

Full Length Research Paper

Effects of Malaysian medicinal plants on macrophage functions *in vitro* study

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The effects of aqueous leaf extract of eight local medicinal plant species on the phagocytic and antibacterial potential of mouse peritoneal macrophages against *Staphylococcus aureus* were investigated. The mouse macrophages cultures were incubated with various concentrations (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) of plant extracts in different sets of test tubes at 37°C for one hour. After incubation, their phagocytic and antibacterial effects on *S. aureus* were determined. Results have shown that the extracts of *Orthosiphon stramineus*, and *Andrographis paniculata* inhibited the phagocytic activity and intracellular killing of *S. aureus* compared with controls. Activated cells had more phagocytic activity and intracellular killing than normal macrophages. There were no differences in the viability of macrophage cells treated with extracts and those without extracts (control).

Key words: Aqueous plant extracts, murine macrophages, phagocytosis.

INTRODUCTION

Acanthaceae and *Lamiaceae*, families are groups of plants that have been widely used in natural medicine. *Andrographis paniculata* Nees (*Acanthaceae*) is widely used in the traditional medicine in Southeast Asia and China to treat various diseases, including diabetes, hypertension and cancer (Kumar et al., 2004). The plant leaves are known to contain diterpenes, flavonoids, stigmaterols, andrographolide together with several other diterpenoids (Siripong et al., 1992). Andrographolide, deoxyandro-grapholide, and neoandrographolide, the major diterpenoids of the *A. paniculata* leaves, have shown cytotoxic activity against human epidermoid carcinoma and leukemia cells (Siripong et al., 1992). The herbal extract of *A. paniculata* is useful as an anti-inflammatory (Siripong et al., 1992; Kumar et al., 2004; Sheeja et al., 2006) antioxidant (Tripathi and Kamat, 2007), antiviral (Calabrese et al., 2000), anticancer (Kumar et al., 2004), antimicrobial (Singha et al., 2003, Rattanachaikunsopon and Phumkhachorn, 2009),

hepatoprotective (Trivedi et al., 2007), immunostimulatory (See et al., 2002), anti-diabetic (Reyes et al., 2006), and hypotensive (Zhang and Tan, 1996) agents.

Orthosiphon stramineus, Benth (*Lamiaceae*) is among the popular medicinal plants used in traditional medicine for curing various diseases especially those affecting urinary tract, diabetes mellitus, eruptive fever, epilepsy, gallstone, hepatitis, rheumatism, tonsillitis, hypertension, syphilis, gonorrhea, renal calculus and menstrual disorder and to improve physical strength (Awale et al., 2003; Olah et al., 2003; Sriplang et al., 2007). In Malaysia, the tea prepared from the leaves is taken as a beverage to improve health and for treatment of kidney and bladder inflammation, gout and diabetes (Wanger, 1982).

O. stramineus is effective for alleviating hyperglycemia and improving lipid profile in diabetic rats (Sriplang et al., 2007), and it possesses antioxidant and hepatoprotective effects (Yam et al., 2007). Phytochemical screening of *O. stramineus* extract showed several chemically active constituents, such as terpenoids, polyphenols and sterols (Tezuka et al., 2000). The polyphenols have antioxidant activities (Fuhrman et al., 1995), which have been reported to be effective in reducing oxidative stress by

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inhibiting the formation of lipid peroxidation products in biological systems (Hollman and Katan, 1999). The purpose of this study was to investigate the effects of aqueous crude extracts of eight local medicinal plants on the phagocytic activity and the intracellular killing of *S. aureus* by mouse peritoneal macrophage *in vitro*.

MATERIALS AND METHODS

Experimental animals

Pathogen-free adult male 7 - 9 weeks BALB/c mice weighing 30 - 40 g used for this study were obtained from the animal house, Faculty of Medicine, University of Malaya. The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Malaysia, and Ethic No. PM/27/07/2009/MAA (R).

Preparation of plants extract

Crepis japonica, *O. stramineus*, *Baeckea frutescens*, *Piper betal*, *Piper sarmentosum*, *Hibiscus rosa sinensis*, *Elephantopus scaber* and *A. paniculata* were obtained from Ethno Resources Sdn Bhd, Selangor Malaysia, and identified by comparison with the Voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur. Leaves of each of the plants washed with distilled water, dried in an incubator (50°C) to constant weight for 1 week and then ground to powdered form after 1 week.

The dried powdered plants were successively extracted with water. Fifty grams of blended plants were weighed and placed into 1000 ml flask. Distilled water was added in a ratio of 1:20. After that, they were heated and stirred on a hot-plate for 3 h (80°C). Next, they were allowed to cool down using filter paper and a filter funnel to filter them. This step was followed by rotor vaporizer to remove the water. The aqueous extract was then submitted to lyophilisation by a freeze-dryer, to produce powdered forms of the extract and stored at -20°C until used (Rattanachaikunsopon and Phumkhachorn, 2009).

Preparation of bacteria

Bacterial cultures

Staphylococcus aureus were obtained from Medical Microbiology Unit, University Malaya Medical Centre (UMMC). Single colony of *S. aureus* from blood agar plate was aseptically transferred to sterile Brain Heart Infusion (BHI) broth and incubated overnight at 37°C for multiplication.

Bacterial counts

BHI broth containing *S. aureus* was centrifuged at 1000 g, 4°C for 15 min and discard supernatant. A Pellet was washed twice with sterile phosphate buffer saline pH 7.2, centrifuged at 1000 g, for 15 min to isolate and purify *S. aureus*. Re-suspend the pellet and adjust the concentration of bacteria to 4×10^5 bacteria/ml of cold RPMI 1640 medium (media used as supporting growth for many types of cell culture) by counting with a haemocytometer at 40 x magnifications.

Bacterial opsonisation

Normal mice serum (NMS) was incubated in water bath at 56°C for 30 min, to inactivate complement and centrifuge, 500 g, 10 min at 4°C to remove debris and then dilute NMS to 20% with RPMI 1640 medium. Incubate an equal volume of bacterial suspension in RPMI 1640 and diluted NMS for 30 min at 37°C on a rotary platform (this will allow attachment of IgG Fab to the surface of the bacteria). Serum was removed by centrifugation and opsonised bacteria were re-suspended to a concentration of 4×10^6 bacteria/ml RPMI 1640 medium (Tomita et al., 1981; Cuffini et al., 1987).

Collection of peritoneal macrophages

Mice were sacrificed by cervical dislocation. Resident peritoneal cells were obtained by washing the peritoneal cavity with warm PBS (37°C). The peritoneal lavages were collected in sterile test tube (this tube must be kept on ice and chilled before use since macrophages will adhere to the glass at room temperature). Macrophages were washed once with cold PBS and re-suspended in cold RPMI 1640. Cell viability was determined by Trypan blue exclusion method and was greater than 98%. The numbers of viable macrophages in this suspension was determined using haemocytometer chamber and adjust to 4×10^5 /ml RPMI 1640 in 10% inactivated calf serum. Elicited peritoneal cells were harvested from mice injected intra-peritoneally with 3 ml of sterile 2% starch solution in PBS 3 days before sacrificed to stimulate peritoneal macrophages (Ito et al., 1981).

Measurement of phagocytic capability and intracellular killing of bacteria by macrophage

The 200 µl of the cell suspension (resident or elicited macrophages) was placed into each well of TC chamber slide (Nanc) and was incubated for 2 h at 37°C, in an atmosphere of 95% air, 5% CO₂. Media and unattached cells were removed by gentle aspiration and wash with 100 µl warm RPMI 1640. Add 200 µl of various concentrations of plant extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) each was added into wells of TC chamber slide, except the control (add 200 µl of RPMI 1640).

TC chamber slide was incubated for one hour then 200 µl of opsonised bacteria (10:1 bacteria to cell ratio) were added into all the wells. TC chamber slide was incubated for 45 min at 37°C, in an atmosphere of 95% air, 5% CO₂ for phagocytosis and intracellular killing of bacteria to occur. The content of the well was aspirated and discarded, and washed wells twice with warm RPMI 1640 medium.

The 200 µl of gentamaicin (50 µg/ml) was added into all the wells and was incubated for 15 min to kill the extra-cellular and non-specifically attached bacteria. Wells and gasket were detached from the glass slide and were washed twice with warm RPMI 1640. The slide was stained with previously prepared acridine orange (0.14 mg/ml RPMI 1640) for 45 sec, and acridine orange was aspirated and discarded and washed briefly with warm RPMI 1640 to remove excess stain. The slide was then counter-stained with previously prepared crystal violet for one minute (prepared by adding 5 mg crystal violet to 10 ml PBS, shake vigorously and filter with Whatmans filter paper).

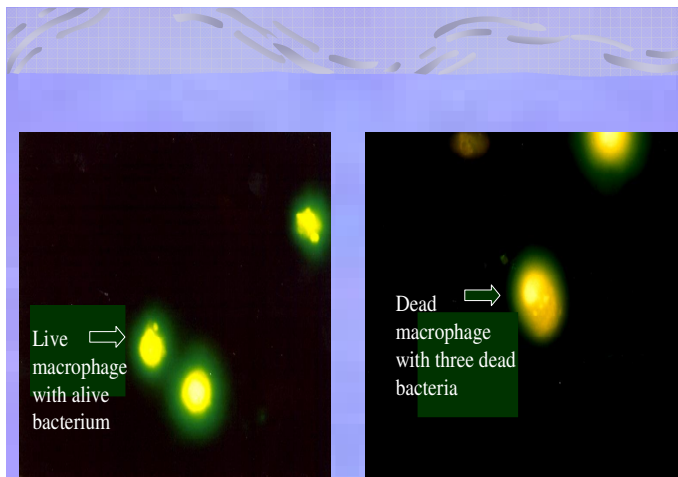
The slide was aspirated and crystal violet was discarded and washed briefly with warm RPMI 1640 to remove excess stain. The slide was air-dried and examined under fluorescence microscopy. Cell counts were performed under x 1000 magnification using oil immersion. The number of live and dead bacteria in 100 cells was counted by systemic scanning of each well (intracellular bacteria fluoresce green when viable and red when non-viable). Cells that had ingested at least 4 bacteria were considered positive. All

Table 1. Effect of *A. paniculata* on the phagocytic capability and intracellular killing of bacteria by murine peritoneal macrophages after incubation with different dilutions of plant extract.

<i>A. paniculata</i> extract concentration (mg/ml)	% Phagocytic cells		% Intracellular killing	
	Resident peritoneal cells (%)	Starch elicited peritoneal cells (%)	Resident peritoneal cells (%)	Starch elicited peritoneal cells (%)
3.2	20	32	6(1.2)	10(3.2)
1.6	29	44	7(2.03)	13(5.7)
0.8	43	50	10(4.3)	16(8)
0.4	50	59	12(6)	22(12.98)
0.2	62	68	18(11.16)	29(19.72)
Control	74	80	24(17.76)	38(30.04)

Table 2. Effect of *O. stramineus* on the phagocytic capability and intracellular killing of bacteria by murine peritoneal macrophages after incubation with different dilution of plant extracts.

<i>O. stramineus</i> extract concentration (mg/ml)	% Phagocytic cells		% Intracellular killing	
	Resident peritoneal cells (%)	Starch elicited peritoneal cells (%)	Resident peritoneal cells (%)	Starch elicited peritoneal cells (%)
3.2	22	30	5(1.1)	9(2.7)
1.6	33	40	5(1.7)	11(4.4)
0.8	45	49	8(3.6)	15(7.4)
0.4	53	57	11(5.8)	20(11.4)
0.2	60	64	14(8.4)	26(16.6)
Control	74	80	24(17.76)	38(30.04)

**Figure 1.** Shows the effect of aqueous plant extracts on the phagocytic capability and intracellular killing, fluorescence microscope (100 x).

measurement was carried out in duplicate (Drevets and Campbell, 1991; Salmah et al., 2004).

Percentage of bacteria phagocytised by macrophage = number of macrophages having one or more bacterial cell/100 macrophages count.

Percentage of intracellular killing of bacteria by macrophages = total number of bacteria that fluoresce red in all macrophages/total number of bacteria counted in all macrophages.

RESULTS

The effect of different concentrations of aqueous plant extract on the phagocytic capability and intracellular killing of bacteria is shown in Tables 1 and 2; and Figure 1. Extracts of *O. stramineus* and *A. Paniculata* caused inhibition of phagocytosis and intracellular killing of bacteria by murine peritoneal macrophages even at the lowest concentration tested. Between treated cells, these inhibitory effects were higher in elicited cells than normal resident cells. The average percentages of phagocytic bacteria were lower in treated (elicited and resident) cells than those of normal cells. Also there were differences observed in the viability of total number of cells between treated and control cultured cells according to the trypan blue exclusion method employed in any of the extract concentrations tested. There were no differences in the average percentages of the phagocytic activity among treated (elicited and resident) cells with the remaining plant extracts. The average phagocytic activity has a range of (66 - 74) with the same dilutions used as in Tables 1 and 2.

DISCUSSION

The present study has clearly demonstrated that aqueous extracts of *A. paniculata* and *O. stramineus* exert inhibitory effect on the phagocytic capability and intracellular killing of bacteria by murine peritoneal macrophages *in vitro* culture. Similar results have been reported with extracts from other plant families (Courreges et al., 1994; Benencia et al., 1995). However, in contrast to our results, several researchers using other plant extracts have shown no phagocytic functions (Simons et al., 1990; Benencia et al., 1994). Other researchers have demonstrated that some plant extracts have the ability to stimulate phagocytic function (Atal et al., 1986; Sharma et al., 1994). Using trypan blue exclusion method, results of the current study clearly indicate that there were no differences in the percentage of viability of cells observed between treated and controls. These findings are consistent with previous reports that have shown lack of toxicity of these extracts in different cell cultures (Andrel et al., 1985; Cordoba et al., 2006). Results of the present study have shown that the extracts inhibit the ingestion of bacteria. Though macrophages are critical for the control and elimination of a wide number of pathogens, they can also produce tissue damage associated with generation of toxic oxygen products (Badwey and Karnovsky, 1980). In conclusion, starch activated macrophages have more phagocytic activity and intracellular killing of *S. aureus* than normal macrophage. *O. stramineus* and *A. Paniculata* extracts showed inhibitory effect on phagocytic activity and intracellular killing, and no differences in the viability of macrophage cells treated with extracts and those without extracts (control).

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