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In vitro antimicrobial activity of *Muntingia calabura* extracts and fractions

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The aim of the present study is to determine the *in vitro* antimicrobial activity of various extracts, partitions and fractions of *Muntingia calabura* (Elaeocarpaceae) leaves against a selected panel of microorganisms. The leaves of *M. calabura* were soaked separately in the aqueous, chloroform and methanol solvent systems in the ratio of 1:20 (w/v) for 72 h and these procedures were repeated three times. Antimicrobial testing was carried out using the micro -broth dilution method. The microbes targeted were *Staphylococcus aureus* 25923, *S. aureus* 33591 (a multi-drugs resistant *S. aureus* (MRSA) isolate), *Escherichia coli* 35218, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* 10231 and *Microsporum canis* ATCC 36299. The methanol extract inhibited MSSA (MIC = 1250 µg/ml; MBC = 1250 µg/ml) and MRSA (MIC = 1250 µg/ml; MBC = 1250 µg /ml) and considered the most effective extract and was further partitioned sequentially using the aqueous, petroleum ether and ethyl acetate. The ethyl acetate partition exhibited effective antibacterial activities with the MIC/MBC value of 156 and 313 µg/ml against *S. aureus* 25923 and *S. aureus* 33591, respectively. The ethyl acetate partition underwent fractionation process and yielded 15 fractions (A1-A15) of which only fractions A9 to A15 effectively inhibited the growth of *S. aureus* 25923 and *S. aureus* 33591 with MIC/MBC values ranging from 78 to 2500 µg/ml.

Key words: *Muntingia calabura*, *Elaeocarpaceae*, antimicrobial activity, micro-broth dilution, ethyl acetate fraction.

INTRODUCTION

Discovery of new antimicrobial compounds with diverse chemical structures and novel mechanisms of action becomes an urgent attention currently. The development of resistant strains of bacteria has increased the need for new antibiotics (Eloff, 1998). Higher plants, which are able to produce photosynthesis, produce hundreds to thousands of diverse chemical compounds with different biological activities (Hamburger and Hostettmann, 1991). It is believed that these compounds have an important ecological role. They can work as pollinator attractants and as chemical defenses against insects, herbivores and microorganisms (Harborne, 1990). These anti-microbial compounds produced by plants are active against plant and human pathogenic microorganisms (Sarac and Ugur, 2007). There are several reports in the literature regarding the antimicrobial activity of plant crude extracts and the bioassay-guided fractionation of those extracts that yielded active principles (Rabe and Van Staden, 2000; Palombo and Semple, 2001; Portillo et al., 2001; Srinivasan et al., 2001; El-Seedi et al., 2002; Zgoda-Pols et al., 2002).

Muntingia calabura is widely cultivated and has become one of the common roadside trees in Malaysia. It is known locally in Malay as 'Kerukup Siam'. It is native to the American continent and is widely cultivated in warm areas of Asian region, including Malaysia (Chin, 1989). Various parts of this tree have several documented

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medicinal uses in both Southeast Asia and tropical America (Kaneda et al., 1991; Nshimo et al., 1993). The roots have been employed as an emmenogogue in Vietnam and as an abortifacient in Malaysia. In the Philippines, the flowers of this species have been used to treat headaches, and as an antidyspeptic, antispasmodic and diaphoretic. Infusions of the flowers of this plant are drunk as a tranquillizer and tonic in Colombia (Perez-Arbelaez, 1975; Kaneda et al., 1991).

Scientifically, several types of flavonoids and flavones have been isolated and identified from this plant (Keneda et al., 1991; Su et al., 2003; Chen et al., 2005) with the first two authors also reported on their anti-tumor activity. The aqueous extract of this plant also possesses opiod-mediated antinociception (Zakaria et al., 2007, 2005a). In addition, the *M. calabura* leaves extracts also possesses anti-inflammatory and anti-pyretic properties (Zakaria et al., 2007a, b, 2008), antibacterial activity (Zakaria et al., 2006) and antistaphyloccocal activity (Zakaria et al., 2007d) . Therefore, the main objective of this study is to search for the active fraction with strong antimicrobial activity which could serve as a good candidate for the development of new antimicrobial agents.

MATERIALS AND METHODS

Plant materials

The leaves of *M. calabura* were collected from its natural habitat in Shah Alam, Selangor, Malaysia in January 2008. The leaves have been previously identified by a botanist at Universiti Putra Malaysia (UPM) Serdang Selangor, and a voucher specimen (SK 964/04) has been preserved at the Herbarium of the laboratory of Natural product, Institute of Bioscience, UPM, Selangor, Malaysia.

Preparation of *M. calabura* extracts

The leaves of *M. calabura* were air-dried on the laboratory bench at room temperature for two weeks. The dried leaves were then ground into small particles, weighed (1 kg) and soaked in chloroform, methanol and aqueous in the ratio of 1:20 (w/v) for 72 h. To remove solid plants material, the first supernatant was filtered using cotton and followed by a filter paper (Whatman No.1, Whatman Ltd., Kent, UK). The filtrate of methanol and chloroform were concentrated by evaporation in a vacuum rotavapor at 40°C while the aqueous extract was subjected to the freeze-drying process. All the extracts then assayed for antimicrobial activity.

Preparation of *M. calabura* partitions and fractions

The preparation of partitions from the most effective extract, which is, in this case, the methanol extract, was carried out by sequential extraction of the crude methanol extract with petroleum ether, ethyl acetate and aqueous solvents. Each partition was then subjected to antimicrobial assays against *Staphylococcus aureus* 25923 and *S. aureus* 33591 and the most effective partition, in this case, the ethyl acetate partition, was subjected to the fractionation process. The ethyl acetate extract was fractionated by vacuum liquid chromato-graphy (VLC) using silica gel 60 (1.07747 Merck, Germany). The column was eluted by stepwise elution with hexane and ethyl acetate and finally with methanol to give thirty-five fractions. Analytical thin layer chromatography (TLC) on silica gel 60 F_{254} plates (Merck, Germany) was used to identify the similar fractions. The fractions having the same chromatograms were combined. The obtained fractions were subjected to the antimicrobial assay.

Microorganisms tested

Microorganisms tested in this study were obtained from Forest Research Institute of Malaysia (FRIM) namely, *S. aureus* ATCC 25923, *S. aureus* ATCC *33591* (A multi-drugs resistant *S. aureus* (MRSA) isolate), *Esherichia coli ATCC 10536*, *Pseudomonas aeruginosa ATCC 27853*, *Candida albicans ATCC 10231* and *Microsporum canis ATCC 36299.*

Antimicrobial screening

The dry *M. calabura* extracts and fractions were evaluated against the test microorganisms using liquid micro-dilution method described by Society of Japanese Chemotherapy (1990) with slight modification to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Test samples were dissolved in dimethyl sulphoxide (DMSO) except for the aqueous extract of *M. calabura* that was dissolved in distilled water. A 100 µl volume of the extract sample was transferred to the first well of each row, and serial 2-fold dilutions were performed; the remaining 100 µl was discarded. A 100 µl volume of working suspension was added to each well. A number of wells were reserved in each plate for sterility control, inoculum viability (no extract added) and the DMSO inhibitory effect. The final volumes in wells were 100 µl. A standard MHB was employed for the bacterial assays. The samples for antimicrobial tests were incubated at 35°C for 24 h, while the antifungi tests were performed at 25°C over 24 h. The growth of the microorganisms was determined by turbidity. Clear well indicated absence of microorganism growth. The MIC values were defined as the lowest concentration of test samples that completely inhibited the visible growth. The MBC was determined using methyl thiazolyl tetrazolium chloride (MTT). The lowest concentration showing no growth was identified as the MBC. The tests were carried out in triplicate.

RESULT AND DISCUSSION

Preliminary antimicrobial screening of *M. calabura* (Table 1) showed that the aqueous and chloroform extracts did not give significant inhibitory effects against all test microorganisms since the MIC/MBC values were > 5000 µg/ml. However, the methanol extract gave moderate inhibitory effect against *S. aureus* 25923 and *S. aureus* 33591 with the MIC/MBC value 1250 and 2500 µg/ml respectively. None of the crude extracts were effective against the test fungi tested, *C. albicans* and *M. canis*.

Based on the result, the methanol extract was considered to be the most active extract and it was further partitioned with aqueous, petroleum ether and ethyl acetate to separate polar and non polar compounds. These partitions of methanol extract were also subjected to the antimicrobial testing. Interestingly, only the ethyl acetate partition followed by the aqueous partition gave positive antibacterial activity with the MIC/MBC values ranging from 156 to 2500 µg/ml (Table 2). Therefore, 10 g of ethyl acetate extract was

Table 1. The MIC and MBC values of various extracts of *M. calabura*.

Extracts	Assay (µg/ml)	Inhibitory potential					
		MSSA	MRSA	Ec	Pa	Ca	Mc
Aqueous	MIC	> 5000	> 5000	> 5000	> 5000	> 5000	> 5000
	MBC	> 5000	> 5000	> 5000	> 5000	> 5000	> 5000
Chloroform	MIC	> 5000	2500	> 5000	> 5000	> 5000	> 5000
	MBC	> 5000	2500	> 5000	> 5000	> 5000	> 5000
Methanol	MIC	1250	2500	> 5000	> 5000	> 5000	> 5000
	MBC	1250	2500	> 5000	> 5000	> 5000	> 5000

MSSA: *S. aureus* ATCC 25923; MRSA: *S. aureus* ATCC 33591; Ec: *E. coli*; Pa: *P. aeruginosa*; Ca: *C. albicans*; Mc: *M. canis*.

Table 2. The MIC and MBC values of partitions of methanol extract of *M. calabura*.

Extracts	Assay (µg/ml)	Inhibitory potential	
		***S. aureus* 25923**	***S. aureus* 33591**
Petroleum ether	MIC	> 5000	> 5000
	MBC	> 5000	> 5000
Aqueous	MIC	625	1250
	MBC	625	1250
Ethyl acetate	MIC	156	313
	MBC	156	313

further fractionated by vacuum liquid chromatography (VLC) using silica gel 60 to give 35 fractions. These fractions were pooled together according to their chromatogram similarity to yield fifteen fractions labeled as A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14 and A15. The fractions were again tested against *S. aureus* 25923 and *S. aureus* 33591 and the findings revealed that only fractions A9 until A15 exhibited antimicrobial activity against the bacteria with the MIC and MBC values range from 78 to 2500 µg/ml (Table 3). Fraction A10 which showed the maximum inhibitory potential (MIC/ MBC 78 µg/ml) will be further purified to determine the active compound responsible for this activity.

Staphylococci have been reported to be one of the most commonly encountered pathogens in clinical practice. Furthermore, *S. aureus* has also been reported to be a major cause of nosocomial infections, food poisoning and a wide-range of other disorders (Rubin et al., 1999). There has been an alarming increase in nosocomial staphylococcal infections by multiple drug resistance strains of *S. aureus* in recent years (Al-Masaudi et al., 1991; Hiramatsu et al., 1997). Therefore, there is a need to find new antimicrobial compounds, particularly antistaphylococcal agents. The lower MIC/MBC conferred by fraction for the two strains of *S. aureus* compared to extracts and partitions (2500 to 78 µg/ml) could be due to the fact that the fractions consist of pure compounds while the extracts as well as the partitions contained various types of compounds, which included the ineffective fractions A1 until A8.

Findings by Zakaria et al. (2006) have demonstrated the presence of flavonoids, triterpenes, saponins steroids and tannins in *M. calabura* leaves. Several mechanisms of action could be suggested with regards to those groups of chemical compounds, particularly flavonoids and tannins, which are presence in the *M. calabura* extracts. Some of the flavonoids that favor polar solutes entry, like rutin and quercetin, bind to the bacteria's structural membrane proteins called porines, causing changes in the tridimentional conformation exposuring the hydrophilic character of the pore, which lead to an easier passage of other polar bioactive compounds via diffusion (Alvarez et al., 2006). The ability of tannins to form chelates with metal ions, particularly iron, which lead to the disruption of the *S. aureus* membrane, could be one of the possible factors that contribute to its antimicrobial activity (Akiyama et al., 2001). In addition, tannins, like tannic acid, has a greater relative binding efficiency to iron and may act with iron from the medium

Table 3. The MIC and MBC values of various fractions isolated from the ethyl acetate fractions of *M. calabura* methanol extract.

Fractions	*S. aureus* ATCC 25923		*S. aureus* ATCC 33591	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
A1	> 5000	> 5000	> 5000	> 5000
A2	> 5000	> 5000	> 5000	> 5000
A3	> 5000	> 5000	> 5000	> 5000
A4	> 5000	> 5000	> 5000	> 5000
A5	> 5000	> 5000	> 5000	> 5000
A6	> 5000	> 5000	> 5000	> 5000
A7	> 5000	> 5000	> 5000	> 5000
A8	> 5000	> 5000	> 5000	> 5000
A9	2500	2500	2500	2500
A10	78	78	313	313
A11	156	156	313	313
A12	156	156	625	625
A13	156	156	625	625
A14	156	156	625	625
A15	156	156	625	625

to form chelates and deplete iron from microorganisms. It is well known that aerobic microorganisms require iron to perform a variety of functions, like reduction of ribonucleotide and formation of haem (Chung et al., 1998). Besides, tannins of the catechin group have also been shown to exhibit antimicrobial activity via mechanisms that involved damage to the membrane, for example the leakage of 5,6-carboxyfluorescin from phosphatidyl choline liposomes (Ikigai et al., 1993). In conclusion, the present study successfully isolated fractions with antistaphylococcal activity from the methanol extract of *M. calabura*. Further studies are being planned in our laboratory to identify the chemical structure of compounds presence in the effective fractions.

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