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The *in vitro* Antibacterial Activity of *Corchorus olitorius* and *Muntingia calabura* Extracts*

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Abstract: The present study was carried out to evaluate the possible antibacterial activity of aqueous (AEMC), methanol (MEMC) and chloroform (CEMC) extracts of *Muntingia calabura* as well as methanol (MECO) and chloroform (CECO) extracts of *Corchorus olitorius* using the *in vitro* disc diffusion methods. The sterilized blank discs (6 mm diameter) was impregnated with 20 µL of the respective extract (the concentrations were 10,000, 20,000, 40,000 and 50,000 ppm for *C. olitorius* and 10,000, 40,000, 70,000 and 100,000 ppm for *M. calabura*) and tested against *Salmonella enteritidis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Vibrio parahemolyticus*, *Pseudomonas aeruginosa* and *Salmonella typhi*. The AEMC was effective in inhibiting the growth of all bacteria at the concentration of 70,000 ppm; the MEMC was effective in inhibiting the growth of *C. freundii*, *K. pneumoniae*, *V. cholerae*, *V. parahemolyticus* and *S. typhi*; and the CEMC was ineffective at all concentrations tested. However, the MECO and CECO were ineffective against all bacteria tested. Except for *P. aeruginosa*, the standard antibiotic chloramphenicol (30 µg µL⁻¹) was found to give inhibition zone of more than 20 mm against all bacteria tested. Based on this study, we concluded that *M. calabura*, but not *C. olitorius*, possesses a potential antibacterial activity against the selected microorganisms and this may provide a basis for the isolation of compounds of biological interest from *M. calabura*.

Key words: *Muntingia calabura*, *Corchorus olitorius*, antibacterial activity, aqueous extract, methanol extract, chloroform extract

Introduction

Herbal remedies are widely known to be used in the treatment of many infectious diseases throughout the history of mankind. Plant materials continue to provide a major source of natural therapeutic remedies and play an important role in health care in many developing countries (Czygan, 1993; Ody, 1993). With the raising problems of side effects and limited efficacy (Gupta *et al.*, 1998;

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Corazo *et al.*, 1999) new, safer and more effective antibiotics need to be developed and produced and researchers are nowadays turning to natural products, especially from plants (Nitta *et al.*, 2002; Souza *et al.*, 2003) as their main source of bioactive compounds with antibacterial properties.

Muntingia calabura L. and *Corchorus olitorius* L., also known locally to the Malays as *Kerukup siam* and *Senaung betina*, are plants of the family Elaeocarpaceae (Chin, 1989) and Tiliaceae (Zeghichi *et al.*, 2003), respectively. Scientifically, several types of flavonoids and flavonones has been isolated and identified from *M. calabura* by Kaneda *et al.* (1991), Su *et al.* (2003) and Chen *et al.* (2005) with the first two authors also reported on their anti-tumour activity. On the other hand, the seeds of *C. olitorius* have been reported to contain essential oil with estrogenic activity (Watt and Breyer-Brandwijk, 1962) as well as several cardiac glycosides (Negm *et al.*, 1980). Recent study has also shown that the *M. calabura* and *C. olitorius* aqueous extracts possesses opioid-mediated antinociception (Zakaria *et al.*, 2004, 2005a) and antibacterial activity (Zakaria *et al.*, 2005b, c). The latter activity was observed only in the aqueous and methanol, but not chloroform, extracts of *M. calabura* leaves and the methanol and chloroform extracts of *C. olitorius* leaves when they were tested against *Corneybacterium diphtheria*, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, *Staphylococcus epidermidis*, *Kosuria rhizophila*, *Shigella flexneri*, *Escherichia coli*, *Aeromonas hydrophila* and *Salmonella typhi*, respectively.

The basis for carrying the present study was attributed to our observations and finding of report, such as the one published by Lin *et al.* (1999), on plants with other pharmacological activities, like pain or inflammation relieving properties, that also showed antibacterial activity and to the problems related to the presently available antibiotics as mentioned earlier. Based on our preliminary reports on both plants ability to inhibit the growth of several selected Gram positive and Gram negative bacteria (Zakaria *et al.*, 2005b, c), the aim of the present study was to extent the screening procedure of both plants against a selected group of Gram negative bacteria available in our laboratory and to compare on those plants effectiveness as antibacterial agents.

Materials and Methods

Materials

M. calabura and *C. olitorius* leaves were collected from Shah Alam, Selangor, Malaysia in January-February 2005 and voucher specimens, SK 964/04 and SK 963/04, were deposited at the Herbarium of Institute of Bioscience, Universiti Putra Malaysia, Selangor, Malaysia.

Microorganisms tested in this study were *Salmonella enteritidis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Vibrio parahemolyticus*, *Pseudomonas aeruginosa* and *Salmonella typhi*.

Methods

Fresh leaves of *M. calabura* and *C. olitorius* were oven-dried for 24 hours at 40°C according to the methods described by Somchit *et al.* (2003) but with slight modifications. The leaves of *M. calabura* and *C. olitorius* were then ground into small pieces under sterilized condition and the former was then extracted separately with aqueous, methanol and chloroform while the latter was extracted using only methanol and chloroform. All extraction was carried out in the ratio of 1: 20 (w/v) for 24 h by using the Soxhlet apparatus. The aqueous extract of *M. calabura* was kept at -80°C for 48 h and then freeze-dried for 72 h while the resultant extraction of methanol and chloroform of *M. calabura* and *C. olitorius* were completely evaporated by using rotary evaporator machine

(Somchit *et al.*, 2003). The obtained dried crude aqueous (AEMC), methanol (MEMC) and chloroform (CEMC) extracts of *M. calabura* were prepared into 10,000 ppm, 40,000 ppm, 70,000 ppm and 100,000 ppm concentrations while the methanol (MECO) and chloroform (CECO) of *C. olitorius* were prepared into 10,000, 20,000, 40,000 and 50,000 ppm concentrations by dissolving the dried AEMC in distilled water (DH₂O) and, the dried MEMC, CEMC, MECO and CECO in dimethyl sulfoxide (DMSO). Twenty micro liter of the respective extract were then loaded into empty sterilized blank discs (6 mm diameter, Oxoid, UK) and left to dry at room temperature under sterilized condition prior to subjection to antibacterial assay. In addition, commercial antibiotic discs (Chloramphenicol; 30 µg µL⁻¹) were used for comparison.

Preparation of Microorganism Culture

The above-mentioned bacteria were incubated at 37°C±0.5 for 24 h after injection into nutrient broth. Mueller Hinton Agar (MHA) (Oxoid, UK), sterilized in a flask and cooled to 40-50°C was poured, in the volume of. Fifteen microliter, into sterilized Petri dishes (diameter of 9 cm) and allowed to harden under room temperature. This is followed by homogenous distribution of 0.1 mL bacteria cultures (10⁶ bacteria per mL) onto medium in Petri dishes. Discs loaded with extracts were then positioned on the solid agar medium by pressing slightly (Sundar, 1996). Petri dishes were placed in incubator according to their respective growth temperature and condition for 18 to 24 h. At the end of the period, inhibition zones formed was measured in mm. The study was performed in triplicate and the formation of the inhibition zones were compared with those of antibiotic discs.

Results and Discussion

In term of the number of bacteria growth inhibited, the AEMC was more effective than the MEMC while the CEMC was not effective against any of the bacteria tested. The range of growth inhibition was between 7-13 mm with the AEMC and MEMC were more effective against *C. freundii*, *V. cholerae* and *S. typhi* (Table 1).

On the other hand, Table 2 showed that neither the MECO not the CECO produced any significant antibacterial activity against all the tested bacteria.

The present study demonstrated the potential antibacterial properties of *M. calabura*, but not of the *C. olitorius*, which is in contrast to our previous report (Zakaria *et al.*, 2005c) on the presence of antibacterial properties in *C. olitorius*. Present finding on the failure of MECO and CECO to inhibit Gram negative bacteria growth was concomitant with the previous report (Zakaria *et al.*, 2005c).

Furthermore, the ineffectiveness of CEMC and the ability of AEMC and MEMC, to inhibit bacteria growth were also in line with our previous finding (Zakaria *et al.*, 2005b). However, the lower than 13 mm in diameter of inhibitory zone obtained with both the AEMC and MEMC were not expected since the same extracts have been found to give inhibitory zone between 13-20 mm in the previous study (Zakaria *et al.*, 2005b).

The data obtained for both *M. calabura* and *C. olitorius* in the present study should not be use to conclude on their effectiveness as antibacterial agents since we have proven on their ability to inhibit bacteria growth in previous reports as mentioned earlier. The lower value obtained in term of the diameter of inhibitory zone might be attributed to the types of bacteria used. In term of the Gram negative bacteria like *S. enteriditis* and *S. typhi*, the presence of lipopolysaccharide (LPS) and their association to the systemic infections caused by these types of bacteria has been a major focus during the last decade, leading to a Journal of Endotoxin Research and several International Symposia

Table 1: The antibacterial activity of aqueous, methanol and chloroform extracts of *Muntingia calabura* determined by disc diffusion method

Bacteria	Concentration (ppm)											
	AEMC				MEMC				CEMC			
	10K	40K	70K	100K	10K	40K	70K	100K	10K	40K	70K	100K
<i>S. enteriditis</i>	-	+	+	+	-	-	-	-	-	-	-	-
<i>C. freundii</i>	-	++	++	++	-	+	+	++	-	-	-	-
<i>E. aeruginosa</i>	-	-	+	+	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	-	-	-	+	-	+	+	++	-	-	-	-
<i>V. cholerae</i>	-	+	++	++	-	++	++	++	-	-	-	-
<i>V. parahemolyticus</i>	-	-	-	-	-	-	+	+	-	-	-	-
<i>P. aeruginosa</i>	-	-	+	+	-	-	-	-	-	-	-	-
<i>S. typhi</i>	-	-	+	+	-	+	++	++	-	-	-	-

IZ = Inhibition zone (mm), - No inhibition zone + IZ ≤ 9.0 mm ++ 9.0 mm < IZ ≤ 13.0 mm, +++ 13.0 mm < IZ ≤ 16.0 mm ++++ 16.0 mm < IZ ≤ 20.0 mm +++++ 20.0 mm < IZ, Except for *P. aeruginosa* (IZ = 10 mm), Chloramphenicol gave inhibition zone of ≤ 20 mm against all bacteria

Table 2: The antibacterial activity of methanol and chloroform extracts of *Corchorus olitorius* determined by disc diffusion method

Bacteria	Concentration (ppm)							
	MECO				CECO			
	10K	20K	40K	50K	10K	20K	40K	50K
<i>S. enteriditis</i>	-	-	-	-	-	-	-	-
<i>C. freundii</i>	-	-	-	-	-	-	-	-
<i>E. aeruginosa</i>	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	-	-	-	-	-	-	-	-
<i>V. cholerae</i>	-	-	-	-	-	-	-	-
<i>V. parahemolyticus</i>	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	-	-	-	-	-	-
<i>S. typhi</i>	-	-	-	-	-	-	-	-

IZ = Inhibition zone (mm), -No inhibition zone (mm), Except for *P. Aeruginosa* (IZ = 10 mm), Chloramphenicol gave inhibition zone of ≤ 20 mm against all bacteria

(Levin *et al.*, 1993; Whitfield, 1995; Maskell and Allen, 1997). Although the data on LPS and invasion of gut epithelia are unclear and sometimes contradictory, several reports have correlated the differences in virulence with the capacity of LPS to activate complement through the alternate pathway (Grossman and Leive, 1984; Saxen *et al.*, 1984, 1987). However, recent studies have demonstrated that the long chain LPS only plays a secondary role in invasiveness of Gram negative bacteria like *S. enteriditis*, at least for rabbit gut epithelia (Martin *et al.*, 2000). The poor antibacterial activity of both *M. calabura* and *C. olitorius* could also be associated to finding made by Ernst *et al.* (1999). Ernst *et al.* (1999) have reported that LPS is involved in intracellular survival of salmonellae, probably by interacting with antibacterial peptides. Whether the same LPS interact with the antibacterial compounds present in those extracts needs further extensive researches, which is not the objective of the present study.

Furthermore, based on the present and previous findings it is plausible to suggest that the bioactive compound(s) responsible for the *M. calabura* observed antibacterial activity possessed a broad spectrum activity and this type of activity can also be seen with standard antibiotics such as tetracycline, streptomycin, cephalosporins and ampicillin (Cheesbrough, 1994). On the other hand, the bioactive compound(s) responsible for the antibacterial activity of *C. olitorius* as previously reported (Zakaria *et al.*, 2005c), but not seen in the present study, could be due to its narrow spectrum

of activity as can be seen with standard antibiotics like penicillin G, erythromycin, clindamycin and gentamicin (Cheesbrough, 1994). The reason for the former suggestion was that both extracts of *M. calabura*, AEMC and MEMC, were effective against both types of Gram positive and Gram negative bacteria while the reason for the latter suggestion was that both extracts of *C. olitorius*, MECO and CECO, produced significant antibacterial activity only against the Gram positive, but not Gram negative, bacteria as can be seen from the present and the previous studies (Zakaria *et al.*, 2005c). In addition, the broad antimicrobial action of the AEMC and MEMC could be ascribed to the anionic components such as nitrate, sulphates, chloride and thiocyanate beside other water-soluble components that are naturally occurring in most plant materials (Darout *et al.*, 2000). However, the ineffective effect of MECO and CECO could be due to little diffusion properties of both extracts in the agar or because fresh plants contain active substances, which may be affected or disappeared by the steps of extraction methods (El Astal *et al.*, 2005).

In comparison to the standard antibiotic chloramphenicol ($30 \mu\text{g } \mu\text{L}^{-1}$), the antibacterial activity of AEMC and MEMC were less promising and seems to be in line with the Peruvian, or even the Malays, folklore medicinal used that does not include *M. calabura* in the treatment of infectious diseases (Chin, 1989). Furthermore, the lower activity could be explained by the fact that the extracts used are crude extracts and not the purified compounds as chloramphenicol.

The AEMC and MEMC showed better killing action than the CEMC, which tend to support our earlier report (Zakaria *et al.*, 2005b) and thus should be used for further investigations to distinguish its components and their individual antimicrobial effect. Our recent findings have validated the use of *M. calabura* leaves for the treatment of some Gram negative bacterial infections, which have been linked to bacterial food poisoning. Although identification of chemical constituents is not part of the objective of this study, the present of various types of flavonoids, flavonones and flavans as reported by Kaneda *et al.* (1991), Su *et al.* (2003) and Chen *et al.* (2005) are believed to contribute to the observed activity of *M. calabura* (Diaz *et al.*, 1988; Ogunleye and Ibitoye, 2003). Other than that, the presence of saponins (Pretorius *et al.*, 2003), tannins (Diaz *et al.*, 1988; Ogunleye and Ibitoye, 2003) and glycosides (Chukwurah and Ajali, 2000), which are main constituents of leaves of many plants, could also be associated with the antibacterial activity of *M. calabura*. Further studies are being carried out in our laboratory to isolate the responsible constituents for further analysis. In addition, the results also showed that the polar compound(s), believed to be present in AEMC and MEMC, rather than the non-polar ones (present in CEMC) were more effective against the Gram-negative bacteria.

As reported earlier (Zakaria *et al.*, 2005c) the AECO was not used in the present study because of the sticky mucilaginous mucus released once the leaves were soaked in DH_2O . The sticky AECO takes time to dry and thus would provide a condition that will promote bacteria growth instead of inhibiting it. Although the present of acidic polysaccharide in AECO have been reported by Ohtani *et al.* (1995), their pharmacological effects including antibacterial activity, has never been reported or investigated. The polysaccharide has been reported to contain high amount of uronic acid and consisted of rhamnose, glucose, galacturonic acid, glucuronic acid as well as a methyl group.

Finally, we concluded that these results provided a basis for isolation of antibacterial compounds of interest from *M. calabura*, especially using the polar solvents like water or methanol. Further study is being carried out to isolate and identify the antibacterial compounds present in both extracts of *C. olitorius*.

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