **PAC2** by column chromatography on silica eluted with dichloromethane-hexane gradient solvent systems yielded six sub-fractions (**PAC2.1**-**PAC2.6**),

among which only sub-fraction **PAC2.2** has a workable amount (153.1 mg). HPLC analysis indicated that such fraction contain three major components.

Meanwhile, eight sub-fractions (**PAC4.1**-**PAC4.8**) were obtained from the purification of fraction **PAC4.4** through a silica column using gradient mixtures of dichloromethane-hexane and methanol-dichloromethane as solvent systems. Only three sub-fractions (**PAC4.3**, 69.7 mg; **PAC4.4**, 57.4 mg; and **PAC4.8**, 61.3 mg) have workable amounts needed for the cytoxicity evaluation.

Brine shrimp lethality test results for sub-fractions **PAC2.2**, **PAC4.3**, **PAC4.4** and **PAC4.8** are presented in Tables 3 and 4.

Table 3. Brine Shrimp Larvae Mortality after Exposure* to Different Sub-fractions of *Handalamay*

Sub-fraction	Dose (ppm)	Mortality (%)
PAC2.2	1000	**88.42**
	500	**78.31**
	300	**64.62**
	100	**32.08**
PAC4.3	1000	**83.80**
	500	**63.30**
	300	**46.20**
	100	**26.40**
PAC4.4	1000	**83.10**
	500	**61.40**
	300	**27.10**
	100	**9.60**
PAC4.8	1000	**83.10**
	500	**53.80**
	300	**35.80**
	100	**9.60**

*6-hrs for **PAC4.3** and 24-hrs for **PAC2.2**, **PAC4.4**, **PAC4.8**

Table 4. Estimated LC_{50} Range* of the Different Sub-fractions of *Handalamay*

Sub-fractions	LC_{50} range, ppm
PAC2.2	168.42-165.98
PAC4.3	277.58-280.02
PAC4.4	412.77-414.83
PAC4.8	415.17-417.23

*Acute for **PAC4.3** and Chronic for **PAC2.2**, **PAC4.4** and **PAC4.8**

The results show that among the sub-fractions evaluated, **PAC4.3** was the most cytotoxic in as much as it only took six-hour exposure to a relatively low concentration (277.58.42-280.02 ppm) of **PAC4.3** to kill 50% of the brine shrimps. Meanwhile, the other three sub-fractions took twenty-four hours to kill 50% of the brine shrimps. Moreover, among the three, **PAC2.2** was the most toxic, followed by **PAC4.4** and **PAC4.8.**

All the sub-fractions of both **PAC2** (**PAC2.1**-**PAC2.6**) and **PAC4** (**PAC4.1**-**PAC4.8**) possess antioxidant potentials as they all showed positive results against the free radical DPPH.

Table 5 summarizes the results of the infrared spectroscopic measurements on selected sub-fractions of **PAC2** and **PAC4**, respectively**.**

Table 5. Infrared Spectroscopic Profiles of **PAC2** and **PAC4** Sub-fractions

Sub-fraction	Functionalities Indicated
PAC2.1	C=C (vinylic)
PAC2.2	C=C (vinylic)
PAC2.4	C=C (vinylic), C=O (carboxyl)
PAC2.5	C=C (vinylic), C=O (carboxyl)
PAC2.6	C=C (vinylic)
PAC4.3	C=C (vinylic), C=O (carboxyl), OH (hydroxyl)
PAC4.4	C=C (vinylic), C=O (carboxyl)
PAC4.6	NO_2 (nitro), C=O (carbonyl)
PAC4.7	NO_2 (nitro), C=O (carbonyl)
PAC4.8	C=C (vinylic), C-O (ether)

The results indicate that **PAC2.1**, **PAC2.2** and **PAC2.6** have similar functionality, only the vinylic group while **PAC2.4** and **PAC2.5** contain both the vinylic and carboxyl groups.

For the **PAC4** sub-fractions, all contain the carboxyl functionality while the vinylic functional group is present in **PAC4.3**, **PAC4.4** and **PAC4.8**. Sub-fractions **PAC4.6** and **PAC4.7** have the nitro group, **PAC4.3** has hydroxyl and **PAC4.8** has an ether functionality.

The presence of these different functionalities in the different sub-fractions account for the free radical scavenging activities they exhibited against DPPH.

C. Purification, Antioxidant Screening and IR profiling of the Chloroform Fractions of *Lipang Aso*

Fractions **LMC10** and **LMC11** obtained from the previous chromatographic separation of the chloroform extract **LMC** of *Lipang aso* leaves were subjected to purification.

The purification of **LMC10** through silica column chromatography eluted with gradient mixtures of methanol-dichloromethane yielded the five sub-fractions **LMC10.1**-**LMC10.5**, among which, **LMC10.3** and **LMC10.5** exhibited high purity (>90%) in the HPLC analysis. These were sent to Japan for spectral analysis. However, the data obtained were broad and complex that their structures could not be absolutely determined. Nevertheless, initial analyses indicate that the compounds may be polymeric in nature.

The purification of **LMC11** by silica column chromatography using dichloromethane-hexane and methanol-dichloromethane solvent systems yielded six sub-fractions (**LMC11.1**-**LMC11.6**). Sub-fraction **LMC11.4** was further purified by reversed-phase HPLC to give a steroid elucidated to be **24-ethylcholesterol** by NMR spectral measurements.

The other **LMC11** sub-fractions exhibited radical scavenging activity against DPPH. Moreover, IR spectral analysis showed that the vinylic and carbonyl groups are present in all of sub-fractions except for **LMC11.3** which only contains the vinylic functionality.

The sub-fractions were not subjected to any further cytotoxicity evaluation due to their insufficient amounts.

D. Phytochemical Screening of the Leaves of *Ipil*, *Handalamay* and *Lipang Aso*

Qualitative and quantitative analyses for the presence of tannins, phlobatannins, saponins, flavonoids, terpenoids, cardiac glycosides and alkaloids were conducted on the air-dried leaves of the three plants and the results are presented in Table 6.

Table 6. Phytochemical Screening Results on the Leaves of *Ipil*, *Handalamay* and *Lipang Aso*

Phytochemical	**Indication (Amount in % weight)**		
	Ipil	***Handalamay***	***Lipang aso***
Tannins	+	+	-
Phlobatannins	-	-	-
Saponins	+ (0.802)	+ (2.31)	+ (0.0467)
Flavonoids	+ (11.28)	+ (3.03)	+ (3.68)
Terpenoids	+	-	+
Cardiac Glycosides	-	-	-
Alkaloids	+ (0.7)	+ (1.88)	+ (0.124)

+ = Present
- = Absent

The results indicate that the leaves of the three plants all contain saponins, flavonoids and alkaloids. Saponin and alkaloid contents are highest in *Handalamay* while *Ipil* contains the highest amount of flavonoids.

Although *Ipil* and *Handalamay* contain tannins, the amount was below the detectable limit of the instrument used. Terpenoids are also present in *Lipang Aso* and *Ipil* but at non-detectable amounts.

Summary and Conclusions

Purification of the chloroform fraction **IBC4** of *Ipil* yielded four sub-fractions. Sub-fractions **IBC4.2** and **IBC4.4** were highly toxic to brine shrimps with chronic and acute LC_{50} range values of 146.94-149.42 ppm and 59.43-61.93 ppm, respectively. All the sub-fractions except **IBC4.2** possess antioxidant potentials. IR spectroscopic analysis results indicated the presence of the vinylic, carboxyl, nitro and hydroxyl groups in the sub-fractions. Phytochemical screening of the *Ipil* leaves revealed the presence of tannins, saponins, flavonoids, terpenoids and alkaloids with the alkaloids present in the highest quantity.

Six and eight sub-fractions were respectively obtained from the purification of the *Handalamay* chloroform fractions **PAC2** and **PAC4**. Cytotoxicity evaluation of four selected sub-fractions showed that **PAC4.3** was the most lethal since it only took six hours for it to kill 50% of the test animals (acute LC_{50} range = 277.58-280.02 ppm). Meanwhile, the chronic LC_{50} ranges for **PAC2.2, PAC4.4** and **PAC4.8** are 168.42-165.98 ppm, 412.77-414.83 ppm and 415.17-417.23 ppm, respectively. These three sub-fractions are still considered highly cytotoxic in as much as their values are below 1000 ppm. All sub-fractions tested exhibited radical-scavenging activity against the free radical DPPH. Vinyl, carbonyl, hydroxyl, nitro and ether functionalities are indicated in the sub-fractions. The chemical constituents found in the leaves of *Handalamay* include tannins, saponins, flavonoids and alkaloids with the flavonoids present in highest amount.

Purification of the chloroform fractions **LMC10** and **LMC11** yielded five and six sub-fractions, respectively. Sub-fractions **LMC10.3** and **LMC10.5** are suspected to be polymeric in nature based on initial NMR spectral analysis while an isolate obtained from the further purification of sub-fraction **LMC11.4** was determined to be a steroid, **24-ethylcholesterol**. Meanwhile, the other **LMC11** sub-fractions exhibited antioxidant potentials and were shown to mostly contain the vinyl and carboxyl functional groups. Phytochemical analysis indicated the presence of saponins, flavonoids, terpenoids and alkaloids with the flavonoids in highest quantity.

References

1. Esperanza, L.M. and Kitche, G. O. Inventory of Medicinal Tree Species in the Secondary Growth Forest of Sitio Tagkiling, Anticala, Butuan City as Utilized by the Locals. 2005 NORMICIST Research and Development In-house Review. Northern Mindanao State of Science and Technology, Butuan City, Philippines. **2005**.

2. Quisumbing, E. Medicinal Plants of the Philippines. Katha Publishing Co., Philippines. **1978**.

3. Gabona, M.G. Triterpenoids and Other Metabolites from the Hexane Extract of *Pipturus arborescens*. MS Thesis, De La Salle University, Manila, Philippines. **2000**.

4. Rosal, R.R. Bioassay-guided Isolation and Structure Elucidation of Some Metabolites from *Pipturus arborescens*. MS Thesis, De La Salle University, Manila, Philippines.**1995**.

5. Peteros, N.P. and Uy, M.M. Antioxidant and Cytotoxic Activities and Phytochemical Screening of four Philippine Medicinal Plants. *J. of Med. Plants Res.*, 4(5), 407-414: **2010**.

6. Miya, T. S. et. al. Laboratory Guide in Pharmacology, 4th Ed., Burgess Publishing: Minneapolis, **1973**.

7. Moon J.K. and Shibamato T. Antioxidant Assays for Plant and Food Components. *J. Agric. Food Chem.*, 57(5): 1655-1666. **2009**.

8. Edeoga, et. Al. Phytochemical Constituents of Some Nigerian Medicinal Plants. *African J. of Biotech.*, 4(7): 685-688. **2005**.