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Effects of Plant Extracts on the Phagocytosis of *E. coli* by Peritoneal Macrophage *in vitro*

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Abstract: Eight plant species were picked randomly and their aqueous extracts have been screened to know their effects on the phagocytic capability and intracellular killing of *E. coli* bacteria by rat peritoneal macrophages. Macrophage cultures were incubated with different concentration of each plant extract for 1 hour. Among these aqueoud extracts, *Ageratum conyzoides* and *Malastoma melabathricum* inhibited the phagocytic capability and intracellular killing of *E. coli* compared with controls. Elicited (activated) cells have more phagocytic capability and intracellular killing than the resident (normal) macrophages. There were no differences in the viability of cells between treated cells with extract and controls.

Key words: *Ageratum conyzoides, Malastoma melabathricum,* rat macrophages, phagocytosis

Introduction

The Astreaceae and Melastomataceae are groups of plants that have been widely used in natural medicine. Among others, anti-inflammatory, spasmolytic, gastroprotective, anti-conceptive, anti-bacterial, anti-helmintic and anti-hypertensive activities have been reported (Yamamoto *et al.*, 1991; Abena *et al.*, 1993; Cheng *et al* 1993; Olajide, 1999; Perumal Samy *et al* 1999; Alen *et al.*, 2000; Silva *et al.*, 2000; Okunade, 2002; Jagetia *et al.*, 2003; Shirwaikar *et al.*, 2003). The anti-bacterial and anti-helmentic activities of some members of these families, such as *Ageratum conyzoides* and *Melastoma melabathricum* have been explained by their action on the immune system (Perumal Samy *et al* 1999; Alen *et al.*, 2000).

Ageratum conyzoides L., is an annual herb with long history of traditional medicine uses in many countries in the world, especially in the tropical and subtropical regions. A wide range of chemical compounds including alkaloids, flavonoids and terpentoids has been isolated from this species. Extracts and metabolites from these plant extracts have been found to possess pharmacological and insecticidal activities (Okunade, 2002). Such extracts also used for mental disorders and infectious diseases, cepgalgia, enteralgia and fever and contraceptive properties (Albena *et al.*, 1993).

A diminution in the phagocytic capability response of rat peritoneal macrophages was observed when these cells were treated with certain different plant extracts from other families (Van der Nat *et al.*, 1989; Courreges *et al.*, 1994; Benencia *et al.*, 1999). The plant ectract also inhibit the binding of opsonized particles to macrophages thus indicating that the observed anti-phagocytic effects is possibly due to failure of opsonized particle to bind these cells (Benencia *et al.*, 1999).

In the present study an attempt was, therefore, made to investigate the effects of certain plant extracts on the phagocytic capability and intracellular killing of *E. coli* by rat peritoneal macrophages, because currently there was no reports on such studies.

Materials and Methods

Experimental Animals: Pathogen-free adult *Spargue Dawley* rats aged 12 week-old weighing 180-220 grams were used throughout the experiments.

Preparation of Plants Extracts: Water-soluble extracts of *Cerpis Japonica, Elephantopus scaber, Ageratum conyzoides, Orthosipan aristatus, Melastoma melabathricum, Piper betel, Piper sarmentosum* and *Baeck frustescens*, were collected and identified by ethnobotanist. For the preparation of extracts, plants were washed with distilled water and dried in oven (50°C) separately. After fully dried (one week), plants were ground to powder form. Crude aqueous extracts was prepared, filtered and dried under vacuum in a rotary evaporator. The remaining resinous products were freeze-dried and stored at -20°C until used.

Preparation of Bacteria

Bacterial Cultures: *E. coli* grew colonies on blood agar, transfer colonies into Brain Heart Infusion (BHI) broth and cultured overnight at 37°C.

Bacterial Counts: BHI broth, centrifuge at 1000g, 4°C for 15 minutes and supernatant discarded. Wash pellet twice

centrifugally with PBS pH 7.2 at 1000g, 4°C for 15 minutes. Re-suspend and adjust concentration of bacteria to 4 X 10^6 bacteria/ml cold RPMI 1640 by counting with a haemocytometer at 400X magnification.

Bacterial Opsonization: Denature complement by immersing normal rat serum (NRS) in a 56oC water bath for 30 minutes. Centrifuge at 500g for 10 minutes at 4°C to remove debris and dilute NRS to 20% with RPMI 1640. Incubate an equal volume of bacterial suspension in RPMI 1640 and diluted NRS for 30 minutes 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 opsonized bacteria was re-suspended to a concentration of 4 X 10^6 bacteria/ml RPMI 1640 medium.

Collection of Peritoneal Macrophages: Rats were sacrificed by overdose of diethyl ether. 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 rube 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 the cold RPMI 1640. Cell viability, determined by trypan blue exclusion method, was always greater than 98%. The numbers of viable macrophages in this suspension was determined using haemocytometer chamber and adjusted to 4 X 10^6/ml RPMI 1640 in 10% inactivated calf serum.
In the same way elicited peritoneal cells were harvested from rat injected intra-peritoneally with 3 ml of sterile 2% starch solution in PBS 3 days before scrifice.

Measurement of Phagocytic Capability and Intracellular Killing of Bacteria by Macrophages: Place 200 μl of cell suspension residence or elicited macrophages) into each well of TC chamber slide (Nanc) and incubate for 2 hours at 37°C, in at atmosphere of 95% air, 5% CO_2 (during this step cells will adhere to the slides). Remove media and unattached cells by gentle aspiration and wash with 100 μl warm RPMI 1640.
Add 200 μl of different concentration of the each plant extract into each well except the controls (add 200 μl of RPMI 1640), after 1 hour incubation add 200 μl of opsonized bacteria (10:1 bacteria to cell ratio) into all the wells of slide. Incubate for 45 minutes at 37°C, in at atmosphere of 95% air, 5% CO_2 for phagocytosis and intracellular killing of bacteria to occur. Aspirate and discard contents of wells, wash wells 2 X with warm RPMI 1640.
Add 200 μl of gentamicin (50 μg/ml) into all the wells incubate for 5 minutes to kill the extra-cellular and non-specifically attached bacteria.
Detach wells and gasker from the glass slide and wash 2 X with warm RPMI 1640. Stain with previously prepared acridine orange (0.14 mg/ml RPMI 1640) for 45 second, aspirate and discard acridin orange and wash briefly with warm RPMI 1640 to remove excess stain. Counter stain with previously prepared crystal violet for 1 minute (prepared by adding 5 mg crystal violet to 10 ml PBS, shake vigorously and filter with Whatmans filter paper). Aspirate and discard crystal violet and wash briefly with warm RPMI 1640 to remove excess stain. Air-dry the slide and examine under fluorescence microscopy. Cell counts are performed under x1000 magnification using oil immersion. Count number of live and dead bacteria in 100 cells 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 measurements were carried out in duplicate.
Determine % phagocytosis (% phagocytosis = Number of macrophages having one or more bacteria/100 macrophages count). Determine % intracellular killing (% intracellular killing = total number of bacteria that fluoresce red in all cell/total number of bacteria counted in all cells.,

Result

Effect of plant extracts treatment on peritoneal macrophages:
Phagocytic capability and intracellular killing of bacteria: The effect of different concentration of aqueous plant extracts on the phagocytic capability and intracellular killing of bacteria is shown in Table 1 and 2.
Extracts of *A. conyzoides* and *M. melabathricum* caused inhibition of phagocytosis and intracellular killing of bacteria by rat 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 percentage of phagocytic bacteria were lower in treated (elicited and resident) cells than those of control cells.
Also there were no 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 concentration 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 ranges between (66-74) with the same dilations used as in Table 1 and 2.

Table 1: Effect of A. conyzoides extract on the phagocytic capability and intracellular killing of bacteria by rat peritoneal macrophages after incubation with different dilution of plant extract

A. Conyzoides extract concentration (mg/ml)	% Phagocytic cells		% intracellular killing	
	Resident peritoneal cels	Stache elicited Peritoneal cells	Resident peritoneal cells	Strach elicited peritoneal cells
3.2	20%	32%	6(1.2%)	10(3.2)
1.6	29%	44%	7(2.0%)	13(5.7%)
0.8	43%	50%	10(4.3%)	16(8%)
0.4	50%	59%	12(6%)	22(13%)
0.2	62%	68%	18(11.2%)	29(9.7%)
Control	74%	80%	24(17.8%)	38(36%)

Table 2: Effect of M. melabthricum extract on the phagocytic capability and intracellular killing of bacteria by rat peritoneal macrophages after incubation with different dilution of plant extracts

M. melabthricum extract concentration (mg/ml)	% Phagocytic cells		% intracellular killing	
	Resident peritoneal cels	Stache elicited Peritoneal cells	Resident peritoneal cells	Strach 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(17.8%)	38(36%)

Discussion

The present study clearly demonstrates that aqueous extracts of *A. conyzoides* and *M. melabathricum* exert inhibitory effect on the phagocytic capability and intracellular killing of bacteria by rat peritoneal macrophages *in vitro* cultured. Similar results have been obtained with plant extracts from other families (Van der Nat *et al.*, 1987; Correges *et al.*, 1994; Benencia *et al.*, 1995). However, , in contrast to our results, several researchers using other plant extracts have not show any influence on the phagocytic function (Simons *et al.*, 1990; 't Hart and Simons, 1992; Benencia *et al.*, 1994). Also, several workers using other plant extracts have shown that these extracts appeared to stimulate phagocytic function (Atal *et al.*, 1986; Sharma *et al.*, 1994).

The results of the current study also clearly indicate that there were no differences in the percentage of viability of cells observed between treated and controls according to trypan blue exclusion methods. These data are consistence with previous reports showing the lack of toxicity of these extracts indifferent cell culture (Andrei *et al.*, 1985; Cordoba *et al.*, 1991).

The results of the present study showed that the extracts inhibited 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 *et al.*, 1980).

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