

Pharmacognostical, Phytochemical, Antioxidant and Antimicrobial Activity of *Hedyotis hedyotide*

Mukesh S. Sikarwar*, Loh Chye Wei, Neeraj Paliwal

Department of Pharmacy, AIMST University, Malaysia

*Corresponding author: Mukesh S. Sikarwar, Associate Professor, Pharmaceutical Chemistry Unit, Faculty of Pharmacy, AIMST University, Bedong, Malaysia. Email: mukeshsikarwar@gmail.com

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Abstract

This research was conducted to study the pharmacognostical, phytochemical, antioxidant and antimicrobial activity of *Hedyotis hedyotide* leaf extract.

Macroscopy, microscopy, phytochemical analysis, thin layer chromatography, antioxidant and antimicrobial activity were carried out. Total phenolic and total flavonoid content were estimated. The antioxidant activity was determined by DPPH radical scavenging assay. Agar well diffusion and dilution method were used to determine zone of inhibition and MIC against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus pumilus* and *Micrococcus luteus*.

Preliminary phytochemical analysis revealed the presence of anthraquinone glycosides, sterols, phenolic compounds and alkaloids. Physicochemical studies revealed the ash value of 16.50%. Water-soluble and alcohol-soluble extractive values were higher than other extracts which were 14% and 11% respectively. Loss on drying was found to be 11.33%. The plant extract showed total phenolic content of 5.93 mg GAE/g at 1 µg/ml. For flavonoid content, plant extract showed 7.75 mg RE/g at 2000 µg/ml. In DPPH radical scavenging activity test, extract shown weak antioxidant activity. Extract antimicrobial activity was seen against *Staphylococcus aureus* at concentration of 40, 60, 80, 100 mg/ml and *Bacillus pumilus* at 60 and 80 mg/ml. The zone of inhibition produced by both species were within the range of 1 to 3 cm diameter. As compared to zone of inhibition produced by penicillin, *Hedyotis hedyotide* has weaker antimicrobial strength than that of penicillin.

From this study, it can be concluded that *Hedyotis hedyotide* leaf extract possess weak antioxidant activity and mild antimicrobial activity against few microorganism's species.

Keywords: Antimicrobial activity; Antioxidant activity; *Hedyotis hedyotide*

Introduction

In Malaysia, Malaysians have been no strangers to the potential of herbal plants. Our lush rainforests are reported to be home to over 2,000 plants with medicinal value, and in most countryside around the country, the older generation still use myriad brews, poultices and pastes derived from herbal plants, for everything from physical ailments to beauty concerns [1]. Despite of herbal products have been utilised largely by Malaysian, herbal medicines industry is still lack of proper scientific data and regulations

to push it forward to become a profitable industry. This explained why most of us are familiar with potential benefits of herbal medicines but yet few of us would really give them credibility. As such we can see that Malaysia has a huge potential for herbal medicines due to its mega biodiversity. Malaysia herbal industry has been seen as a new source of economic growth and categorized as one of the Entry Point Project (EPP) in early 2011. The NKEA EPP [1] project works together with the current national health policy to enhance traditional herbal medicine towards integrative medicine for the improvement and betterment of quality of life [2].

Hedyotis hedyotide is commonly known as White Ox Creeper (Figure 1). The synonyms of *Hedyotis hedyotide* is *Old-*

enlandia hedyotide. In Chinese, it is known as niu bai teng (牛白藤). The classification of *Hedyotis hedyotide* is as follows:



Figure 1: *Hedyotis hedyotide* collected from Gurun, Kedah.

Kingdom	:	Plantae
Order	:	Gentianales
Family	:	Rubiaceae
Subfamily	:	Rubioideae
Genus	:	<i>Hedyotis</i>
Species	:	<i>Hedyotis hedyotide</i>

Hedyotis hedyotide can grow to height of 10 to 45 cm. It needs full sunlight or partial shade in hot areas. Its germination time is two or three weeks. It also needs moist soil with pH 5.0 to 6.0. *Hedyotis hedyotide* needs regular watering throughout its growth.

Hedyotis hedyotide has been traditionally used for the treatment of arthritis, cold, cough, gastro-enteritis, headstroke, etc. But few studies have screened the active compounds from extracts of *H. hedyotide*. Chen JC, et al. (2015) studied the chemical constituents from stems of *Hedyotis hedyotide* and their immunosuppressive activity. Eleven compounds were obtained and identified as vomifoliol, betulonic acid, betulinic acid, betulin, 3-epi-betulinic acid, ursolic acid, β -sitosterol, stigmast-4-en-3-one, 7 β -hydroxysitosterol, (3 β ,7 β) -7 methoxystigmast-5-en-3-ol and morindacin [3].

Xiao Ping Hu, et al. (2011) reported five new anthraquinone glycosides, hedanthrosides and two new iridoid glycosides, hederidosides, along with two known anthraquinones and four known iridoids from the stems of *Hedyotis hedyotide* (DC.) [4].

Hedyotis hedyotide has been used in traditional Chinese medicine for the treatment of autoimmune diseases. Also, it possesses other pharmacological activities, including anti-tumor, anti-

viral, anti-inflammatory, and anti-atherosclerotic plaque activities [5]. Until now there wasn't much research done on *Hedyotis hedyotide*, so study conducted to explore this plant antioxidant and antimicrobial potential.

Materials and Methods

Plant of study which is *Hedyotis hedyotide* was collected in 14 October 2018 from Gurun, Kedah, Malaysia. The leaves were collected and dried in the oven for five days at 45°C in hot air oven. After the leaves were completely dried and blended into fine powder. Powder was passed through sieve No. 40 (420 micrometer) to ensure that the powder particle size is uniform.

Herbarium voucher specimen of *Hedyotis hedyotide* was prepared using standard procedure and then submitted to Faculty of Pharmacy of AIMST University, Malaysia.

The selected plant was examined for its macroscopy study like shape, texture, colour, surface, and other macroscopic characteristics.

In microscopic study, leaf of the selected plant and its powder were studied for various microchemical test and microscopical parameters using standard methods.

In physicochemical analysis, extractive values with different solvents, loss on drying, total ash value, foreign organic matter, fluorescence analysis and thin layer chromatography was carried out using the procedures mentioned in reference text.

Extraction was done by using Soxhlet extraction. After extraction, the solvent was removed by using rota vapour and dried extract was preserved for further studies.

Phytochemical Screening of the plant extract was carried out to detect various phytochemicals present in plant by following reference methods as cited in pharmacopoeial standards.

Total Phenolic Content

Several dilutions of standard gallic acid were prepared which include, 1, 2, 4, 6 and 8 μ g/ml. The total phenolic contents of the ethanolic extracts of sample were estimated by using the Folin-Ciocalteu reagent and 2.5% sodium carbonate. After serial dilution, 200 μ l of FC reagent and 4ml of 2.5% sodium carbonate solution were added to 200 μ l of various concentration of gallic acid. The samples were incubated at room temperature for an hour. The absorbance of each concentration was measured at 750 nm using UV spectrophotometer and subsequent calibration curve was constructed. For the extract sample procedure was followed like gallic acid preparation.

Phenolic content was determined for ethanolic leaves extract by using the formula,

Total Phenolic Content, $C = (A/B) \times \text{dilution factor}$, where

C= expressed as mg GAE/g dry weight of the extract

A= the equivalent concentration of gallic acid established from calibration curve (mg)

B= dry weight of the extract (grams)

Total Flavonoid Content

Rutin was used as standard stock solution. Serial dilution was performed from stock solution to form different concentration of 5, 10, 20, 40, 60, 100, 200 and 300 µg/ml. 0.5 ml of various concentration of rutin solution, 1.5 ml 95% ethanol, 0.1 ml of 10% AlCl₃ solution, 0.1 ml of 1M potassium acetate solution and 2.8 ml of distilled water were added to respective test tubes. All tubes were incubated at room temperature for an hour. After incubation, absorbance of various concentration of rutin solution and ethanolic extract were determined at maximum wavelength of 415 nm. Calibration curve was constructed. For the extract sample procedure was followed like rutin preparation. Flavonoid content was calculated as follow:

Total Flavonoid content, C= (A/B) x dilution factor, where,

C= expressed as mg RE/g dry weight of extract

A= the equivalent concentration of rutin established from the calibration curve (mg)

B= dry weight of extract (grams)

DPPH (1,1-Diphenyl-2-picryl hydrazyl) Radical Scavenging Assay

In DPPH assay, butylated hydroxytoluene (BHT) was used as standard stock solution. Serial dilution was made from the stock solution to make various concentration of 10, 20, 40, 60, 80, 100, 120, 140, 250, 300, 350 and 400 µg/ml respectively. The DPPH scavenging activity of the sample was estimated using 0.1 mM DPPH reagent by dissolving 3.94 mg of DPPH into 100 ml of 95% ethanol. Then, to 2.5 ml of each of the concentration of BHT solution, 3 ml of DPPH reagent was added.

As for the extract sample, stock solution was prepared similar to BHT preparation. Serial dilution was made into various concentration which include 10, 20, 40, 60, 80, 100, 120, 140, and 5000 µg/ml. Similarly, to 2.5 ml of each of the concentration of ethanolic leaves extract solution, 3 ml of DPPH reagent was added. Control solutions were prepared by adding 2.5 ml of 95% ethanol and 3 ml of DPPH reagent. All the test tubes were shaken well and incubated in dark for 30 minutes. Absorbance for both BHT and ethanolic leaves extract were determined by using wavelength of 518 nm in UV spectrophotometer and calibration curve was constructed. Antioxidant activity usually expressed as IC₅₀ which can be calculated from standard graph plotted. Percentage scavenging activity of ethanolic extract can be calculated as follow:

Percentage scavenging activity= [(Ac-As)/Ac] x 100, where,

Ac= absorbance of control reaction

As= absorbance of extract samples

Antimicrobial Study

Agar well diffusion method

In this study, 6 species of microorganisms were tested against *Hedyotis hedyotide* leaf extract, which were *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus pumilus* and *Micrococcus luteus*. These microorganisms were inoculated into sterile nutrient broth (100 ml each) respectively. Then they were incubated for 24 hours with 37°C. In the meantime, sterile nutrient agar plate was also made. Next, plant extract was prepared with various concentrations (i.e. 100, 80, 60, 40, 20 and 10 mg/ml), by dissolving 200 mg of leaf extract into 2 ml of distilled water mixed with 5% DMSO. Also, 10% penicillin solution and 5% DMSO solution were prepared to be used as control.

The next day after incubation, the agar plate surface was inoculated with 0.1 ml microbial inoculum and spreaded over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer. Next, a volume of 50 µL of various concentration of plant extract and control solution were added into the well respectively. Then the agar plates were kept in refrigerator for an hour for the diffusion of antimicrobial agents into the agar. Next, they were incubated 37°C for 24 hours. This study was done in duplicate. After the incubation, zone of inhibitions was observed and measured. At least 8 readings were obtained [6].

Dilution method (Determination of MIC value)

In this method, microorganisms to be tested were inoculated into sterile nutrient broth and incubated 37°C for 24 hours. The next day, the microbial inoculum was centrifuged and resuspended in nutrient broth to obtained standard McFarland microbial culture. Plant extract with 10000 µg/ml concentration was prepared by dissolving 50 mg extract in 5ml distilled water with 5% DMSO.

Initially, five tubes were taken. The first tube was added with 0.2 ml plant extract solution and 1.8 ml standard McFarland microbial culture. For the second tube, 1 ml of solution from the first tube was transferred to it and added with 1 ml of standard McFarland microbial culture. Similarly, for the third tube, 1 ml of solution from second tube was transferred to it and added with 1 ml standard McFarland microbial culture and same goes to the fourth and fifth tubes. This is 2 folds dilution method. For this study, it was carried out in duplicate, so ten tubes were required for each species of microorganism studied. Also, positive control and negative control tubes were prepared by adding 2 ml of standard

McFarland microbial culture and 2 ml of nutrient broth into each test tube respectively. Finally, all the tubes were incubated 37°C for 24 hours and any growth of microorganism were observed.

Results

Herbarium Voucher Specimen Accession Number was assigned as AIMST/FOP/33. In morphology, leaves were light green in colour and 6-10 cm in length 2-3 cm width. Pattern of leaves were opposite with interpetiolar stipule, pinnate venation, ventral surface of leaves was shiny and waxy whereas dorsal surface was pale green. Odour was characteristic and strong.

Microscopic studies showed the presence of cuticle, palisade cells, lignified annular type xylem vessel, reticulate type xylem vessel, vascular bundle with xylem & phloem, parenchymatous cells, oil cells, single layer epidermis, chlorenchyma, mucilage, starch grains, needle shape calcium oxalate crystals after treating 60% H₂SO₄ and anomocytic stomata.

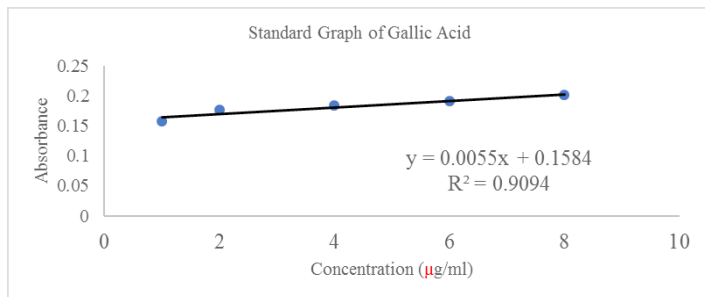
In physico-chemical study, extractive value results for alcohol-soluble, water-soluble, chloroform-soluble and petroleum ether-soluble extractive were found to be 11, 14, 7 and 2% respectively. Loss on drying was found to be 11.33%. Total ash value was 16.50%. foreign organic matter was 2.40%. No fluorescence was shown by drug in fluorescence analysis. TLC results the presence of 5 compounds with R_f values of 0.264, 0.375, 0.528, 0.194 and 0.431 in Butanol: Acetic Acid: Water 4: 1: 0.5 solvent system (Table 1).

Concentration (µg/ml)	Absorbance	Total Phenolic Content (GAE/g)
1	0.191	5.93 mg
2	0.198	7.2 mg

Table 1: Concentration and corresponding absorbance of *Hedyotis hedyotidea* leaf extract.

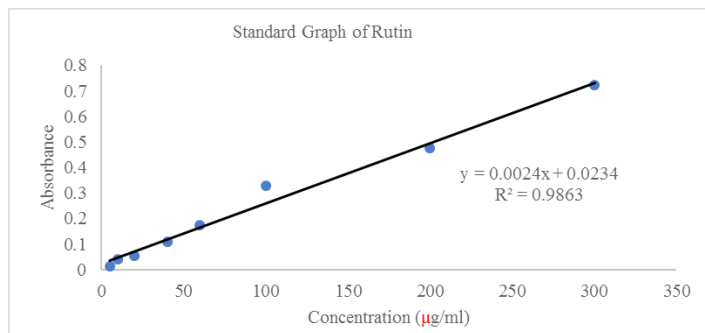
Phytochemical analysis results shown the presence of carbohydrates, protein, anthraquinone glycosides, steroids, flavonoid, alkaloids, tannins and phenolic compounds.

Total Phenolic Content



Graph 1: Standard Graph of Gallic Acid.

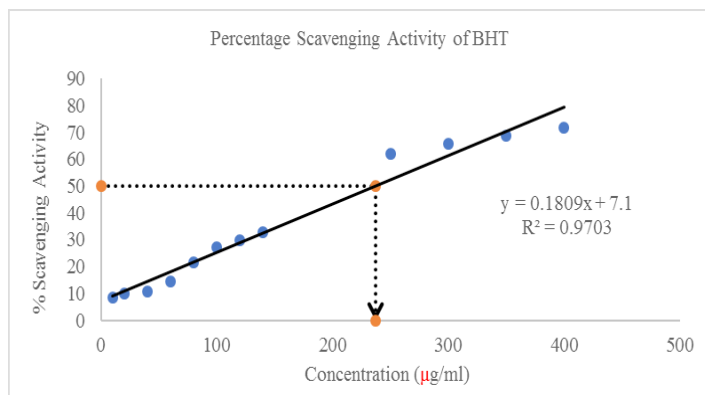
Total Flavonoid content



Graph 2: Standard Graph of Rutin.

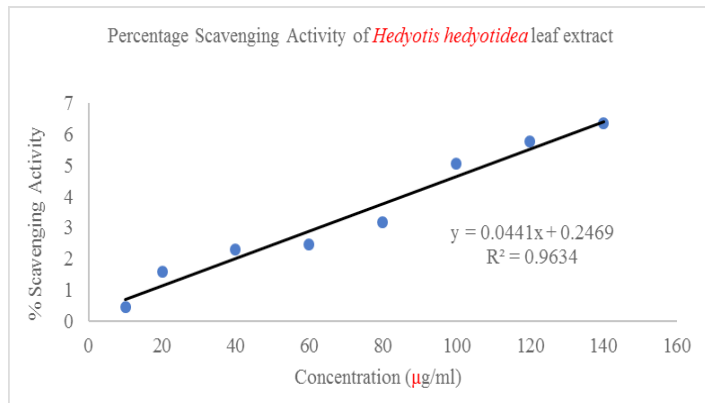
Antioxidant Studies

DPPH scavenging activity

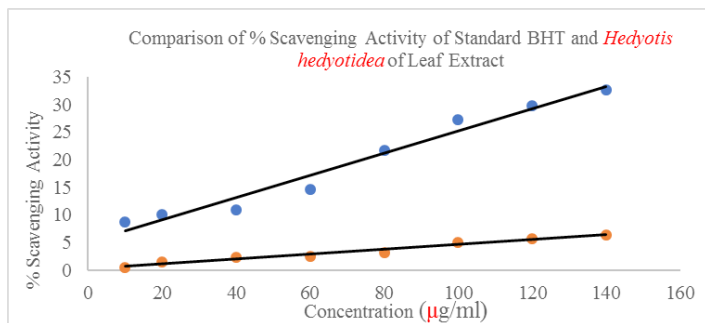


Graph 3: Percentage scavenging activity of BHT.

IC₅₀ of BHT was found to be 237.15 µg/ml



Graph 4: Percentage scavenging activity of *Hedyotis hedyotidea* leaf extract.



Graph 5: Comparison of percentage scavenging activity of standard BHT and *Hedyotis hedyotideae* leaf extract.

IC₅₀ of BHT was found to be 237.15 µg/ml

Antimicrobial Study

Agar well diffusion method (Determination of zone of inhibition)

Four species of microorganism *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Micrococcus luteus* shown negative results whereas two species of microorganism *Staphylococcus aureus* and *Bacillus pumilus* shown zone of inhibition.

For *Staphylococcus aureus* penicillin (control) shown zone of inhibition with average diameter of 6.86 cm whereas plant extract 40 mg/ml shown average diameter of 1.74 cm 60 mg/ml average diameter of 2.46 cm, 80 mg/ml average diameter of 3.11 cm and 100 mg/ml shown average diameter of 1.56 cm of inhibition.



Figure 2: Antimicrobial effect of plant extract on *Staphylococcus aureus* by diffusion method.

For *Bacillus pumilus* penicillin (control) shown zone of inhibition with average diameter of 5.34 cm whereas plant extract 60

mg/ml shown average diameter of 1.92 cm and 80 mg/ml shown average diameter of 2.12 cm of inhibition.

For *Bacillus pumilus* penicillin (control) shown zone of inhibition with average diameter of 5.34 cm whereas plant extract 60 mg/ml shown average diameter of 1.92 cm and 80 mg/ml shown average diameter of 2.12 cm of inhibition.



Figure 3: Antimicrobial effect of plant extract on *Bacillus pumilus* by diffusion method.

Discussion

Herbarium voucher specimen of *Hedyotis hedyotideae* was prepared and submitted to the Herbaria of Faculty of Pharmacy (FOP), AIMST University, Malaysia. Herbarium voucher specimen accession number AIMST/FOP/33 was assigned to the specimen.

Morphology of the plant was observed by the help of magnifying glasses. The plant has close resemblance to plants within the family of Rubiaceae (i.e. opposite leaves with stipule, interpetiolar stipules).

Microscopical structures observed in *Hedyotis hedyotideae* leaf are vascular bundle (xylem and phloem), epidermis, collenchyma, parenchyma, palisade cells, cuticle, chlorenchyma, needle shape crystals, starch grains, oil cells and mucilage. In powder microscopy, structures observed are fibers, annular xylem, uncoiled xylem, and reticulate xylem. Besides *Hedyotis hedyotideae* leaf are having anomocytic type of stomata. Anomocytic stomata is the stoma surrounded by a limited number of subsidiary cells which are quite alike the remaining epidermal cells. The accessory or subsidiary cells are five in number.

Extractive values give idea about the nature of the chemical constituents present in a crude drug. It is also useful for the estima-

tion of constituents extracted with the solvent used for extraction. In this study, water-soluble extractive value was found to be highest followed by alcohol-soluble extractive hence this study also assisted us in selection of solvent used for extraction of the plant constituents by using Soxhlet extraction.

Loss on drying is a gravimetric method and it determines the amount of volatile matter of any kind (including water) that can be driven off from the plant sample. Next, the moisture content of 10% and below is the recommended value for drying of leaves and for powder production [7]. In this study, the moisture content is 11.33% which is higher than it should be. This could be because of insufficient drying of leaf powder and there is greater amount of moisture content.

Ash values used to determine quality and purity of crude drug and to establish the identity of it. After complete burning, ash value obtained for leaves of *Hedyotis hedyotide* was 16.50%. A high ash value indicates that it may be due to the consequences of contamination, substitution, adulteration, or carelessness in preparing the drug or drug combinations. It was found that the individual drugs usually have total ash values in the range from 4.18 to 14.47% w/w [8]. In this study, the percentage showed higher than average amount which could be due to the fact that the plant has relatively higher inorganic matter content. Ash values would have been better if done with muffle furnace.

Foreign organic matter is parts of the organs of the crude drug other than those named in definition and description of drug are defined as foreign organic matter. From this study, foreign organic matter is 2.40% which mostly consists of soils and spider web adhered to the leaf surface.

Fluorescence is the phenomenon exhibited both in visible and UV- light by various chemical constituents present in the plant material. Fluorescence analysis of *Hedyotis hedyotide* was carried out. It was found that the plant powder fluoresces when added with glacial acetic acid under wavelength of 365 nm of UV light. Acetic acid-water solution excited by Ultra Violet (UV) light when the exciting light wavelength is longer than 246 nm [9]. Orange red fluorescence which was observed indicates the present of complex nitrogenous compounds and phytochemical analysis result is also support this finding by showing positive results for alkaloids.

Samples of all the fresh leaf extracts were prepared with ethanol solvents 70% were spotted onto the TLC plate as a single spot with capillary tubes. After trying different solvent systems by trial and error method, a suitable solvent system was selected. Among these, two solvent systems showed some separation under ultraviolet light which were Butanol: Ethanol: Water (5:1:1.1) and Butanol: Acetic acid: Water (4:1:0.5). Seven compounds were separated with R_f values of 0.225, 0.366, 0.264, 0.375, 0.528, 0.194 and 0.431.

Phytochemicals analysis showed the presence of reducing sugars, gums, proteins, deoxysugar which is component of glycoside, anthraquinone glycosides, steroids, flavonoids, alkaloids and phenolic compounds. In addition to these, *Hedyotis hedyotide* is well-known with its iridoid glycosides and triterpenoids.

Total phenolic content is an assay to determine the quantity of polyphenol in a sample. The basic mechanism is an oxidation/reduction reaction between the Folin-Ciocalteu reagent and phenolic compounds present in the sample which results in the formation of a blue colour complex that absorbs radiation and allows quantification by UV spectrophotometry at 750 nm. The reaction forms a blue chromophore, where the maximum absorption of the resulting chromophores depends on the alkaline solution and the concentration of phenolic compounds in the sample. The higher the amount of phenols in the sample, the higher the intensity of the blue colour. *Hedyotis hedyotide* extract showed total phenolic content 5.93 mg GAE/g at concentration of 1 μ g/ml while 7.20 mg GAE/g at concentration of 2 μ g/ml. This indicates that leaf extract contains some amount of polyphenols content. Low contents of phenolic compounds indicated that these compounds contribute to mild antioxidant activity. Plant polyphenols with antioxidant capacity could scavenge reactive chemical species as well as minimise oxidative damage resulting from excessive light exposure [10].

Flavonoids are determined by using reagents 10% Aluminium chloride along with 1M potassium acetate, ethanol and water mixed with plant samples. Formation of an acid stable complex between the aluminium ion and the C-4 keto groups and either C-3 or C-5 hydroxyl group of flavones and flavonols results in yellow colour formation. *Hedyotis hedyotide* showed total flavonoid content of 1.083 mg RE/g at concentration of 1000 μ g/ml while total phenolic content of 7.75 mg RE/g at concentration of 2000 μ g/ml. This indicates that leaf extract does contain some amount flavonoids.

In DPPH radical scavenging activity assay 0.1 mM DPPH reagent was used. By comparing the percentage scavenging activity standard BHT (butylated hydroxytoluene) and percentage scavenging activity of *Hedyotis hedyotide* leaf extract, it was found that *Hedyotis hedyotide* was having much lesser antioxidant activity than that of BHT. IC_{50} for standard BHT was found to be 237.15 μ g/ml.

In Antimicrobial studies, six species of microorganisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus pumilus* and *Micrococcus luteus* were taken. However, only two species *Staphylococcus aureus* and *Bacillus pumilus* shown positive results with significant zone of inhibition surrounded the well containing plant extract. In this study, 10% penicillin was used as control. Also, 5% DMSO (Dimethyl Sulfoxide) was used as solubilizing agent to assist the plant extract to be dissolved completely in water. *Hedyotis hedyotide* leaf ex-

tract has antimicrobial activity against *Staphylococcus aureus* and *Bacillus pumilus* but it has weaker strength compared to that of penicillin drug which is already available in market.

This method was used to determine Minimum Inhibition Concentration (MIC) value produced by *Hedyotis hedyotide* leaf extract. In this study, leaf extract with concentrations of 1000, 500, 250, 125 and 62.5 µg/ml were used. As results, all the tubes showing turbidity in the solution (negative results). Higher concentration of leaf extract is needed for further investigation. As there are no inhibition was shown, there are no further MBC (microbial bactericidal) test for this study.

Conclusion

All standardization parameters for leaf of the plant were found within pharmacopoeial standards. The phytochemical analysis of *Hedyotis hedyotide* leaf revealed that it contains anthraquinone glycosides, sterols, phenolic compounds and alkaloids. Based on the results, it was found that *Hedyotis hedyotide* possess weak antioxidant and thereby further validation by more antioxidant assays are needed. Also, *Hedyotis hedyotide* is having antimicrobial effect against *Staphylococcus aureus* and *Bacillus pumilus* but the antimicrobial strength is weaker than penicillin. Besides further investigations on MIC and MBC were needed by using higher concentration of plant extracts.

References

1. Ganesan S (2015) Growing our herbal industry. The Star Online.
2. Farizah A, Azlan M, Nooraishah S, Fadzilah A (2014) Issues and Challenges in the Development of the Herbal Industry in Malaysia. Prosiding Perkem 10: 227-238.
3. Chen JC, Fang JB, Wang XG (2015) Chemical constituents from stems of *Hedyotis hedyotide* and their immunosuppressive activity 40: 2357-2362.
4. Xiao Ping Hu, Shu-Wei Zhang, Shan-Shan Liu, Li-Jiang Xuan (2011) New anthraquinone and iridoid glycosides from the stems of *Hedyotis hedyotide*. Helvetica Chimica Acta 94: 675-685.
5. Zhou YQ, Weng XF, Dou R, Tan XS, Zhang TT, et al. (2016) Betulin from *Hedyotis hedyotide* ameliorates concanavalin A-induced and T cell-mediated autoimmune hepatitis in mice. Acta Pharmacol Sin 38: 201-210.
6. Mounyr Balouiri, Moulay Sadiki, Saad Koraichi Ibensouda (2016) A review on methods for in vitro evaluating antimicrobial activity. Journal of Pharmaceutical Analysis 6: 71-79.
7. Raja KS, Taip FS, Zakuan Azmi MM, Islam Shishir MR (2019) Effect of pre-treatment and different drying methods on the physicochemical properties of *Carica papaya* leaf powder. Journal of the Saudi Society of Agricultural Sciences 18: 150-156.
8. Chandel HS, Pathak AK, Tailang M (2011) Standardization of some herbal antidiabetic drugs in polyherbal formulation. Pharmacognosy research 3: 49-56.
9. Caiqin Han, Ying Liu, Yang Yang, Xiaowu Ni, Jian Lu, et al. (2009) Study on fluorescence spectra of molecular association of acetic acid-water. Chinese Optics Letters 7: 357-360.
10. Piluzza G, Bullitta S (2011) Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. Pharmaceutical Biology 49: 240-247.