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Research Paper

Determination of Minimum Inhibitory Concentration of *Euphorbia hirta* (L.) Extracts by Tetrazolium Microplate Assay

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ABSTRACT

Euphorbia hirta has been used widely in traditional Malay medicine as a treatment against infectious pathogens. This ethnomedicinal plant is also well known for its diverse biological activities. The aim of the present study is to determine the antibacterial and antifungal activities of *E. hirta* extracts against 13 microbial strains. Tetrazolium microplate assay was employed in order to quantitatively assess the antimicrobial activities of *E. hirta* extracts by determining the Minimum Inhibitory Concentration (MIC) values obtained at initial concentration of 1mg/ml. This assay used p-iodonitrotetrazolium chloride (INT) as color indicator to evaluate the growth of test microorganisms which were treated with *E. hirta* extracts with low range concentrations of 1-0.015mg/ml. The ethanolic and methanolic extracts of *E. hirta* aerial part exhibited strongest antimicrobial activity against *Salmonella typhi* and *Pseudomonas aeroginosa* with MIC values of 0.031mg/ml and 0.062mg/ml respectively. The flower and leaves extracts showed moderate antimicrobial activity whereas no activity was found in stem extract. Dichloromethane and ethyl acetate extracts demonstrated mild activities with MIC values in the range between 1-0.5mg/ml. Preliminary phytochemical analysis of the plant extracts showed the presence of tannins, alkaloids, flavonoid, terpenoids, cardiac glycosides, saponins, reducing sugar and steroids. None of the extracts display acute cytotoxicity against Vero cells. Our results revealed the extracts of *E. hirta* plant posse's potential antimicrobial therapeutic effect against various pathogenic microorganisms. Based on the broad spectrum antimicrobial activities exhibited by this plant, we believe this report validates the safe use of this plant in ethnomedicine for treating various infectious diseases.

Keywords: *Euphorbia hirta*; Antimicrobial activity; MIC.

INTRODUCTION

Infectious diseases represent a serious health problem today and account for one third of all deaths worldwide. Antimicrobials of plant origin have enormous therapeutic potential as they are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with

synthetic antimicrobials (Tomoko, et al., 2002). In Malaysia, more than one hundred plant species are reported to have medicinal properties and amongst is *Euphorbia hirta* with traditional claims for curing pathogen infection.

Euphorbia hirta L. (Family-*Euphorbiace*) is alternatively known by its Malay names as ara tanah, gelang susu, and lanchang. *E. hirta* is also commonly known by many vernacular names such as cats' hair, asthma weed, basri dudhi, malnommee, and fei yang cao. The plant has been used widely in traditional Malay medicine as a treatment for skin problems, gastrointestinal disorders, particularly intestinal parasitotosis, amoebic dysentery, diarrhoea, and ulcer. The plant is also used in bronchial and respiratory disorders including asthma, bronchitis, and hay fever.

E. hirta is well documented for its biological activities such as anxiolytic (Lanthers, et al., 1990), antifungal (Masood and Rajan, 1991), diuretic, antihypertensive (Johnson, et al., 1999), antidiarrhoeial, antimalarial (Tona, et al., 2004), anthelmintic (Adedapo, et al., 2005), antibacterial (Sudhakar et al., 2006), anti-inflammatory (Singh, et al., 2006) and antioxidant, anticancer / antiproliferative (Mothana, et al., 2009).

The present research was aimed at investigating the antimicrobial efficacy and traditional claims of ethnomedicinal plant, *E. hirta* in treating infectious ailments thus report the minimum inhibitory concentration (MIC) of the extracts using tetrazolium microplate assay method with initial concentration of 1000µg/ml (1mg/ml).

MATERIAL AND METHODS

***Plant collection and authentication*:** The fresh plant of *E. hirta* was obtained from several areas of Penang City, Malaysia. The plant was authenticated by Mr. Shanmugam, the botanist of the School of Biological Sciences, University Science Malaysia, where a voucher specimen (13580) was deposited in the Herbarium Unit of the school.

***Extraction of plant material*:** Various parts of *E. hirta* mainly, leaves, stem, flower and aerial part of the plant were air-dried and grounded into fine powder and macerated individually by ratio of 10g of ground plant material in 100ml of organic solvents. The powdered leaves, stem, and flower of *E. hirta* were extracted solely with methanol at room temperature. Extraction was done for 3 days under occasional shaking and the process was repeated three times. The aerial part of *E. hirta* was extracted separately with different solvent systems in order of increasing polarity. The solvents used were hexane, dichloromethane, ethyl acetate, methanol, and ethanol. The combined extracts obtained were filtered and concentrated to dryness with a rotary evaporator (Rotavapor® R-200 Buchi, Switzerland) under reduced pressure. The extracts obtained were eventually freeze-dried (FreeZone®, MO) to remove any residual water and stored at 4^{o}C until use.

Antimicrobial Assay

***Microbial Strains, culture medium and inocula preparation*:** Thirteen species of test microorganism which selected to study were originally clinical isolates obtained from Department of Medical Microbiology and Parasitology (JTMP), School of Medical Sciences, University Science Malaysia, Kelantan. Gram-negative species which involved in this study were *Enterobacter aerogens, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi, Shigella dysenteriae* and Gram-positive species were *Staphylococcus aureus* and *Bacillus subtilis*. Fungi species used in this study include *Aspergillus niger, Candida albicans* and *Candida glabrata*. Their identities were confirmed by culturing on the specific media followed by biochemical test using API

system as previously reported (Mbaveng, et al., 2008). Bacterial strains stock cultures were kept on Nutrient Agar (Difco, USA) at 4^{o}C. *Candida albicans, Candida glabrata* and *Aspergillus niger* was kept on Potato Dextrose Agar (Difco, USA) at 4^{o}C. All the microbial strains were sub-cultured on a fresh appropriate agar plate 24h prior to antimicrobial test. Inocula were prepared by transferring several single colonies of microbes to a sterile broth. The microbial cell suspension was mixed to homogeneity to give a final density of 5 x 10^5 CFU/ml and these were confirmed by viable counts. The infective dose for most microorganism are 10^5 CFU/ml. *Aspergillus niger* spores were obtained in vitro from monoconidial cultures after incubation (7 days, 24^{o}C) on Potato Dextrose Agar, harvested in sterile distilled water containing 0.1% Tween 80 and stored at 4^{o}C until used as inoculum.

***Preparation of crude extracts and antibiotics*:** The plant extracts were dissolved in 50% dimethylsulfoxide (DMSO) in sterile Mueller Hinton broth (MHB) for bacterial isolates and Potato Dextrose broth (PDB) for fungal isolates respectively in order to obtain a stock concentration of 20mg/ml. The stock solution was further diluted using respective broth in ten-folds to obtain working concentration of 2mg/ml. The final concentration of DMSO in the well was ensured to be less than 2%. Preliminary analyses with 2% (v/v) DMSO affected neither the growth of the test organisms nor the change of tetrazolium color due to this growth. Gentamycin, Amoxycillin, Vancomycin, Chloramphenicol and Penicillin were prepared to a final concentration of 0.1mg/ml and served as the positive drug control against bacterial strains. Amphotericin B (0.1mg/ml) and Miconazole (0.1mg/ml) were used as positive control against fungal isolates. Preparation of commercial drugs exceeding the concentration of 0.1mg/ml is regarded as not potent antibiotic.

***Tetrazolium Microplate Assay*:** The minimum inhibitory concentration (MIC) of test microorganisms and reference antibiotics were determined by using tetrazolium microplate assay which were slightly modified from serial broth microdilution method as previously described by Eloff (1998a). This assay was performed using flat bottom 96-well clear microtitre plates. The wells in column A of each row were left blank and the last seven wells from column B to H were filled with 100µl of sterilized MHB (bacterial isolates) and PDB (fungal isolates). Working solution of plant extracts were added to the wells in column A and B of each row and an identical two-fold serial dilution were made from column B to the column G. The last wells in column H was served as drug-free controls. An appropriate solvent blanks (DMSO) were included as negative control. Lastly, 100µl of bacterial inoculum were added in all the wells from column A to H and mixed thoroughly to give final concentrations ranging from 1mg/ml-15.625µg/ml. Tests were done in triplicates. The cultured microplates were sealed with parafilm and incubated at 37°C for 24h for bacterial and yeast species meanwhile plates with fungal culture were incubated at 28°C for 48h. The MIC of samples was detected following addition (50µl) of 0.2mg/ml p-iodonitrotetrazolium chloride in all the wells (INT, Sigma-Aldrich, USA) and incubated at 37°C for 30 min. Microbial growth were determined by observing the change of color p-iodonitrotetrazolium chloride (INT) in the microplate wells (pinkish-red formazan when there is growth and clear solution when there is no growth). MIC was defined as the lowest sample concentration showing no color change (clear) and exhibited complete inhibition of bacterial growth. MIC value <0.5mg/ml was defined as potential strong activity.

***Preliminary Phytochemical Analysis*:** A portion of each crude extract (1ml) that was subjected to biological screening was used for the identification of the major

secondary metabolites such as terpenoids, tannins, alkaloids, cardiac glycosides, anthroquinones, phlobatannins, saponins, flavonoids, reducing sugar and steroids by the protocols described by Trease and Evans (1996), Wagner and Bladt (2009).

Cytotoxicity Assay

***Vero cell line*:** Vero cells are mammalian cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*) are highly recommended for screening chemical toxicity in vitro (ISO, 1996). The cell line was initiated from kidney of a normal adult African green monkey on March 27th, 1962, by Yasummura and Kawakita at the Chiba University, Japan American Public Health Association, 1992.Vero cells were maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2raM), penicillin (100units/ml) and streptomycin (100µg/ml). The cells were cultured at 37°C in a humidified 5% CO_2 incubator.

***MTT Test*:** The in vitro cytotoxicity of the extracts was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) assay reported by (Mosmann, 1983). Concentrations ranging from 100-6.25µg/ml of the plant extracts were prepared from the stock solutions by serial dilution in RPMI-1640 to give a volume of 100µl in each well of a microtiter plate (96-well). In the MTT test, 100-150µg/ml is the fixed highest in-test concentration. Higher concentrations than this range generally result in a markedly increased incidence of false-positives and insignificant activity. Later, 100µl of Vero cell suspension at $1x10^6$cells/ml was filled in each well. Controls that contained only Vero cells were included for each sample. The assay for each concentration of extract was performed in triplicates and the culture plates were kept at 37^oC with 5% (v/v) CO_2 for one day. After 24h of incubation, 20µl of 0.5% w/v MTT was added to each well and allowed to further 4h incubation. After 4h of incubation, 100µl of DMSO was added to each well to dissolve the formazan crystals. Absorbance was read at 554 nm using a Bio-TEK Microplate Scanning Spectrophotometer. The cytotoxic effect was expressed as IC_{50}, the concentration of extract that reduces cell viability to 50% of the control.

RESULTS

***Antimicrobial activity*:** MIC values of all the extracts tested against 13 clinical isolates were summarized in Table 1. The ethanolic extract of *E. hirta* aerial part showed the strongest antimicrobial activity compared to all other extracts. The inhibitory activity varied significantly against all thirteen clinical isolates with MIC value ranged between 0.031-1mg/ml. In addition to that, the MIC value exhibited by this extract against *Salmonella typhi* was lower compared to standard antibiotic, Amoxycillin and Chloramphenicol used in this study. The methanolic extract of *E. hirta* aerial part displayed second highest antimicrobial activity against *Pseudomonas aeroginosa*. The flower and leaves extracts inhibit the growth of all the tested clinical isolates to a moderate extent with MIC value ranging from 1 to 0.125mg/ml.

***Phytochemical analysis*:** Major secondary metabolites of *E. hirta* extracts were screened (Table- 2). All the extracts revealed the presence of alkaloids. Antraquinones and phlobatannins were absent in all the extracts.

***Cytotoxicity*:** The in vitro cytotoxicity assay (Table 3) revealed all the extracts were non-toxic to Vero cells (IC50 >100µg/ml).

DISCCUSSION

According to Rios and Recio (2005), many papers made a common mistake in claiming positive activity for slight dilutions or excessively high concentrations for

antimicrobial tests. Therefore the quantity of the plant extracts used for antimicrobial susceptible experiments were suggested to be as lower as 1mg/ml and are considered to possess significant antimicrobial activity in the case where MIC values are lower than 100µg/ml (Gibbons, 2004; Rios and Recio, 2005). Some researchers have tested the susceptibility of several pathogenic microorganisms on *E. hirta* extracts. However their results of MIC values were generally very high except for one author Sudhakar et al., (2006) reported the MIC values of ethanol extract of aerial part lower than 1mg/ml against few microorganisms using tube dilution method. Early observation by El-Mahmood (2009), displayed MIC values for aqueous, methanol and hexane extracts of *E. hirta* leaves were in the range between 25 to 100mg/ml against *E. coli, K. pneumonia, S. typhi, P. mirabilis* and *S. dysentriae.* These findings are comparable to data reported by Chika et al., (2007) who have found inhibitory effect of *E. hirta* ethanolic leaves extracts on *E. coli*, *Staph aureus*, *P. aeruginosa* and *B. subtilis* with the MIC values of 58.09, 22.55, 57.64, and 74.61mg/ml respectively. In contrast, Mohammad et al., (2010), reported antimicrobial activity of methanolic leaf extracts of *E. hirta* on *E. coli, S.aureus, C. albicans* and P. *mirabilis* with the lowest MIC values of 3.12, 12.5, 3.12 and 50 mg/ml respectively. The reported MIC values are very high compared to the results obtained from present study on *E. hirta* methanolic leaves extracts against *E. coli, S.aureus, C. albicans* and P. *mirabilis.*

Therefore, due to its distinctive antimicrobial activity proven by previous studies with higher MIC values, the various extracts of *E. hirta* were analyzed in more detail through tetrazolium microplate assay method. This colorimetric assay represents an alternative approach to determine MIC economically and yields greater reproducible result. The application of tetrazolium salt in the assay as colorimetric indicator have enhanced the sensitivity and accuracy of MIC determination since the formazan derivatives produced by bacteria or fungi can be quantified (Masoko, et al., 2007). In the present study, the ethanol and methanol extract of *E. hirta* aerial part showed the most prominent antimicrobial activity against *S.typhi* and *P.aeruginosa* respectively. The MIC values obtained were substantially very low compared to previous studies carried on this plant.

A clear correlation between the presence of phytochemicals and antimicrobial activities of these extracts was apparent. The broad spectrum antimicrobial action displayed by some of these extracts could be attributed to the presence of pronounced antimicrobial phytoconstituents such as terpenoids, tannins, flavonoids and alkaloids (Table 2). As was previously noted by Navarro et al., (1996) the substantial antimicrobial effects of plant extracts were due to the presence of secondary metabolites. Flavonoids are known to be synthesized by plants in response to microbial infection. Previous phytochemical investigation on *E. hirta* has led to the isolation of various secondary metabolites. Such as, flavonoids (Tona, et al., 2009), tannins (Yoshida, et al., 1988), triterpenes (Martinez, et al., 1999), phenolic acids, and amino acids (Lanthers, et al., 1991). Completely all the microorganism tested in this study were non-susceptible towards the stem extract at the initial concentration of 1mg/ml. Based on the ethnomedicinal property of this plant, it is the sap or latex secreted from the stem part of this plant is often used traditionally against cuts and warts as well as for fungus infection on the skin. The higher MIC value obtained (<1mg/ml) from stem extract can be best explain by the absence of secondary metabolites such as tannins and flavonoids as shown in Table 2. The absence of this phytochemicals could be due to the drying process undertaken during extract preparation. Therefore this finding supports the folkaric usage of this plant by

applying latex to the wounded area from fresh cut stem since air drying process possibly diminish all the responsible secondary metabolites with antimicrobial properties. The plant aerial part which was extracted in nonpolar solvents system exhibited weak inhibitory effect toward test microorganisms. This could be due to the fact that most of antimicrobial compounds such as flavonoids and terpenoids are polar constituents and they can't be extracted using non-polar solvent system. As a result, hexane extract appeared to be the least active extract compared to all other extracts as its inhibition potency was been observed only on *Proteus vulgaris* (MIC,1mg/ml). Dichloromethane and ethyl acetate extracts however displayed mild activity with selective antimicrobial action against the clinical isolates with MIC values varied from 1 to 0.5mg/ml. Dichloromethane and ethyl acetate are polar solvents but their polarities are less if compared to methanol and ethanol. As a consequence, few polar compounds with antimicrobial activity would be present in that particular extracts and thus produce very mild antimicrobial activity.

The in vitro cytotoxicity investigation (Table 3) using all the extracts against Vero cells showed that the extracts are not toxic. Only plant extracts with an IC_{50} value below than 20µg/ml can be accepted as cytotoxic extracts (Wall, et al., 1987). The results therefore indicates this plant is proven very harmless to consume and useful as topical medicines in the treatment against infectious diseases caused by pathogens. This report validates the safe use of this plant in ethnomedicine for treating various infectious diseases.

CONCLUSIONS

We can concluded that *E. hirta* plant extracts posses potential antimicrobial effect against wide array of pathogenic microorganisms with MIC values lower than 1mg/ml compared to previous findings by other authors with larger MIC values with different conventional method.

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Table -1A: Minimum inhibitory concentration (mg/ml) of *E. hirta* extracts.

Microorganism	*E. hirta* extracts							
	L (MeOH)	St (MeOH)	Fl (MeOH)	Ae (MeOH)	Ae (EtOH)	Ae (Hex)	Ae (DCM)	Ae (EtOAc)
Enterobacter aerogens	1	>1	1	1	1	>1	1	>1
Escherichia coli	0.5	>1	0.5	0.5	0.5	>1	0.5	1
Klebsiella pneumonia	0.5	>1	0.5	1	1	>1	1	1
Proteus mirabilis	0.5	>1	0.5	0.5	0.5	>1	0.5	1
Proteus vulgaris	0.5	>1	0.25	0.5	0.25	1	0.5	0.5
Pseudomonas aeruginosa	0.125	>1	0.125	0.062	0.125	>1	1	1
Salmonella typhi	0.5	>1	0.125	0.5	0.031	>1	0.5	0.5
Shigella dysenteriae	0.5	>1	1	>1	0.5	>1	0.5	>1
Staphylococcus aureus	0.25	>1	1	0.125	0.5	>1	1	1
Bacillus subtilis	0.5	>1	0.25	0.25	0.25	>1	0.5	0.5
Candida albicans	1	>1	1	0.5	0.25	>1	0.5	0.5
Candida glabrata	0.5	>1	0.5	0.25	0.5	>1	0.5	0.5
Aspergillus niger	1	>1	0.5	1	0.5	>1	1	1

- L: Leaves; St: stem; Fl: Flower; Ae: Aerial; MeOH: Methanol; EtOH: Ethanol; Hex: Hexane; DCM: Dichloromethane; EtOAc: Ethyl acetate
- (-): Not tested

Table -1B: Minimum inhibitory concentration (MIC; mg/ml) of standard antibiotics.

Microorganism	Standard antibiotic					
	Amox	Gent	Chl	Van	Amp β	Micon
Enterobacter aerogens	-	0.062	0.015	-	-	-
Escherichia coli	-	0.015	0.015	-	-	-
Klebsiella pneumonia	-	0.015	0.015	-	-	-
Proteus mirabilis	-	0.015	0.031	-	-	-
Proteus vulgaris	-	0.5	0.5	-	-	-
Pseudomonas aeruginosa	-	0.015	-	-	-	-
Salmonella typhi	0.062	0.015	0.062	-	-	-
Shigella disenteriae	-	0.015	0.1	-	-	-
Staphylococcus aureus	0.1	-	-	0.015	-	-
Bacillus subtilis	0.1	-	-	0.015	-	-
Candida albicans	-	-	-	-	0.015	0.031
Candida glabrata	-	-	-	-	0.031	0.062
Aspergillus niger	-	-	-	-	0.062	0.062

- Amox: Amoxycillin; Gent: Gentamicin; Chl: Chloramphenicol; Van: Vancomycin; Amp β: Amphoterecin β; Micon:Miconazole.
- (-): Not tested

Table- 2: Phytochemical constituents of various extracts of *E. hirta.*

Extract	Te	Ta	Fla	Al	Cg	An	Ph	Sa	Rs	Ste
L (MeOH)	-	+	+	+	+	-	-	+	+	+
St (MeOH)	+	-	-	+	-	-	-	-	+	-
Fl (MeOH)	+	+	-	+	+	-	-	-	+	-
Ae (MeOH)	-	-	+	+	-	-	-	-	-	-
Ae (EtOH)	+	+	+	+	+	-	-	+	+	+
Ae (Hex)	-	-	-	+	-	-	-	-	+	-
Ae (DCM)	-	-	-	+	-	-	-	-	+	-
Ae (EtOAc)	-	-	-	+	-	-	-	-	+	-

- Te: Terpenoids; Ta: Tannins; Fla: Flavonoids; Al: Alkaloids; Cg: Cardiac glycosides; An: Antraquinones; Ph: Phlobatannins; Sa; Saponins; Rs: Reducing sugar; Ste: Steroids
- L: Leaves; St: stem; Fl: Flower; Ae: Aerial; MeOH: Methanol; EtOH: Ethanol; Hex: Hexane; DCM: Dichloromethane; EtOAc: Ethyl acetate;
- + Present ; - Absent

Table- 3: In vitro cytotoxic activity (IC_{50}; n=3) of *E. hirta* extracts tested against Vero cell line.

Extract	IC_{50} (µg/ml)
L (MeOH)	98.6
St (MeOH)	103.2
Fl (MeOH)	82.5
Ae (MeOH)	131.9
Ae (EtOH)	126.0
Ae (Hex)	140.8
Ae (DCM)	128.3
Ae (EtOAc)	119.4

- L: Leaves; St: stem; Fl: Flower; Ae: Aerial
- MeOH: Methanol; EtOH: Ethanol; Hex: Hexane; DCM:Dichloromethane; EtOAc: Ethyl acetate