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Standardization of *Euphorbia hirta* with Chemical Compounds Identification (GC-MS)

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Abstract

The present study was intended to evaluate the various pharmacognostical procedures in the leaves of *E. hirta,* (Euphorbiaceae). The various pharmacognostical parameters were carried out as per WHO guidelines procedure i.e., macroscopic and microscopic evaluation, heavy metal analysis, functional group analysis by Fourier Transform Infrared (FTIR) spectroscopy and assay. The study was extended with analyzing the chemical compounds identification in the extract of *E. hirta* by using GC-MS. The assay part of standardization involved determination of the antioxidant activity (DPPH assay) which could help assesses the chemical effects and establishes curative values. The extracts showed an antioxidant activity above 50%, independent of the extraction time. Six major peaks in the range of 600 - 1500 and 2800 - 3400 cm^{-1} were observed in the FTIR spectra. The chemical constituent aspect of standardization involves quantification of the main chemical components in *E. hirta*. The GC-MS method used for quantification of 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid in the extract was rapid, accurate, precise, linear (R^2 = 0.9981), rugged and robust. The concentrations of heavy metals determined in *E. hirta* extract are well below the permissible limit. The result of the pharmacognostical standardization of this plant serves as a reference piece and helps in future identification and authentication of this plant specimen.

Key words: *E. hirta*; FTIR fingerprints; heavy metals, microscopical; macroscopical standardization.

Introduction

Studies on natural products have been gaining importance with the realization that plants provide a source of useful chemicals that may be used directly or as templates for the development of drugs useful for defense or protection against various diseases. In consideration for the commercialization of formulations based on medicinal plants, quality control standards of various medicinal plants used in traditional medicine are becoming more important. The standardization of extracts is of great importance in order to guarantee their quality. Standardization as defined by American Herbal Products Associations refers to the body of information and controls necessary to produce materials of reasonable consistency. This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing process [1]. Quality control is crucial to ensure the safety and correct handling of herbal medicines. There have been numerous reports on the toxicity, the misidentification and substitution of plant species. Herbal products have been reported to contain heavy metals and synthetic remedies or non-prescription drugs [2]. Hence the current study was carried out for the standardization of *E. hirta* with respect to authenticity, assay and chemical constituent analysis including heavy metals content.

Euphorbia hirta L., belongs to the family Euphorbiaceae. It comprises about 300 genera and 5,000 species, common to tropical countries. *E. hirta* is a medicinal plant used for treating asthma, chronic bronchial disorders, acute nasal catarrh, hay fever and emphysema [3]. The sedative, anxiolytic, analgesic, antipyretic and anti-inflammatory properties of *E. hirta* have been reported in the literature [4]. Furthermore, studies revealed that *E. hirta* posses galactogenic, anti-anaphylactic, antimicrobial, antioxidant, anticancer, antifeedant, anti-platelet aggregation and anti-inflammatory, aflatoxin inhibition, antifertility, anthelmintic, antiplasmodial, antiamoebic, antimalarial, and larvicidal activities [5]. The plant is collected from the wild sources and varies in constituents and efficacy due to its geographical diversity. Improper collection and storage condition lead to the deterioration of the raw material. Keeping in view the above mentioned problems, it was important to standardize the crude extracts of *E. hirta* to establish a quality and identity profile of the plant for the purpose of overall quality assurance of this

medically important plant. Since there are no reports in the literature regarding the standardization of *E. hirta* crude extracts, in the present investigation an attempt has been made to standardize them by using macroscopic and microscopic characteristics, authenticity, biological activity assay and chemical constituent analysis.

Materials and Methods

Chemicals and reagents

All the chemicals and reagents used were of analytical grade, purchased from Sigma Chemical Co. (St Louis, MO, USA) and Merck (Darmstadt, Germany).

Plant sample

Sample of *E. hirta* was collected in January 2011, from Universiti Sains Malaysia campus, Pulau Pinang, Malaysia and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia where a sample (voucher number 11215) has been deposited.

Preparation of the plant extract

A hundred grams of all plant part powder was extracted by maceration in 400 ml of methanol for 4 days with frequent agitation. The mixture was filtered through clean muslin cloth followed by double filtration with Whatman No. 1 filter paper and the filtrate was concentrated by rotary evaporator with vacuum at 50°C, poured in glass Petri dishes and brought to dryness at 60°C oven. The percentage yield of the crude extract was determined for each part and was 17.1%. The obtained paste like mass was then stored in parafilm sealed Petri-dishes in dark cabinet. The extracts were reconstituted by dissolving in methanol to the required concentrations. The reconstituted extracts were maintained at 2- 8°C.

Qualitative investigation

The macroscopic features of the fresh leaves, stem and roots were determined using the methods of Evans [6]. Anatomical sections, a surface preparation of the fresh leaves, stem and roots sample for the microscopy was carried out according to methods outlined by Brain and Turner [7]. Morphological examination of the *E. hirta* leaf specimen was used to identify the leaf structures. Leaf tissue was fixed in 10% buffered formalin. After fixation, the tissue was dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin wax. Multiple 5 mm sections from the block were mounted on slides and stained with 0.5% methylene blue and examined under a light microscope for identification of leaf structure.

Fourier transforms infrared (FTIR) analytical methods

Infrared spectra were recorded on a Shimadzu FTIR Spectrometer 8000 series, between 4,000–500 cm^{-1}. All determinations were performed in triplicate. For liquid samples, a drop of methanol extracts of *E. hirta* was placed between two plates of KRs-5. The drop forms a thin film between the plates. KRs-5 (TlBr-TlI) is a gorgeous red crystal commonly used for attenuated total reflection prisms for IR spectroscopy. It is also used as an infrared transmission window in gas and liquid sample cells used with FTIR spectrophotometers in place of Potassium Bromide (KBr) or Cesium Iodide (CsI) for analysis of aqueous samples that would attack KBr or CsI optics. It has a wide transmission range and is virtually insoluble in water (8).

Gas chromatography–mass spectrometry (GC–MS) analysis

The GC/MS analysis was done on a thermo gas chromatograph - mass spectrometer (model Shimadzu 2010) equipped with DB-5 capillary column (30 m long. 0.25 mm i.d., film thickness 0.25 μm). The column temperature program was 60 °C for 0 min, with 10°C per min increases to 270 °C; which was maintained for 30 min. The carrier gas was helium at a flow rate of 1 mL/min with split 10:1. The detector and injector temperatures were both maintained at 270 °C. The quadrupole mass spectrometer was scanned over the range 28–400 amu at 1 scan s-1, with an ionising voltage of 70 eV, an ionisation current of 150 Ma and an ion source temperature of 200 °C [15]. In order to determine the Kovats index of the components, a mixture of alkenes (C9–C24) was added to the crude extract before injecting in the GC–MS equipment and analysed under the same conditions as above. The compounds were identified by computer searches in the commercial libraries of the National Institute of Standard and Technology (NIST) and by their Kovats retention indexes [9].

Heavy Metal Analysis

Atomic absorption spectrometry (AAS) is used for the determination of the amount or concentration of specific heavy metals. AAS uses the phenomenon that atoms in the ground state absorb light of a specific wavelength, characteristic of the particular atom, when the light passes through an atomic vapour layer of the element to be determined [10]. The determination of Pb, Cd, Ni and Zn was performed on a Perkin-Elmer 200 atomic absorption spectrophotometer under optimized measurement conditions using hollow cathode lamps. The signals with background correction (deuterium lamp) were measured at optimal flame heights.

DPPH Assay

2,2-Diphenyl-1-picylhydrazyl radical (DPPH) scavenging assay quantitative measurement of radical scavenging properties was carried out in universal bottles. The reaction mixture contained test sample (50 μl) ranging in concentration from 0.5 mg/mL to 6 mg/mL and 0.004% (w/v) DPPH solution in methanol (5mL, 80%

(v/v)). The mixture without test sample was used as black and spiked with 50 ul of black methanol. The commercial antioxidant butylated hydroxytoluene (BHT, Sigma) was used for comparison or as a positive control. Discoloration was measured at 517 nm after baing incubated for 30 min. Measurements were taken in triplicates. DPPH radical's concentration was calculated using the following equation: DPPH scavenging effect (%) = $[(A_0-A1)/A_0] \times 100$, where A_0 is control absorbance and A_1 is the absorbance in the presence of the sample (extract of the *E. hirta*) [9]. The actual decrease in the absorbance induced by the test sample was compared with the positive controls (Butylated hydroxytoluene, BHT). Consistency in the DPPH radical scavenging activity (%) for every 3 months was studied. All the assays were carried out in triplicate. The data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey post test with $\alpha = 0.05$. SPSS version 12 programmer was used for the statistical analysis.

Results and Discussion

Herbarium

Since, plant classifications are constantly changing, the verification of the authenticity of the specimens using the herbarium is becoming an increasingly important step. Voucher specimens help cross-reference these changes to previous plant materials. Hence, in this study an *E. hirta* herbarium was prepared (Fig. 1). *E. hirta* is a recognized source of useful chemicals for the pharmaceutical industries, but authenticity of specimens is important for these industries to avoid the acceptance of wrong plant materials for extracting actives, potentially resulting in a loss of billions of dollars. Therefore, the correctly identified herbarium specimens can be used for comparison of future *E. hirta* collections. In addition, using a herbarium specimen will allow the study of morphological or anatomical details that will help identify field collected plant specimens in the sterile or non-reproductive condition.

Macroscopy and Microscopy

Most of the regulatory guidelines and pharmacopoeias suggest macroscopic and microscopic evaluation of the botanical materials for standardization [11]. Thereby, in this study a macroscopic and microscopic evaluation of the leaf, stem and root of *E. hirta* were done. The leaves stem and roots of *E. hirta* were subjected to macroscopical examination and observations were recorded. The proper examination of the leaves, stem and roots were carried out under sunlight and artificial light sources similar to daylight. The leaves of *E. hirta* were observed to be green darker on the upper surface, opposite leaves, elliptical, oblong or oblong-lanceolate, with a faintly toothed margin. A histology photomicrograph of the identifying features of the leaf is shown in Fig. 2a. Detailed Transverse section

a. TS Leaf, b. TS Root, c. TS Stem [bs, bundle sheath; ck, cork; ct, cortex; cu, cuticle; e, epidermis; pal, palisade layer; pf, pericyclic fibre; ph, phloem; sm, spongy mesophyll; v, vessel; vb, vascular bundle; xy, xylem].

of leaf passing through midrib is deeply and broadly ridged on lower side as compared to upper side with a centrally located meristele and lamina is dorsiventral. Transverse section of the leaf of *E. hirta* through the midrib shows single layered upper and lower epidermis covered with thin cuticle. Upper epidermal cells are polygonal and show paracytic to anomocytic type of stomata, while lower cells are sinuous and contain anomocytic type of stomata. Cells of epidermal cells adjacent to veins are somewhat rectangular and arranged in rows. Centrally located meristele is collateral and surrounded by parenchymatous bundle sheath. Remaining mesophyll tissue is parenchymatous. TS of lamina show 2-3 layers of palisade cells interrupted with collateral vascular bundles. Each vascular bundle surrounded by very large bulbous parenchymatous cells. Transverse section of the root of *E. hirta* shows (Fig. 2b) a wide cortex, a narrow portion of phloem and a wide xylem. Phloem is very narrow and consists of sieve tubes, companion cells and parenchyma. Major portion of root is occupied by xylem, which consists of vessels, tracheids, fibres and xylem parenchyma. Transverse section of the stem of *E. hirta* (Fig. 2c) is almost circular in outline and covered with trichomes. It consists of cortex, groups of pericyclic fibres, a ring of phlem, and xylem encircling pith. Pith is parenchymatous; cells towards centre are larger with intercellular spaces.

FTIR Fingerprinting

The use of FTIR fingerprinting for herbal extract tends to focus on identification and assessment of the stability of the chemical constituents functional groups observed by FTIR analysis. The results of FTIR finger print for the methanolic extract of *E. hirta* is shown in Fig. 3. The results of functional group analysis using FTIR had demonstrated the existence of various functional groups in the *E. hirta* extract. This was verified by the peaks formed during the FTIR study (Figure 3). Six major peaks in the range of 600 - 1500 and 2800 - 3400 cm-1 were observed in the FTIR spectra. Therefore, for future *E. hirta* methanolic extraction, this FTIR spectrum can be used for comparison. Whereby, the further extraction should be standardized to these FTIR fingerprint. Obviously, the FTIR fingerprint can be used to ensure that the functional groups in the new extract are present in reproducible manner

although new extraction has been done. In this way, FTIR fingerprints give information that assist in manufacturing control and assure batch-to-batch consistency of the extraction of *E. hirta*. Moreover, infrared spectroscopy provides a spectral fingerprint that uniquely identifies chemical compounds or extract and their functional group. Fingerprinting is superior to other analytical methods because no two compounds or extract have the same infrared spectrum.

Chemical Constituent Standardization

Along with authentication of species identity and assay, chemical constituent standardization is also required for quality control in the use of plant materials for pharmaceutical purposes. In this study the *E. hirta* crude extract was identified as an extract containing no constituents documented as being determinant or relevant for efficacy, or as having any pharmacological or clinical relevance. In this case, chemically defined constituent (markers) without known therapeutic activity may be used for control purposes [12]. These markers may be used to monitor good manufacturing practice or as an indication for the content of the extract [12]. A standard curve was plotted using the area of the peak
obtained in the GC-MS chromatogram (Fig. 4) against the concentration of 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid (Fig. 5). The GC-MS profile and calibration curve of the compound is given in Figure 4 and 5. This could be applied for the standardization and validation of *E. hirta* crude extract in terms of 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid as a chemical marker. Chromatographic fingerprints and marker compounds are used as reference standards, which indicate the purity, identity, and quality of the herbal drug [13]. Upon GC–MS analysis, chemical composition of the main constituents from methanol extract were identified and shown in Table 1. Identification of the constituents was performed by comparing the recorded mass spectra with the standard mass spectra from the Wiley Library and the National Institute of Standards and Technology (NIST) MS spectral library. The main constituents were considered "identified", when their mass spectral fit values were at the default value of 90% or above. A total of four constituents were identified in the methanol extracts. The major constituents were identified as 2-Butanone, 3,3-dimethyl-1-(methylsulfonyl)-,O-[(methylamino)carbonyl]oxime(1),1,3,4,5 tetrahydroxycyclohexanecarboxylic acid (2), Hexadecanoic acid (3) and 9,12,15- Octadecatrienoic acid (4) with biological activity as shown in the Table 2.

There are at least two significant advantages for GC-MS, that is: (1) with the capillary column, GC–MS has in general very good separation ability, which can produce a chemical fingerprint of high quality; (2) with the coupled mass spectroscopy and the corresponding mass spectral database, the qualitative and relatively quantitative composition information of the herb investigated could be provided by GC–MS, which will be extremely useful for the further research for elucidating the relationship between chemical constituents in herbal medicine and its pharmacology in further research [17].

Heavy Metal Analysis

Another concern related to safety of herbals is presence of contaminants such as heavy metals. The *E. hirta* material was subjected to heavy metal analysis. The results of elemental contents in E. hirta extracts are presented in Table 3. According to WHO [18], the maximum amounts of lead and cadmium n dried plant materials are 10ppm and 0.3ppm respectively, which are based on the ADI values. The dietary limit of Zn is 100 ppm [19]. Zinc is an essential trace element form plant growth and also plays an important role in various cell processes including normal growth, brain development, behavioural response, bone formation and wound healing. EPA has recommended daily intake of Ni should be less than 1 mg beyond which is toxic [20]. The concentrations of heavy metals determined in *E. hirta* extract are well below the permissible limit.

Table 3. Heavy Metal concentration in *Euphorbia hirta* extract

Metal	Metal Concentration (ppm)
Pb	0.154 ± 0.0564
Cd	0.012 ± 0.0088
Ni	0.323 ± 0.0103
Zn	0.763 ± 0.0029

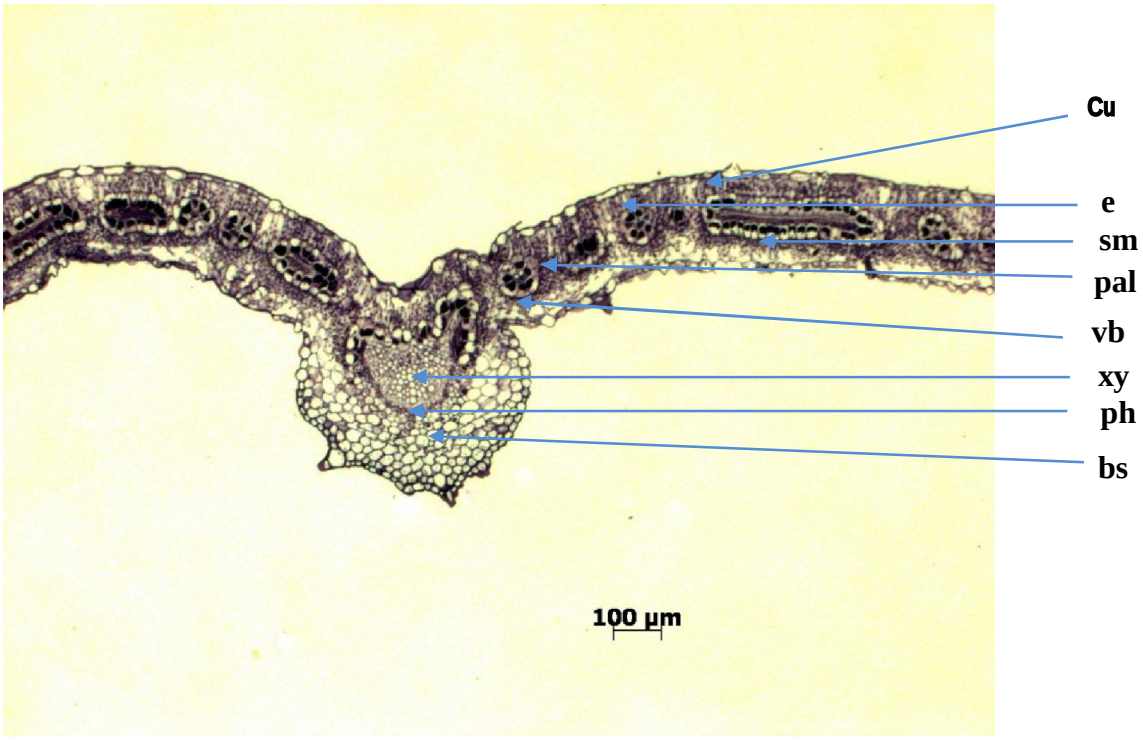
(n=3; values are mean ± S.D.)

The DPPH Radicals Scavenging Activity

Consistency in biological activity is an essential requirement for the effective use of therapeutic agents. It ensures that the extract remains with its therapeutic parameters throughout the shelf life assigned to the extract. Therefore it is crucial to carry out research that confirms that the extract retains its efficacy and biological activity before it is consumed by the pharmaceutical industries. Hence in this study, the antioxidant activity by determining the DPPH radical scavenging value was used for this purpose. DPPH assay is one of the most extensively used antioxidant assay for plant samples. The DPPH assay provides information on the reactivity of the test compounds with stable free radicals. The DPPH method is described as a simple, rapid and convenient method independent of sample polarity for screening of many samples for radical scavenging activity [21]. These advantages make the DPPH method interesting for testing natural source like *E. hirta.* This method is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 517 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour. According to the below analyses, the extract is pharmacologically active and may function as free radical scavenger and metal chelator to finally prevent the generation of free radicals and inhibit the oxidative damages. Table 4 shows that there is a general consistency without statistical significance (always above 50%) in
the DPPH radical scavenging activity for every 3 months. The antioxidant property of *E. hirta* is suspected to be associated with

Fig. 1. The *Euphorbia hirta* herbarium.

a



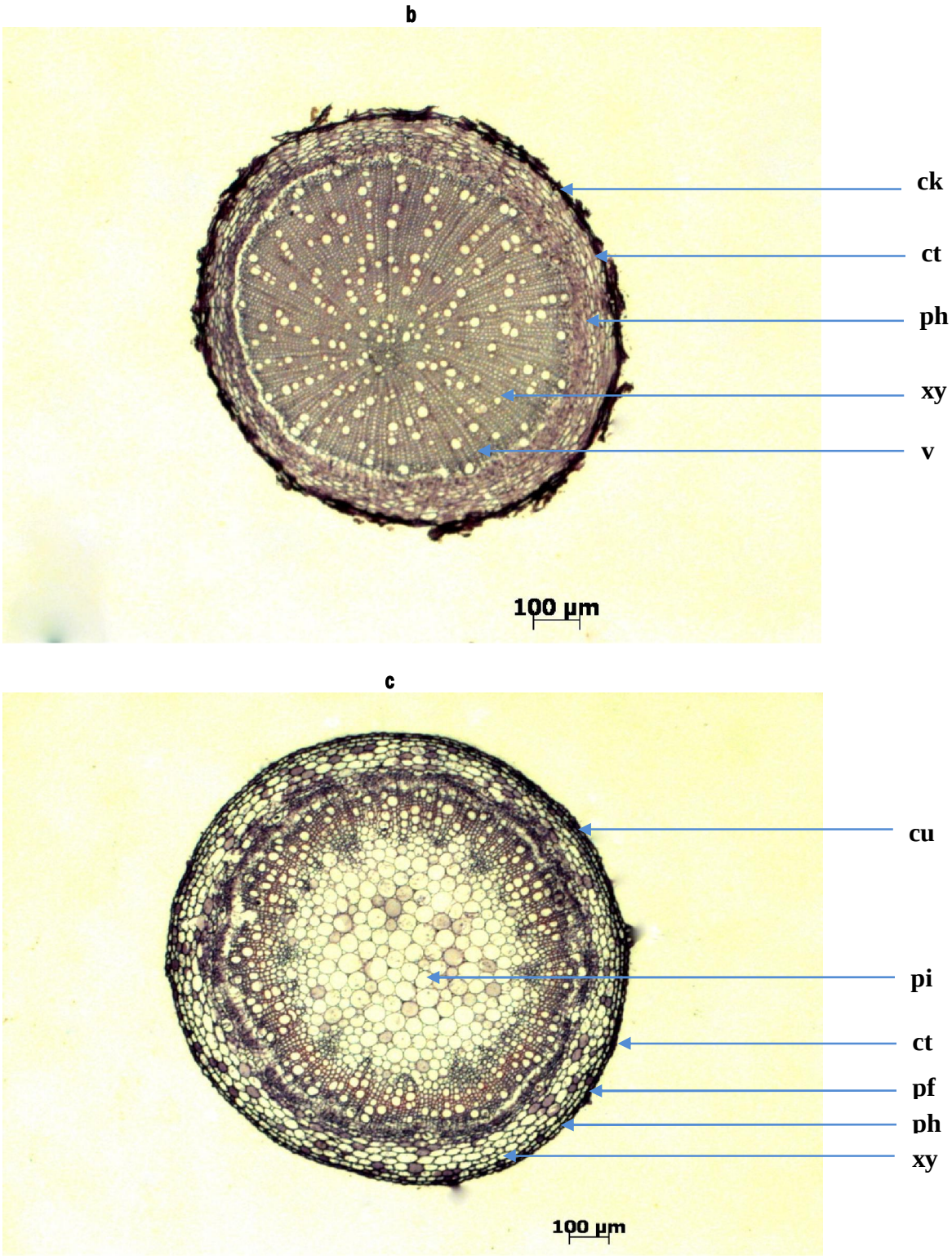


Fig. 2. Microscopy of *Euphorbia hirta*: a. TS Leaf, b. TS Root, c. TS Stem [bs, bundle sheath; ck, cork; ct, cortex; cu, cuticle; e, epidermis; pal, palisade layer; pf, pericyclic fibre; ph, phloem; sm, spongy mesophyll; v, vessel; vb, vascular bundle; xy, xylem].

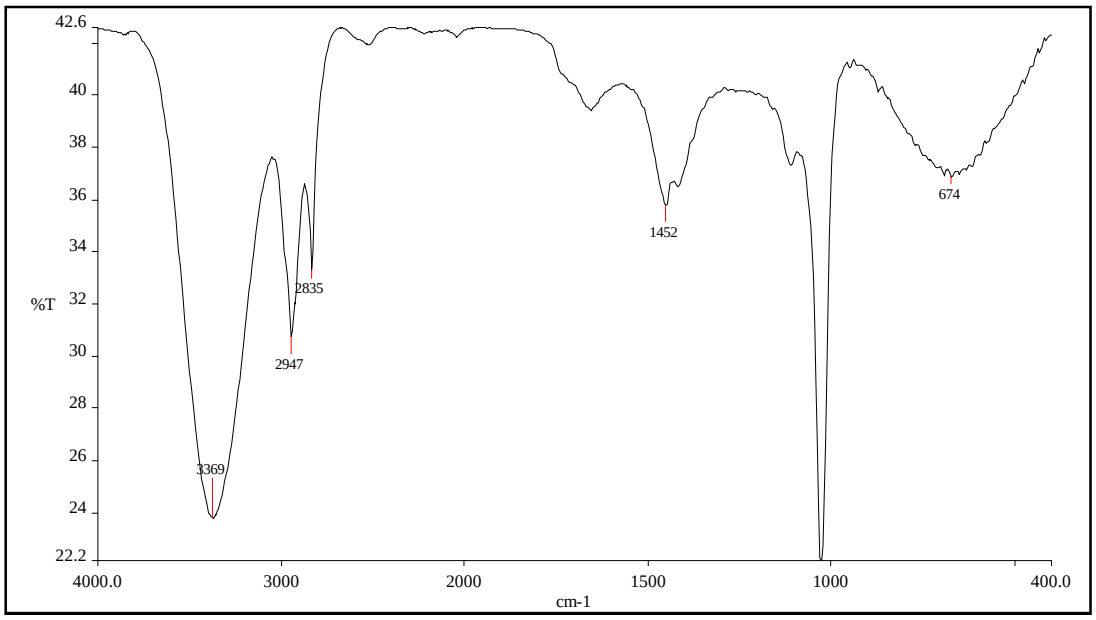


Fig. 3. FTIR spectrum of methanolic extract of *Euphorbia hirta*

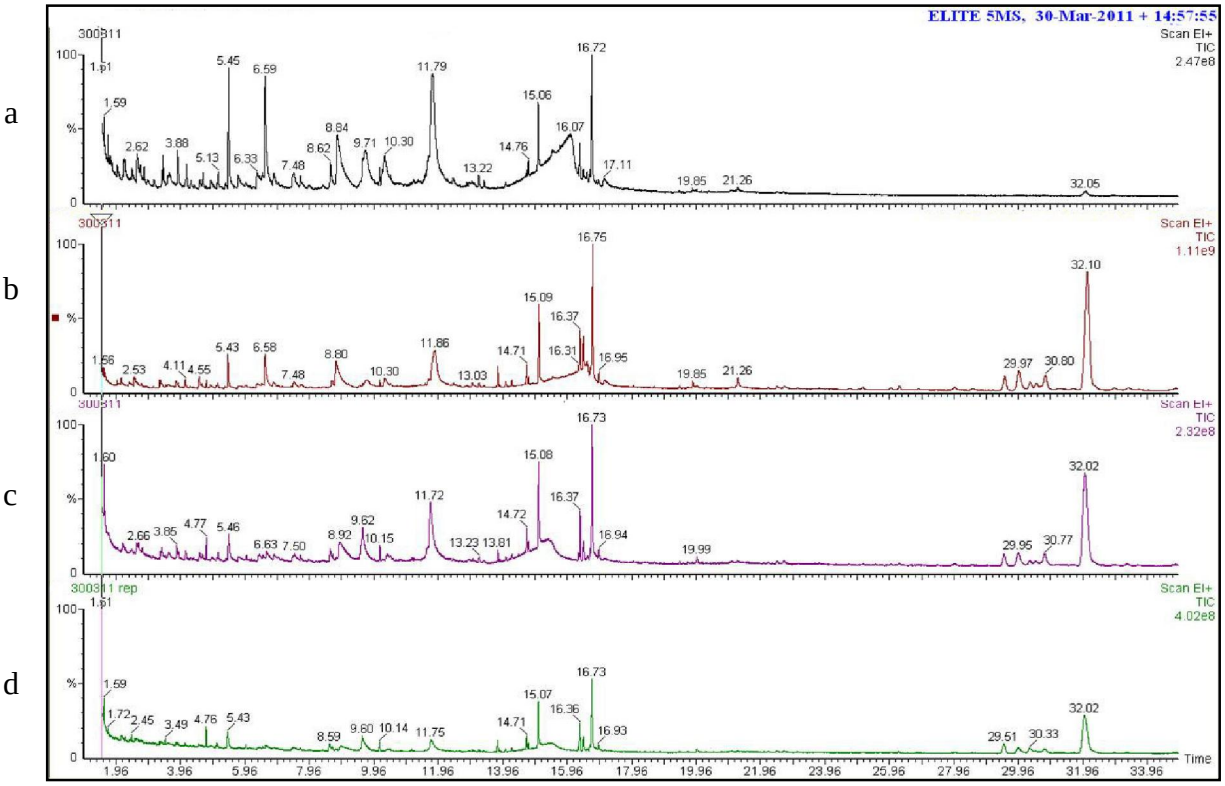


Fig. 4. GC-MS profile of 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid at retention time 11.7 (a=20mg/mL, b=15mg/mL, c=10mg/mL, d=5mg/mL, concentration of the extract)

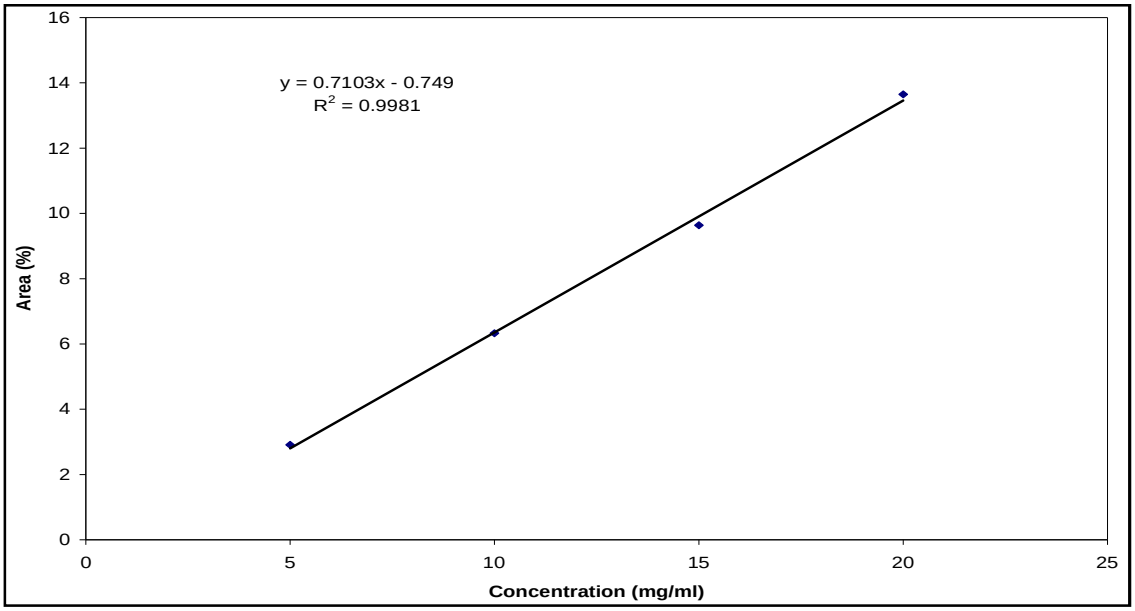


Fig. 5. Calibration curve of 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid by GC-MS.

Table1. The main compounds identified in methanol extract of *E. hirta* crude extract

Retention time (min)	Compound	Molecular formula
1.587-1.599	2-Butanone, 3,3-dimethyl-1-(methylsulfonyl)-, O-[(methylamino)carbonyl]oxime (1)	$C_9H_{18}N_2O_4S$
11.715-11.863	1,3,4,5-tetrahydroxycyclohexanecarboxylic acid (2)	$C_7H_{12}O_6$
15.063-15.088	Hexadecanoic acid (3)	$C_{16}H_{32}O_2$
16.725-16.754	9, 12, 15-Octadecatrienoic acid (4)	$C_{18}H_{30}O_2$

Table 2. Biological activity of major phyto-components identified in *E. hirta* crude extract by GC-MS

Compound	Activity
2-Butanone, 3,3-dimethyl-1-(methylsulfonyl)-, O-[(methylamino)carbonyl]oxime (1) (Thiofanox sulfone)	An insecticide (14).
1,3,4,5-tetrahydroxycyclohexanecarboxylic (Quinic acid)	Astringent, antioxidant (15).
Hexadecanoic acid (3) (Palmitic acid)	Antioxidant, hypocholesterolemic nematicide, pesticide, anti androgenic flavor, hemolytic, 5-Alpha reductase inhibitor (16).
9, 12, 15-Octadecatrienoic acid (4) (a-linolenic acid)	Antiinflammatory, hypocholesterolemic cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic antieczemic, antiacne, 5-Alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary (16).

Table 4. DPPH antioxidant activity of 1mg/mL *Euphorbia hirta* extracts within duration of 3 months

Duration	DPPH radical scavenging activity (%)	
	Extract	BHT
January 2011	52.23 ± 2.21	74.53 ± 2.31
March 2011	53.31 ± 3.24	75.41 ± 3.74
June 2011	52.98 ± 2.75	74.2 8 ± 3.14

(P>0.05; n=3; values are mean ± S.D.)

the 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid, which was detected by the GC–MS analysis in this study (Table 1&2, Figure 4&5). Pero et al. (15) reported the antioxidant activity property of 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid further confirmed this statement.

Conclusion

The present study of *E. hirta* can be concluded that the pharmacognostical standardization and yielded a set of qualitative and quantitative parameters or standards that can serve as an important source of information to as certain the identity and to determine the quality and purity of the plant material in future studies for the first-rate pharmacological activity.

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References

[1]. Gaedcke F, Steinhoff B and Blasius H. (2003) Herbal Medicinal Products: Scientific and Regulatory Basis for Development, Quality Assurance and Marketing Authorisation. Wissenschaftfliche, Stuttgart, CRC Press, Boca Raton, USA. pp. 37–52.

[2]. Razmovski-Naumovski V, Tongkao-on W, Kimble B, Qiao V.L, Beilun L, Li K.M, Roufogalis B, Depo Y, Meicun Y and Li QG.. Multiple Chromatographic and Chemometric Methods for Quality Standardisation of Chinese Herbal Medicines. World Sci. Technol. 2010; 12: 99-106.

[3]. de Padua LS, Bunyapraphatsara N and Lemmens RHMJ. (1999) Plant Resources of South-East Asia No.12(1), Medicinal and poisonous plants 1, Prosea Foundation, Bogor, Indonesia. 268- 326.

[4]. Lanhers MC, Fleurentin J, Dorfman P, Mortier F and Pelt JM. Analgesic, antipyretic and anti-inflammatory properties of Euphorbia hirta. Planta Med.1991; 571: 225-231.

[5]. Anonymous. (2008) Euphorbiahirta L, Available at: http://florabase.calm.wa.gov.au/browse/profile/4629. Access on 31 May 2010.

[6]. Evans WC. (1996) Trease and Evans' Pharmacognosy. 14th ed., W.B. Saunders Company Limited, London, UK. 545–546.

[7]. Brain KR and Turner TD. (1975) The practical evaluation of phytopharmaceuticals. Wright Sciencetechnica, Bristol, UK. 81–82.

[8]. Torey A, Sasidharan S, Latha LY, Sudhakaran S and Ramanathan S. Antioxidant activity and total phenolic content of methanol extracts of Ixora coccinea. Pharma. Biol. 2010; 48: 1119-1123.

[9]. Torey A, Sasidharan S, Yeng C and Yoga Latha L. Standardization of Cassia spectabilis with respect to authenticity, assay and chemical constituent analysis. Molecules. 2010; 15: 3411-3420.

[10]. Ghaedi M, Niknam K, Shokrollahi A, Niknam E, Rajabi HR and Soylak M. Flame atomic absorption spectrometric determination of trace amounts of heavy metal ions after solid phase extraction using modified sodium dodecyl sulfate coated on alumina. J. Hazard. Mater. 2008; 155: 121-127.

[11]. WHO. (1998) Quality Control Methods for Medicinal Plants Materials, Document No. WHO/PHARMA/92.559/rev.1, World Health Organization, Geneva, Switzerland.

[12]. Yadav NP, Thakur M and Dixit VK. (2009) Standardization of herbal/Ayurvedic formulations. In Recent Progress in Medicinal Plants. Vol. 24, Govil, J.N. Singh V.K. (Eds.). Studium Press LLC, Houston, Texas, USA. 25–26.

[13]. Lazarowych NJ and Pekos P. Use of fingerprinting and marker compounds for identification and standardization of botanical drugs: Strategies for applying pharmaceutical HPLC analysis to herbal products. Drug Info. J. 1998; 32: 497–512.

[14]. Chin BH, Tallant MJ, Duane WC and Sullivan LJ. Metabolism of carbamate insecticide thiofanox in

rats. J. Agric. Food Chem. 1980; 28: 1085-1090.

[15]. Pero RW, Lund H and Leanderson T. Antioxidant Metabolism Induced by Quinic Acid. Increased Urinary Excretion of Tryptophan and Nicotinamide. Phytother. Res. 2009; 23: 335–346.

[16]. Praveen Kumar P, Kumaravel S and Lalitha C. Screening of antioxidant activity, total phenolics and GC-MS study of Vitex negundo. Afr. J. Biochem. Res. 2010; 4: 191-195.

[17]. Liang YZ, Xieb P and Chan K. Quality control of herbal medicines. J. Chromatogr. B. 2004; 812: 53–70.

[18]. WHO. (1997) WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues, World Health Organization, Geneva, Switzerland.

[19]. Jones JW. (1998) In Quantitative Trace Analysis of Biological Materials. Chapter 20, McKenzie, H.A., Smythe, L.E. (Eds.). Elsevier Science, New York City, NY, USA.

[20]. McGrath SP and Smith S. (1990) Chromium and Nickel in heavy metals in soils. In Alloway B.J. (ed.). Blackie, Glasgow, UK. 125.

[21]. Koleva II, van Beek TA, Linssen JPH, de Groot A and Evstatieva LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Analysis. 2001; 13: 8-17.