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Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) profiling and phytochemical analysis of *Euphorbia hirta, Gliricidia sepium* and *Moringa oleifera* methanol extracts

Paolo Robert Bueno[1], Michael Russelle Alvarez[1*], Raymond Oliver Cruz[1,2], Richard Macapulay[1], Francis Jayson Vallesfin[1,3] and Francisco Heralde III[1]

[1]College of Medicine, University of the Philippines Manila, Manila City, Philippines
[2]College of Medicine, San Beda College Manila, Manila City, Philippines
[3]College of Arts and Science, University of Perpetual Help System Dalta-Molino Campus, Las Pinas City, Philippines

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ABSTRACT

A number of medicinal plants were studied for their phytochemical properties as possible chemotaxonomic markers. The present study aims to screen and quantify Euphorbia hirta, Moringa oleiferaand Gliricidia sepium leaf methanol extracts for phytochemical content, as well as produce TLC and HPLC profiles for standardization. TLC was carried out in silica gel plates using hexane:ethyl acetate:acetic acid (2:2:1) solvent system and then visualized under UV light and after sulfuric acid staining. HPLC was carried out using a C_{18} column and methanol:water:o-phosphoric acid (20:79.9:0.1) solvent system and detected by 210 nm UV-Vis spectroscopy. Phytochemical screening of the extracts showed the presence of tannins, flavonoids and phenols in all extracts, in addition to alkaloids and anthraquinones found only in E. hirta. Quantification of total alkaloids, flavonoids and phenolics showed that E. hirta had the highest alkaloid content (22.88±0.382 mg reserpine equivalents/g extract), phenolics content (682.8±7.26 mg gallic acid equivalents/g extract) and flavonoids content (229.4±8.61 mg quercetin equivalents/g extract). Moreover, TLC profiles showed 4 bands in the E. hirta extract, 2 bands in M. oleifera and 3 bands in the G. sepium extract. HPLC profiles showed two peaks in the E. hirta extract, 2 in the G. sepium extract and 3 in the M. oleiferaextract. The data presented here could be used for the standardization of methanol extracts of these plants, either for future studies or in herbal drug formulations.

Keywords: *Euphorbia hirta, Gliricidia sepium, Moringa oleifera,* TLC & HPLC profiling, phytochemical analysis

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INTRODUCTION

Due to the growing interest in herbal medications, standardization techniques such as phytochemical analysis and chromatographic techniques, *e.g.* thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC), have been developed to analyze phytochemical constituents of plant extracts and prevent adulteration **[1, 2, 3, 4]**.

E. hirta, traditionally known as *taua-taua*,is a slender stemmed, annual hairy herb from the Family Euphorbiaceae. Traditionally, it is used for treating asthma, chronic bronchial disorders, acute nasal catarrh,skin problems, gastrointestinal disorders, warts, cuts and infections **[5, 6].**

G. sepium, locally called *kakawate*, is a leguminous tree from the family Fabaceae. Traditionally, its leaves are used as mosquito repellent, antibacterial and antifungal agent **[7].**The leaves are also used as poultice on skin erysipelas, bruises and sores, while its bark decoction is used against protozoal diseases **[8].**

M. oleifera, or *malunggay* in Filipino, is a native of the Indian continent but has now been cultivated in tropical and subtropical countries. Almost every part of this tree are used traditionally: its leaves, flowers and fresh pods as foodstuff, while the rest are used as livestock feed **[9].** Moreover, its roots are traditionally applied as plaster to reduce swelling and rheumatism **[10].**

The use of the plants described here in ethnomedicine, as well as their potentials as herbal drug formulations, necessitates the standardization of their extracts. Thus, the present study aims to standardize methanolic extracts of *E. hirta, G. sepium* and *M. oleifera* using phytochemical screening and quantification, as well as with TLC and HPLC profiling.

MATERIALS AND METHODS

Reagents

The reagents and solvents used for the extraction, phytochemical analyses and TLC profiling were analytical grade reagents from Sigma-Aldrich (Philippines). On the other hand, the solvents used for the HPLC profiling were HPLC-grade from Merck (Philippines).

Plant sample procurement and preparation

M. oleifera leaves were collectedfrom Cavite, Philippines, *E. hirta* leavesfrom Manila City, Philippines and *G. sepium* leaves from Isabela, Philippines. Voucher samples were kept at and authenticated by the Botany Division, National Museum, Manila City, Philippines. These were air-dried for a week and then powdered using a homogenizer.

Plant extraction

Extraction was performed via maceration. Plant samples were soaked in methanol at ratio of 10 mL methanol/g plant sample. These were soaked for 48 hours and then filtered, keeping the filtrate. Solvent was removed by rotary evaporation at 37°C until dry. Extracts were finally stored in 4°C until further use.

Phytochemical screening

The extracts were screened for tannins, alkaloids, flavonoids, anthraquinones and phenolics based on the protocols by Bhandary *et al.* (2012)**[11]**. Extracts were reconstituted in methanol at 1 mg/mL and then assayed put into separate reactions, with the components listed in **Table 1**.

Table 1. Phytochemical screening assays, reagents and positive results

Phytochemicals tested	Name of Test	Active reagent	Positive result
Tannins	Ferric chloride test	10% Ferric chloride	Blue-black solution
Alkaloids	Wagner's test	Wagner's reagent	Reddish brown precipitate
Flavonoids	Alkaline reagent test	Sodium Hydroxide	Intense yellow solution
Anthraquinones	Modified Borntrager test	Potassium hydroxide, hydrogen peroxide	Pink solution
Phenolics	Ferric chloride test	5% Ferric chloride	Blue-black solution

Total phenolic content

A volume of 15.4 µL of crude extracts and gallic acid standard (at different concentrations, for the standard curve) were mixed with 61.5 µL of Folin Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 123µL of 7.5% sodium carbonate. The mixtures were allowed to stand at room temperature for 30 min. The absorbance was measured at 765 nm using a FluoStar fluorometer (BMG-LabTech, Philippines) and total phenolic content was calculated in terms of gallic acid equivalents (GAE).

Total flavonoid content

One hundred microliter (100µL) of crude extracts and quercetin standard (at different concentrations, for the standard curve) were mixed with 100 µL of 2% AlCl3. The mixtures were allowed to stand at room temperature for 10 min with intermittent shaking. The absorbance was measured at 415 nm using a FluoStar fluorometer (BMG-LabTech, Philippines) and total flavonoid content was calculated in terms of quercetin equivalents (QE).

Total alkaloid content

Two hundred microliter (200µL) of reserpine standard and crude extracts at different concentrations were mixed with 100 µL of FeCl3 solution and 100 µL of 1,10-phenanthroline solution. The reaction mixture was diluted to 1 mL volume using deionized water. The mixtures were placed in a water bath at 70oC for 30 min. The absorbance was measured at 510 nm using a FluoStar fluorometer (BMG-LabTech, Philippines) and total alkaloid content was calculated in terms of reserpine equivalents (RE).

Thin layer chromatography profiling

Extracts were spotted on thin-layer silica gel plates G60 (Merck, Philippines) and then developed using hexane:ethyl acetate:acetic acid (2:2:1) solvent system. After drying, the plates were visualized using long – wave ultraviolet light, as well as in visible light after staining with 5% sulfuric acid in methanol. Retention factor (R_f) values were calculated for each band observed.

High-performance liquid chromatography profiling

High-performance liquid chromatography was performed on crude extracts only. The HPLC system used was SPD-10AVP/10AVVP (Shimadzu, Philippines). These were carried out using a C_{18} column as the stationary phase and methanol:water:ortho-phosphoric acid (20:79.9:0.1) as the mobile phase, at a flow rate of 1 mL/minute and pressure of 139 kgF/cm2 and temperature 40°C. Elutions were visualized by UV-Vis detection at 210 nm to detect phenolic compounds**[12]**.

RESULTS and DISCUSSION

Table 2. Phytochemical screening results. Legend: '+' = present, '-' = not detected

Sample	Tannins	Alkaloids	Flavonoids	Anthraquinones	Phenols
Euphorbia hirta	+	+	+	+	+
Gliricidia sepium	+	-	+	-	+
Moringa oleifera	+	-	+	-	+

Table 3. Total phenols, flavonoids and alkaloids quantification results. *E. hirta* contained the most phenols, flavonoids and alkaloids of the three extracts

Sample	Total Phenols content (mg GAE/g extract)	Total Flavonoid content (mg QE/g extract)	Total Alkaloid content (mg RE/g extract)
Euphorbia hirta	682.8±7.26	229.4±8.61	22.88±0.382
Gliricidia sepium	247.1±18.0	19.15±2.16	2.612±0.0382
Moringa oleifera	277.2±1.00	33.35±0.601	0.5766±0.0253

Table 4. TLC profiling results. The number of bands produced when visualized under UV light and after sulfuric acid staining were counted and the R_f values determined

Sample	Number of bands	R_f values
Euphorbia hirta	4	0.123, 0.215, 0.769, 0.923
Gliricidia sepium	3	0.123, 0.708, 0.831
Moringa oleifera	2	0.123, 0.938

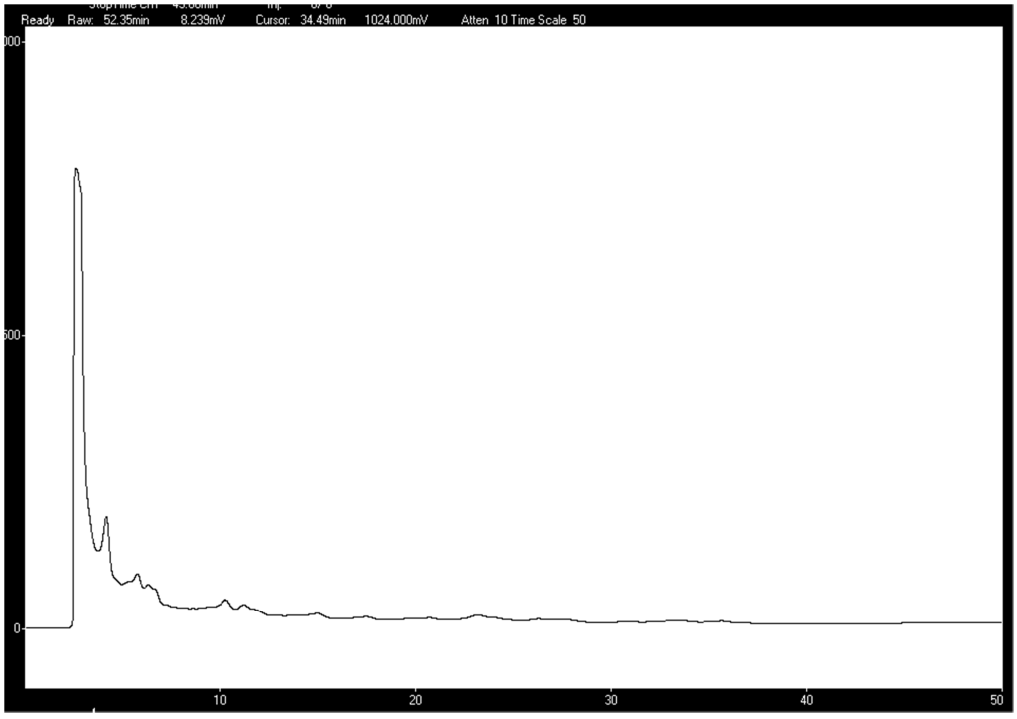

Figure 1. HPLC profile of *E. hirta* methanolic extract. Two prominent peaks can be observed and several other peaks can be observed as well

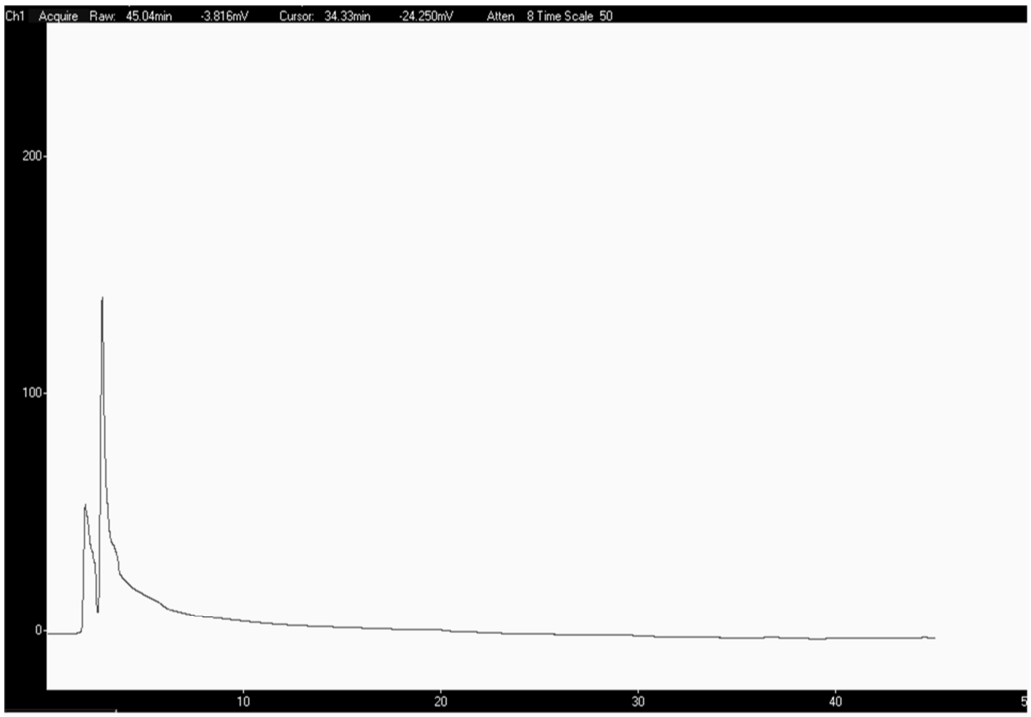

Figure 2. HPLC profile of *G. sepium* methanolic extract. Two prominent peaks can be observed

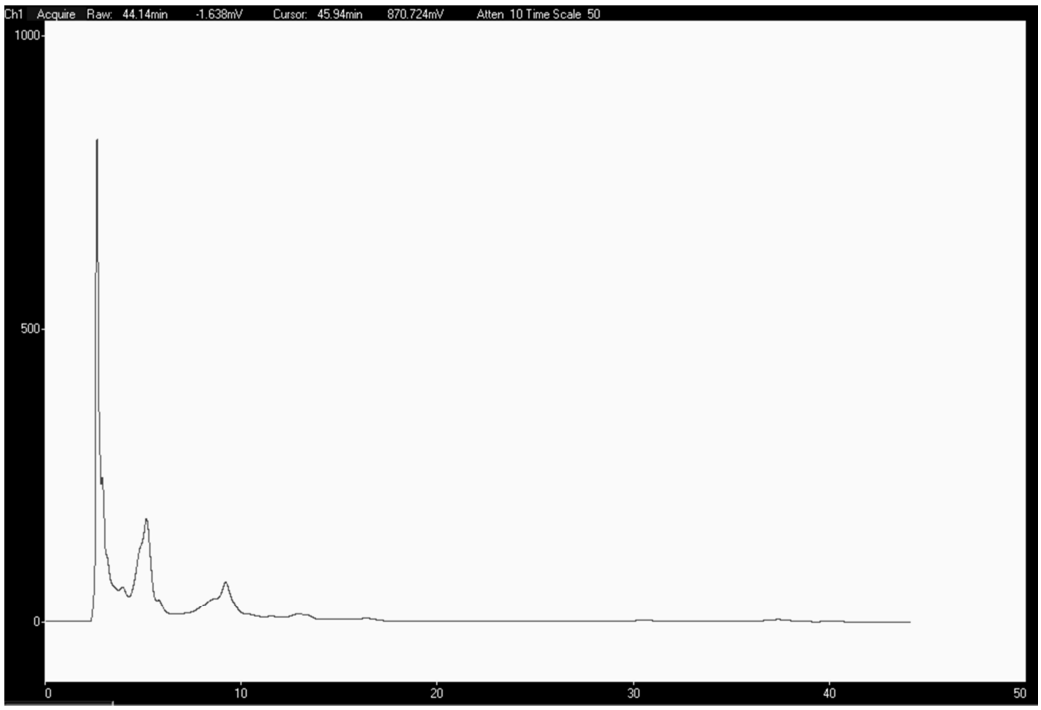

Figure 3. HPLC profile of *M. oleifera* methanolic extract. Three prominent peaks can be observed and several smaller peaks can be observed as well

E. hirta contained the highest amounts of phenolics, flavonoids and alkaloids (**Table 3**). Moreover, it was the only one that contained anthraquinones and alkaloids (**Table 2**). It also had the highest number of peaks in the HPLC profile (**Figure 1**) and bands in the TLC profile (**Table 4**), implying high amount of chemically varied phytochemicals. In recent literature, *E. hirta* extracts have been shown to possess antioxidant activity **[3, 13]**,antimicrobial activity and cytotoxic activity **[6]**, and α-glucosidase inhibition activity **[14].** The high phenolic and flavonoid content of the extract could be correlated to the reported antioxidant activity of *E hirta* in recent literature, since high phenolic and flavonoid content have been previously correlated to high antioxidant and α-

glucosidase activity **[14, 15, 16, 17, 18]**. Additionally, alkaloids have been shown to be responsible for the antimicrobial activities in plants **[19, 20]**.

On the other hand, *G. sepium* contained moderate amount of phenolics and flavonoids and negligible amounts of alkaloids (**Table 3**). In fact, according to the screening test, anthraquinones and alkaloids were not detected (**Table 2**). Although two peaks were observed in the HPLC profile (**Figure 2**), only three bands were observed in the TLC profile (**Table 4**). This could have been due to the difference in the two chromatographic systems and solvent systems used. Antibacterial, larvicidal and antioxidant activities of *G. sepium* leaf extract have been reported previously **[7, 21, 22]**. Since minimal alkaloid content were detected (in this study and in the reported studies as well), other phytochemicals may have been the cause of its activities, such as the phenolics and flavonoids **[23, 24, 25].** Likewise with the *E. hirta* extract, the moderately high phenolic and flavonoid content could have been the reason for *G. sepium*'s antioxidant activity **[14, 15, 16, 17, 18]**.

M. oleifera also contained moderate amounts of phenolics and flavonoids and negligible amounts of alkaloids (**Table 3**). Just like with the *G. sepium* extract, only tannins, flavonoids and phenolics were detected (**Table 2**). Only two bands were observed in the TLC profile (**Table 4**), but three prominent peaks can be observed in the HPLC profile (**Figure 3**). This could be explained by the different systems used in both chromatographic techniques. In recent literature, the antibacterial activity **[9, 26]** and antioxidant activity **[26]**of *M. oleifera* have been reported. Likewise with the two extracts, the phenolics and flavonoid content of *M. oleifera* could explain the extract's antioxidant activity **[14, 15, 16, 17, 18]**. Its potential as a functional food, by evaluating the mineral, vitamin and amino acid content of the leaves have been reported as well **[9, 26]**.

CONCLUSION

Due to the growing interest in herbal drug formulations, standardization of plant extracts using chromatographic techniques and phytochemical quantification have been developed. *E. hirta*, *G. sepium* and *M. oleifera* have been recently reported to possess various pharmacologic activities, as well as nutritional content. The data presented here could be helpful in standardizing extracts of these plants.

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