American Journal of Applied Sciences 8 (3): 201-205, 2011
ISSN 1546-9239

The Phytochemical Contents and Antimicrobial Activities of Malaysian *Calophyllum Rubiginosum*

[1]Suhaib Ibrahim ALkhamaiseh, [2]Muhammad Taher and [3]Farediah Ahmad
[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy,
[2]Department of Pharmaceutical Technology, Faculty of pharmacy,
International Islamic University Malaysia, Kuantan 25200, Malaysia
[3]Department of Chemistry, University Technology Malaysia,
81310 UTM Skudai, Johor Darul Takzim, Malaysia

Abstract: Problem statement: Many species of plants in Malaysia are widely used in folk medicine. However, *Calophyllum* species have been used in traditional medicine for their therapeutic values for many years. Several studies reported that antimicrobial, antifungal, anti-HIV and anti-cancer compounds were isolated from numerous *Calophyllum* species. **Approach:** The stem bark was extracted by EtOH, after with it was fractionated with n-Hexane, Dichloromethane (DCM) and MeOH by using vacuum liquid chromatography apparatus. Phytochemical contents were examined to evaluate the phinolic, flavonoid and flavonol contents. Also the antimicrobial activity was carried out by using disc diffusion and dilution method to evaluate antimicrobial activity of the crude and the fractions respectively. Six references microbial strains of human pathogens were used for examined the anti microbial activity. The two Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*) and two Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*) were used for antibacterial test. Also two fungal strains (*Candida albicans* and *Cryptococcus neoformans*) were used for antifungal test. **Results:** Although the *C. rubiginosum* has phytochemicals as all plants, but it was showed a high content of flavonol in the range of 11.9- 15.2 μg mL^{-1}. The *C. rubiginosum* fractions were showed no activity against gram negative and fungus. However, the non polar and semi polar fractions were showed a result MIC 12.5 μg mL^{-1} against *B.cereus* bacteria. While the MeOH fraction indicated for low or no activity against bacteria and fungus. **Conclusion:** At last, the optimistic result of this study encourage us to go forward for further studies in the future to isolate the active compound of the stem bark of *C. rubiginosum*, where it could lead to a new antibiotic, whereas this species never investigated before.

Key words: *Calophyllum rubiginosum*, phytochemical contents, antimicrobial activities

INTRODUCTION

Plants synthesize a vast range of organic compounds that are traditionally used. These are structurally varied and many are spread among a very limited number of species within the plant kingdom. Their function in plants is now most interest as some appear to have a key role in protecting plants from herbivores and microbial infection. These compounds are also used as dyes, fibres, glues, oils, waxes, flavouring agents, drugs and perfumes and they are viewed as potential sources of new natural drugs, antibiotics, insecticides and herbicides(Buchanan *et al*., 2000; Dewick, 2002; Gomez-Flores *et al*., 2006). Medicinal plants have been the essential for health care throughout the world since the earliest centuries and are still widely used right now. Recognition of their clinical, pharmaceutical and economic value is still growing, although this varies widely between nations. More than 35,000 plant species are still using around the world by various human culture for medicinal purpose. In Malaysia, more than 3000 species of higher plants were reported to have medicinal value and have been used for productions in various traditional health care systems. Plants are important for ethnopharmacological studies and drug development, not only when bioactive phytochemicals are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Newman *et al*.,

Corresponding Author: Suhaib Ibrahim ALkhamaiseh, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, International Islamic University Malaysia, Kuantan 25200, Pahang, Malaysia Tel: +609-5716400

2003; Philip *et al*, 2009). The genus *Calophyllum* belongs to Guttiferae family, which consist of 180-200 different species distributed in the warm humid tropics of the world. Wide phytochemical studies have been reported that *Calophyllum* genus rich in xanthones, coumarins, biflavonoids, chalcones, benzofurans and triterpenes (Pretto *et al*., 2004; Taher *et al*., 2005). Some of these species are commonly employed in folk medicine, where it is used to treat bronchitis, gastric and hepatic disturbances, pain, inflammation, diabetes, hypertension, diarrhea, herpes, rheumatism, varicose, hemorrhoids and chronic ulcer, also it is used for treating toothache and to prevent wound infections by micro-organisms (Cottiglia *et al*., 2004; Pretto *et al*., 2004). As there is no data relative to phytochemical and biological activity of the *rubiginosum* species had been reported. Therefore, the investigation carried on by using in vitro model of relevance to test the phytochemical contents and antimicrobial activity for EtOH crude extract and three main fractions from *C.rubiginosum* stem bark.

MATERIALS AND METHODS

Chemicals: All solvents used were of analytical grade. Hexane, Dichloromethane (DCM) and Methanol (MeOH). Silica gel 60 (230-400 mesh), sodium acetate and Aluminium chloride was obtained from MERCK, Folin-Ciocalteau's reagent, quercetin were purchased from Sigma chemicals. Gallic acid obtained from Alfa Aesar. The media were used in this study for bacteria, Mueller-Hinton Broth medium (MHB) used in MIC method and Mueller-Hinton agar used in disc diffusion method. And for fungus the potato dextrose agar was used for disc diffusion method. Chloramphenicol and Amphotericin B a standard antibiotic used to compare with for bacteria and fungus respectively.

Plant material: The stem bark of *Calophyllum rubiginosum* was collected from Botanical Garden in Melaka, Malaysia in July, 2009. Dr. Shamsul Khamis was identified the sample, botanist from University Putra Malaysia.

Extraction and fractionation: Air-dried and powdered stem bark (1 kg) was macerated in (2.5 L) 98% EtOH for 72 hour. The EtOH extract was filtrated and evaporated under reduced pressure to yield the dark brown gummy (200 g) EtOH crude. A part of EtOH crude (100 g) was chromatographed by VLC (silica gel 230-400 mesh, 1:30 ratio) to obtain three main fractions Hexane, CH_2Cl_2 (DCM) and MeOH.

Phytochemical screening:

Determination the Phenolic Content: Total phenolic content was carried out regarding to the Folin-Ciocalteau's method (Su *et al*., 2009; Ruiz-Cruz *et al*., 2010) with slight modifications. In brief, 0.5 ml of sample (10 mg mL^{-1}) or (standard), mixed with 5.0 mL of Folin-Ciocalteau's reagent (1:10),the mixture was settled in a tube for 5 min and then 4.0 mL of 1M Na_2CO_3 was added. The reaction mixture was incubated in dark at room temperature for 15 min. The 100 µL mixture was moved to 96-microwell plat and the absorbance was taken at 765 nm by using multi-detection microplate reader (Infinite M 200 Nanoquant). The sample was tested in triplicate and a calibration curve with five data points for gallic acid was obtained. The results were compared to gallic acid calibration curve and the total phenolic content of fractions was expressed as µg of gallic acid equivalents per 10 mg of extract:

$(Y = 0.0493X)$ and $R^2 = 0.9952$

Determination the Total Flavonoid: Total flavonoid contents were determined using the method of (Adedapo *et al*., 2008) with slight modification. A volume of 0.1 ml of sample was seated in a 96-microwell plate and mixed with 0.1 ml $AlCl_3$ ethanol (2%) solution. The mixture incubates at room temperature for 1 hour. The absorbance was measured at 420 nm using the multi-detection microplate reader (INFINITE M 200 Nanoquant). The yellow colour indicated flavonoids existence. The three main fractions were evaluated at a final concentration of 10 mg mL^{-1}. The blank was prepared by mixing the sample with ethanol. Total flavonoid content was calculated as quercetin equivalent (µg Que 10 mg^{-1} extract) using the following equation based on the calibration curve:

$(Y = 0.0181X + 0.0215)$, $R^2 = 0.9982$

Determination the Total Flavonol: Total flavonols in the plant extracts were estimated using the method of (Adedapo *et al*., 2008) With slight modification, in brief 50 µL of sample or (standard) mixed with 50 µL of $AlCl_3$ ethanol (2%) solution and 75 µL of sodium acetate (50 g L^{-1}) solutions, the mixture was seated in a 96-microwell plate and incubate for 2.30 hour at room temperature. The absorption measured at 440 nm by using the multi-detection microplate reader (INFINITE M 200 Nanoquant). The main fractions were evaluated at a final concentration of 10 mg mL^{-1}, where the blank was prepared by mixing the sample with ethanol. Total flavonoid content was calculated as quercetin equivalent (µg Que /10 mg extract) using the following equation based on the calibration curve:

($Y = 0.0123X - 0.0067$), $R^2 = 0.9994$.

Antimicrobial methodology:

Microbial strains: Six reference microbial strains of human pathogens were used for the antimicrobial activity. The two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778) and two Gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218) were used for antibacterial test. Also two fungal strains (*Candida albicans* ATCC 10231 and *Cryptococcus neoformans* ATCC 90112) were used for antifungal test.

Disc Diffusion Method: The agar disc diffusion method was employed for the determination of antimicrobial activities of the crude ethanol according to (Philip *et al.*, 2009; Qaralleh *et al*., 2010) with some modification. Briefly, inoculums were containing 10^6-10^7 CFU mL^{-1} was spread on Mueller- Hinton agar plates for bacteria and 10^4 CFU mL^{-1} was spread on potato dextrose agar for fungus strains. Using sterile forceps, the sterile filter papers (6 mm diameter) containing the crude extracts (10 or 20 µg), standard antibiotics (30 µg of chloramphenicol or 100 µg of amphotericin B) or negative control (DMSO) were laid down on the surface of inoculated agar plate. The plates were incubated at 37°C for 24 hour for the bacteria and at room temperature (18-20°C.) for 24-48 hour for yeasts strains. Each sample was tested in triplicate and the zone of inhibition was measured as millimeter diameter.

Microdilution method (MIC and MBC): Minimal Inhibitory Concentration (MIC) was measured according to (Seyydnejad *et al.*, 2010; Qaralleh *et al.*, 2010) by determining the smallest amount of fractions Hexane, DCM or MeOH or (standard) antibiotic needed to inhibit the growth of a test microorganism. This was done by using 96-microwell plates and performed on (Versa MaxTM Tunable) microplate reader. The assay plates were filled with Mueller-Hinton Broth medium (MHB) containing different concentrations of the hexane, DCM or MeOH fractions, Chloramphenicol or solvent control and the test microorganism (10^6-10^7CFU mL^{-1}). After 24 hour incubation periods at 37°C, the turbidity in each well was measured at 600 nm.

Minimal Bactericidal Concentration (MBC) was determined by transferring and spreading the treated culture broth of the wells containing the concentrations equal to and higher than the MIC on agar plates. The lowest concentration of the fractions or the standard antibiotic required to completely destroy test microorganisms (no growth on the agar plate) after incubation at 37°C for 24 hour was reported as Minimum Bactericidal Concentration (MBC).

RESULTS

The Phytochemical Contents was curried out in this study to explore the total phenolic, flavonoid and flavonol contents in three main fractions of C.*rubiginosum*. Table 1 explains these phytochemical contents values for each fraction. It is clearly appearing that all fractions have a considerable amount of flavonol, while the DCM fraction has the highest amount of flavonoid. The MeOH fraction achieves the highest value of phenolic content; also there was a significant amount of flavonoid.

The Antibacterial and Antifungal: The activities of ethanol crude using disc diffusion method are summarized in Table 2 The *C. rubiginosum* crude extract was screened at concentrations of 10 and 20 µg $disc^{-1}$ against six human pathogens. Both gram negative and fungal strains appear to be resistant to the tested concentrations since no inhibition zone was observed while the gram positive bacteria showed moderate sensitivity with no significant difference between the concentrations. Therefore the MIC and MBC were determined using different polarity fractions (hexane, DCM and MeOH) of *C. rubiginosum* against gram positive strains only. Table 3 demonstrates the MIC and MBC values. The Hexane and DCM fractions show MIC values 100 and 12.5 µg mL^{-1} against *S.aureus* and *B.cereus* respectively. The MBC values were 50 and 500 µg mL^{-1} for Hexane fraction, in addition 50 and 250 for DCM fraction. The inhibition growth of microbial at concentration as low as 12.5 µg mL^{-1} was indicated to potential antimicrobial activity of *C. rubiginosum* fractions. Furthermore, the highest activity was obtained against *B. cereus* for the hexane and DCM fractions MIC and MBC was 12.5 and 50 µg mL^{-1}, respectively.

Table 1: The Phytochemistry Screening of Hexane, DCM and MeOH fractions of *Calophyllum rubiginosum.*

Fractions	Phenolic content*	Flavonoid content (◊)	Flavonol content (◊)
Hexane	1.252±0.051	1.241±0.104	13.97± 0.746
DCM	1.398±0.152	13.421±2.475	15.26±0.915
MeOH	3.155±0.182	7.609±1.250	11.83±0.619

*: µg Galic Acid 10 mg^{-1} extract; ◊: µg quercetin 10 mg^{-1} extract

Table 2: Antimicrobial activity of ethanol crude extract using disc diffusion method

	Bacteria				Yeasts	
	S. a	*B. c*	*P. a*	*E. c*	*C. a*	*C. n*
Crude ethanol (10 μg disc^{-1})	9.0±0.7	8.5±0.6	0±0.0	0±0.0	0±0.0	0±0.0
Crude ethanol (20 μg disc^{-1})	10.0±0.6	11.0±0.5	0±0.0	0±0.0	0±0.0	0±0.0
Chloramphenicol (30 μg disc^{-1})	24.5±0.7	26±0.5	33±0.03	37±0.4	ND	ND
Amphotericin B (100 μg disc^{-1})	ND	ND	ND	ND	20±0.7	19±0.4
Negative control	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0

Mean diameter of zone of inhibition in mm± standard deviation including the diameter of the disc 6 mm. *S.a*: *S. aureus; B.c: B. cereus; P.a: P. aeruginosa; E.c: E. coli; C.a: C. albicans; C.n: C. neoformans* Negative control: 100% DMSO; 0: no inhibition zone

Table 3: MIC and MBC of Chloramphenicol control, Hexane, DCM and MeOH fractions.

	S. aureus (μg mL^{-1})		*B. cereus* (μg mL^{-1})	
Fraction	MIC	MBC	MIC	MBC
Hexane	100	500	12.5	50
DCM	100	250	12.5	50
MeOH	>1000	ND	>1000	ND
Chloramphenicol	20	ND	20	ND

ND: Not Determined

DISCUSSION

Medicinal plants considered as greatest resources of novel bioactive substances. A lot of studies indicated that medicinal plant contains methanol soluble compounds. These compounds have a potentially significant in treating human and animal pathogens, including bacteria and fungi (Mohan *et al*., 2008). Phenolic compounds are most abundant secondary metabolites in the plants. Additionally, it has been found in many food and their derivatives (Velioglu *et al*., 1998). Flavonols and flavonoids are a part of phenolic compounds and there are sharing in the physical properties. Numerous phyto-chemicals have demonstrated antibacterial activity and the various mechanisms of action have been described. Phenolics are a large group of compounds that have a variety of antibacterial mechanisms. The subsequent groups have specific mechanisms of action. Simple phenols are worked by substrate deprivation and membrane disrupting. Flavonoids attach to adhesions. The mechanism of action for flavonols is unknown. Other groups like terpenoids are operating by membrane disruption. Alkaloids can intercalate into the cell wall and/or DNA (Iqbal *et al*., 2000). These information support our results in this study where the fractions which high content of flavonoid and flavonol showed a significant result against gram positive bacteria. In this report, no activity was observed against Gram-negative bacteria and yeasts. This is in agreement with many antimicrobial studies, which showed the antimicrobial activity of some species belong to *Calophyllum* genus. Hien Ha *et al* reported that *C. inophyllum* extracts demonstrated promising antibacterial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* while the gram negative *P. aeruginosa* was resistant to this extracts (Ha *et al*., 2009). Pretto and his co-authors showed that all the parts (roots, stems, leaves, flowers and fruits) of *C. brasiliense* exhibited antimicrobial activity against Gram-positive bacteria and no activity was observed against Gram-negative bacteria and yeasts tested (Pretto *et al*., 2004). On the other hand, this difference in susceptibility between the bacteria is related to the outer membrane of gram-negative bacteria. The outer membrane endows the bacterial surface with strong hydro-philicity and acts as a strong permeability barrier (Nikaido and Nakae, 1979). The Hexane and DCM fractions of the stem bark of *C. rubiginosum* showed strong antibacterial activity against *B. cereus*, with MIC values of 12.5 μg mL^{-1}. It is observed that the antimicrobial agents of *C. rubiginosum* consisted of low to moderate polar compounds.

CONCLUSION

Regarding to the results in this study we can conclude that the stem bark of *C. rubiginosum* contains phenolic, flavonoid and flavonol content. However, the phytochemical content available in this plant it might contribute to inhibit the bacteria growth. As illustrated in the result the non and semi polar fractions had the ability to inhibit the gram positive bacterial growth. Therefore, there is a high interest for identifying alternative natural and safe sources of antibacterial agents from plant original. As there no data relative to biological activity of the *rubiginosum* species had been reported, therefore further work deserve to investigate more this species.

ACKNOWLEDGMENT

The researchers thank the Ministry of Higher Education Malaysia for supporting this study through FRGS 0409-103 grant.

REFERENCES

Adedapo, A., F. Jimoh, A. Afolayan and P. Masika, 2008. Antioxidant activities and phenolic contents of the methanol extracts of the stems of acokanthera oppositifolia and adenia gummifera. BMC Complementary Alternative Med., 8: 54. DOI: 10.1186/1472-6882-8-54

Buchanan, B.B., W. Gruissem and R.L. Jones, 2000. Biochemistry and Molecular Biology of Plant. 1st Edn., American Society of Plant Physiologists, USA., ISBN-10: 0943088372, pp: 1367.

Cottiglia, F., B. Dhanapal, O. Sticher and J. Heilmann, 2004. New chromanone acids with antibacterial activity from *calophyllum brasiliense*. J. Nat. Prod., 67: 537-541. DOI: 10.1021/np030438n

Dewick, P.M., 2002. Medicinal Natural Products: A Biosynthetic Approach. 3rd Edn., JohnWiley and Sons, USA., ISBN-10: 0470741686, pp: 550.

Gomez-Flores, R., P. Tamez-Guerra, R. Tamez-Guerra, C. Rodriguez-Padilla and E. Monreal-Cuevase *et al*., 2006. *In vitro* antibacterial and antifungal activities of *nopalea cochenillifera* pad extracts. Am. J. Infec. Dis., 2: 1-8. DOI: 10.3844/ajidsp.2006.1.8

Ha, H.M., V.T. Nguyen, K.Q.C. Nguyen, E.L. Cheah and P.W. Heng, 2009. Antimicrobial activity of *Calophyllum inophyllum* crude extracts obtained by pressurized liquid extraction. Asian J. Traditional Med., 4: 141-146.

Iqbal, A., F. Aqil and M. Owais, 2000. Modern phytomedicine: Turning Medicinal plants into Drugs. 1st Edn., Wiley-VCH, Weinheim, ISBN-10: 3-527-31530-6, pp: 404.

Mohan, S., A.B. Abdul, S.I.A. Wahab, A.S. Al-Zubairi and M.M. Elhassan *et al*., 2008. Antibacterial and antioxidant activities of *typhonium flagelliforme* (Lodd.) blume tuber. Am. J. Biochem. Biotechnol., 4: 402-407. DOI: 10.3844/ajbbsp.2008.402.407

Newman, D.J., G.M. Cragg and K.M. Snader, 2003. Natural products as sources of new drugs over the period 1981-2002. J. Nat. Prod., 66: 1022-1037. DOI: 10.1021/np030096l

Nikaido, H. and T. Nakae, 1979. The outer membrane of gram-negative bacteria. Adv. Microb. Physiol., 20: 163-250. PMID: 394591

Philip, K., S.N.A. Malek, W. Sani, S.K. Shin and S. Kumar *et al*., 2009. Antimicrobial activity of some medicinal plants from Malaysia. Am. J. Applied Sci., 6: 1613-1617. DOI: 10.3844/ajassp.2009.1613.1617

Pretto, J.B., V. Cechinel-Filho, V.F. Noldin, M.R.K. Sartori and D.E.B. Isaias *et al*., 2004. Antimicrobial Activity of Fractions and Compounds from *Calophyllumbrasiliense* (Clusiaceae/Guttiferae). Z. Naturforsch, 59: 657-662. PMID: 15540598

Qaralleh, H., S. Idid, S. Saad, D. Susanti and M. Taher *et al*., 2010. Antifungal and antibacterial activities of four malaysian sponge species (Petrosiidae). J. De Mycologie Médicale, 4: 315-320. DOI: 10.1016/j.mycmed.2010.10.002

Ruiz-Cruz, S., E. Alvarez-Parrilla, L.A. Rosa, A.I. Martinez-Gonzalez and J.J. Ornelas-Paz *et al*., 2010. Effect of Different Sanitizers on Microbial, Seneory and Nutritional Quality of Fresh-Cut Jalapeno Peppers. Am. J. Agric. Biol. Sci., 5: 331-341. DOI: 10.3844/ajabssp.2010.331.341

Seyydnejad, S.M., M. Niknejad, I. Darabpoor and H. Motamedi, 2010. Antibacterial activity of hydroalcoholic extract of *callistemon citrinus* and *albizia lebbeck*. Am. J. Applied Sci., 7: 13-16. DOI: 10.3844/ajassp.2010.13.16

Su, X.Y., Z.Y. Wang and J.R. Liu, 2009. *In vitro* and *in vivo* antioxidant activity of Pinus koraiensis seed extract containing phenolic compounds. Food Chem., 117: 681-686. DOI: 10.1016/j.foodchem.2009.04.076

Taher, M., M.S. Idris, F. Ahmad and D. Arbain, 2005. A polyisoprenylated ketone from *Calophyllum enervosum*. Phytochemistry, 66: 723-726. DOI: 10.1016/j.phytochem.2005.02.013

Velioglu, Y.S., G. Mazza, L. Gao and B.D. Oomah, 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agricul. Food Chem., 46: 4113-4117. DOI: 10.1021/jf9801973