Indian Journal of Experimental Biology
Vol. 55, January 2017, pp.21-26

# Effect of *Tinospora cordifolia* (Guduchi) on the phagocytic and pinocytic activity of murine macrophages *in vitro*

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*Received 23 May 2014*; *revised 17 November 2015*

*Tinospora cordifolia* (Guduchi) is a widely used herb in Ayurvedic system of medicine known to possess immunomodulatory properties. The present study was aimed to study the activation of macrophages after *in vitro* guduchi treatment. The aqueous extract of *T. cordifolia* was found to enhance phagocytosis and pinocytosis *in vitro*. The rate of pinocytosis by macrophages when measured by uptake of horseradish peroxidase was significantly increased after guduchi treatment as compared to medium alone. The macrophages demonstrated an increased phagocytosis to non-infective microorganisms (heat killed yeast) and live infective microorganisms (*E. coli*) after guduchi treatment. The results demonstrate that Guduchi enhances macrophage activation as analyzed by cytochemical parameters.

**Keywords:** Ayurveda, Horse Radish Peroxidase (HRP), Immunostimulant

Lower organisms acquire nutrients primarily by phagocytosis. In *metazoan*, phagocytosis occurs mainly in specialized phagocytic cells such as macrophages and neutrophils. Since then it has evolved into an extraordinarily complex phenomena underlying a variety of critical biological processes. Thus, phagocytosis by macrophages is not only critical for the uptake and degradation of infectious agents and senescent cells but also it participates in development, tissue remodeling, the immune response and inflammation. Macrophages, usually in quiescent form, are critical line of defense involved in tumoricidal and microbicidal functions[2,3]). They become activated appropriately by pathogens or agents termed as biological response modifiers (BRMs). Macrophages have evolved a variety of strategies to internalize particles and solutes, including pinocytosis, receptor-mediated endocytosis, and phagocytosis [4-6].

Pinocytosis usually refers to the uptake of fluid and solutes, and it is closely related to receptor-mediated endocytosis, the specific process through which macromolecules, viruses and small particles enter cells. Pinocytosis and receptor-mediated endocytosis share a clathrin-based mechanism and usually occur independently of actin polymerization. By contrast, phagocytosis, the uptake of large particles (>0.5 µM) into cells, occurs by an actin-dependent mechanism and is usually independent of clathrin. Monocytes/ macrophages and neutrophils have been referred to as professional phagocytes and are efficient at internalizing particles[7]. Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive response.

Concerns such as drug-resistant microorganisms, side effects of modern drugs, and emerging diseases where no medicines are available, have stimulated renewed interest in plants as a significant source of new medicines. Our previous studies have shown enhanced reactive oxygen and nitrogen intermediate levels, enhanced enzyme levels such as lysozyme, NADH-NADPH and enhanced microbicidal as well as tumoricidal activity by macrophages treated with *Tinospora cordifolia* (Guduchi)[8-10]. Studies suggest that *T. cordifolia*, a potent anti-infectious compound can also be used as a biological response modifier in the modulation of the cellular response against neoplasia[11-15]. Here, we investigated the effect of *T. cordifolia* on macrophages activation with respect to their phagocytic and pinocytic activity. Also, we looked at the effect of toxicity, if any, of guduchi on human erythrocytes.

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## Materials and Methods

### Reagents

DMEM with L-glutamine and 25 mM HEPES buffer was purchased from (HiMedia Pvt. Ltd. India), Fetal bovine serum was purchased from Hyclone (Logan, USA) and heat inactivated at 56°C for 45 min. Gentamicin (Ranbaxy Laboratories, Ltd. India) a sterile injectible antibiotic was commercially available as 80 mg/2mL. The plant (guduchi) was obtained from medicinal plant nursery, Pune, Maharashtra. The plant was extracted with 200 mL methanol at 50°C for 8 cycles by soxhlet extraction process. The extract was then concentrated with rotator vacuum evaporator and used for further analysis. The drug guduchi prepared in incomplete RPMI medium was tested for endotoxin contamination by limulus amebocyte lysate assay which showed insignificant levels ($7\times10^{-4}$ ng/mg). Necessary precautions were taken to avoid endotoxin contamination through out the investigation, by using endotoxin free buffers, reagents and sterile water. Horseradish peroxidase (HRP) (Sigma, St. Louis, Mo.) was used at a concentration of 1mg/mL in MEM-20% HIFBS. Triton X-100 was obtained from (Rohm and Haas Co., Philadelphia). All other chemicals and solvents used in this study were obtained from Sigma Chemical Company (St. Louis, USA) and were of analytical grade or the highest grade available.

### Cells

The macrophage J774A.1 cell line obtained from National Center for Cell Sciences (NCCS, Pune) used as source of macrophages, (Origin: BALB/c mouse; Nature: Mature) was grown and maintained in the DMEM enriched with 10% heat inactivated fetal Bovine serum, at 37°C and 5% $CO_2$.

### Viability Assay

Cell viability was determined by the Trypan blue dye exclusion test. Equal volumes of cell suspensions were mixed with 0.4% Trypan blue in PBS, and the unstained viable cells were determined. These cells were further used for phagocytosis and pinocytosis assay in $2\times10^6$ densities per mL in the 24-well tissue culture plates.

### Stimulation of macrophages

The macrophages from late log phase of growth (subconfluent) were seeded in 24-well flat bottom microtiter plates (Tarsons, India) in a volume of 500 µL under adequate culture conditions. Drugs were added in different concentrations in a volume of 500 µL in triplicate. The cultures were incubated at 37°C and 5% $CO_2$ environment. After 24 and 48 h incubation cells were assayed for phagocytosis and pinocytosis.

### Phagocytosis Assay:

*Phagocytosis with dried and killed yeast cells*

Phagocytosis assay was performed following the protocol described elsewhere[15]. Macrophages (cell line J774A.1; $2\times10^6$ cells/mL) were treated with guduchi (80 µg/mL) or LPS (10 µg/mL) or medium alone, on slides (triplicate) and kept for incubation at 37°C with 5% $CO_2$ at different time intervals (24 and 48 h). After incubation the supernatant were removed and each slide was flooded with 100 µL of yeast cell suspension (1 mg/mL yeast was prepared by dissolving 1 mg Baker's yeast in 1 mL PBS) and incubated at 37°C with 5% $CO_2$ for 90 min. After incubation the slides were washed with warm (37°C) PBS (pH 7.4) to remove the nonphagocytosed yeast cells. After washing, the cells were fixed with 100% methanol (single dip), air dried and stained with 0.1% crystal violet for 3-5 min. The slides were air dried and mounted in DPX. The slides were observed under the microscope (bright field, at 40X magnification) and macrophages were counted without any predetermined pattern on each slide to calculate the percent phagocytosis and phagocytic index with the given formulae:

$$\text{Percent phagocytosis (P\%)} = \frac{\text{macrophages showing phagocytosis}}{\text{Total no. of macrophages}} \times 100\%$$

$$\text{Phagocytosis index (PI)} = \frac{\text{Total no. of yeast cell phagocytosed}}{\text{Macrophages showing phagocytosis}} \times \text{P\%}$$

*Preparation of yeast cell suspension*

The yeast cell suspension was prepared by dissolving 5 mg of commercial Baker's yeast in 5 mL of 1x PBS (pH 7.4). Finally, the suspension was heavily vortexed to make single yeast cell suspension.

*Phagocytosis with live bacterial cells* (*E. coli*)

Phagocytosis assay was performed by the method as described in Zeligs *et al.*[17]. Guduchi (80 µg/mL) treated or untreated macrophages (cell line J774A.1) $2\times10^6$ cells/mL were incubated at 37°C with 5% $CO_2$ in 24-well tissue culture plate for 24 h. Macrophages treated with LPS (10 µg/mL) were taken as positive control and macrophages treated with gentamicin (50 µg/mL) were taken as negative control. After incubation the supernatant were removed and each well was flooded with 100 µL of *E. coli* cell suspension containing $2\times10^6$ cells/mL (*E. coli* cell suspension was freshly grown overnight in lysogeny broth) and incubated at 37°C in $CO_2$ incubator for

90 min. After incubation, the wells were washed with warm (37°C) PBS (pH−7.4) to remove the nonphagocytosed bacterial cells. After washing, the cells were lysed with 0.1% SDS and the treated or untreated lysates were plated on agar. After overnight incubation at 37°C, colonies were observed and enumerated.

**Pinocytosis assay (Measurement of pinocytic rate)**

Peroxidase has been shown to be a valuable marker of pinocytosis because it is only taken up into membrane-bound vesicles, it does not adsorb to plasma membrane, and macrophages do not have significant endogenous peroxidase activity[18,19].

Macrophages ($2\times10^6$ cells/mL) were plated in 24-well plastic tissue culture plates and treated with guduchi (80 µg/mL) or LPS (10 µg/mL) or medium alone. These plates were incubated at 37°C in the $CO_2$ incubator for 24 and 48 h. After incubation, the supernatant were discarded and macrophages were treated with 50 mg/0.5 mL of horse radish peroxidase (HRP) in complete medium for 1 h. After the exposure of macrophages to HRP, the cells were washed five times in PBS and then lysed by 0.05% Triton X-100 in distilled water. About 2.5 mL of the assay buffer (consisting of 6.0 mL of 0.1 M sodium phosphate buffer pH 5.0, 0.06 mL of 0.3% $H_2O_2$ and 0.05 mL of 10 mg/mL O-Dianisidine) was added to 0.4 mL of cell lysate, and the resulting reaction mixture was kept at 37°C for 1 h[20,21]. Optical density of supernatants was taken at 460 nm. Following Williams *et al.*[20] a rate of uptake so expressed is termed as endocytic index. The units of endocytic index are mL/$10^6$ cells. Rate of pinocytosis was expressed as nanolitres/hour of reaction[18].

**Estimation of toxicity by human Red Blood Cells lysis assay**

Any drug being developed as therapeutics needs to be checked for its cytotoxicity. For this, RBC haemolysis assay was carried out to evaluate the toxicity of guduchi[22]. RBC suspension was prepared in 10 mM PBS pH 7.4. RBC suspension was incubated with different concentrations of drug ranging from 1×IC10 up to 32×IC10 for different time intervals as 1, 3, 6, 12 and 24 h. RBC suspension containing 2% Triton X-100 was taken as positive control while RBC suspension containing PBS was rated as negative control. After incubation, cells were centrifuged at 2000 rpm for 5 min and supernatant was taken in microtitre plate. Absorbance was measured at 541 nm for estimation of hemolysis as a function of hemoglobin absorbance. Percentage haemolysis was calculated using the following formula:

$$\% \text{ RBC haemolysis} = \frac{\text{Abs. of sample-Abs. of negative control}}{\text{Abs. of positive control}} \times 100$$

**Statistical analysis**

Student *'t'* test was carried out to test the significance between the control and the experimental samples for all the assays performed. All the experiments were repeated thrice in triplicates.

## Results

The guduchi treated macrophages showed enhanced phagocytosis and pinocytosis as compared to macrophages treated with medium Dulbecco's Modified Eagles Medium (DMEM) alone. Guduchi preparation was observed not be harmful for the cells, however, there was enhancement in activation.

**Phagocytosis with dried and killed yeast cells**

Guduchi treated macrophages showed more engulfed yeast cells as compared to macrophages treated with medium alone. Guduchi (4.45±0.12; 5±0.75 at 24 & 48 h, respectively) or LPS (4.5±0.37; 4.5±0.37 at 24 & 48 h, respectively) treated macrophages showed enhanced phagocytic index which was significantly higher than the macrophages treated with medium alone (2.9±0.2 and 3.01±0.2 at 24 and 48 h, respectively) (Table 1).

**Phagocytosis with live bacterial cells (*E. coli*)**

Macrophage cell lysates (J774A.1) obtained after guduchi (80 µg/mL) or LPS (10 µg/mL) treatment showed high number of *E. coli* colonies grown on agar plates indicating the presence of phagocytosed bacterial cells (Fig. 1). Whereas, significantly less *E. coli* colonies were seen on the plates seeded with cell lysate of macrophages treated with medium alone and no colonies were found on plates seeded with cell lysate of macrophages treated with gentamicin (Fig. 2).

**Pinocytosis assay**

Macrophages treated with guduchi (80 µg/mL) showed enhanced pinocytic rate at different time

Table 1—Effect of guduchi on the percent phagocytosis and phagocytic index of J774A.1 cells:

| Treatment [concentration] | Percent Phagocytosis [P%] | | Phagocytic Index [PI] | |
|---|---|---|---|---|
| | 24h | 48h | 24h | 48h |
| Guduchi[80µg/ml] | 89±2* | 90±4** | 4.45±0.12* | 5±0.75** |
| LPS[10µg/ml] | 90±2.5* | 87±2** | 4.5±0.37* | 4.5±0.37** |
| Medium alone | 58±1 | 55±2.75** | 2.9±0.2 | 3.01±0.2 |

[Values given in the table are mean± standard deviation and represent three independent experiments done in triplicate. *$P$ <0.05; **$P$ <0.01. * Significantly increased in guduchi treated over medium alone at 24 h and ** at 48 h]

intervals ($1.48\times10^3\pm0.01$ and $5.1\times10^3\pm0.05$ after 24 and 48 h, respectively). Especially, after 48 h treatment it showed significant increase in the pinocytic rate as compared to macrophages treated with medium alone ($1.43\times10^3\pm0.01$ and $3.4\times10^3\pm0.01$ after 24 and 48 h, respectively). Also macrophages treated with LPS (10 µg/mL) showed significantly increased pinocytic rate ($1.7\times10^3\pm0.01$ and $4.6\times10^3\pm0.01$ after 24 and 48 h, respectively) (Table 2).

**RBC haemolysis assay**

In RBC haemolysis assay, when the drug was incubated for 1h (standard time for haemolysis assay) we found no haemolysis with concentration corresponding to IC10 value. Even after a prolonged incubation with higher concentrations, minor haemolysis was found. After 6 h of incubation with four fold of IC10, only 2.94±0.12% haemolysis was observed for guduchi which is in error range. After 24 h of incubation with 32 fold IC10, we found only 14.746±0.10% of haemolysis (Table 3).

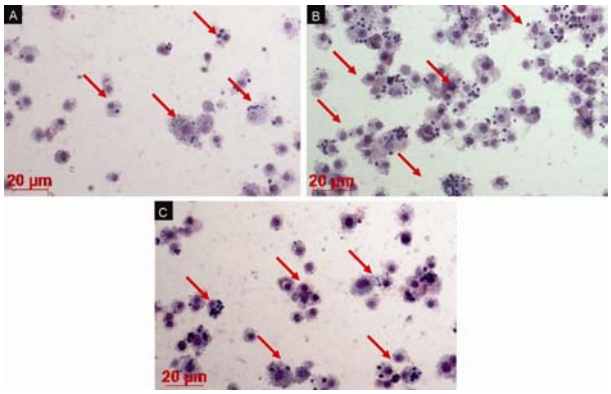


Fig. 1—Phagocytosis by J774A.1 cells treated with (A) Medium alone; (B) Guduchi; and (C) LPS. [Arrows indicating enlarged macrophages with phagocytosed yeast particles]

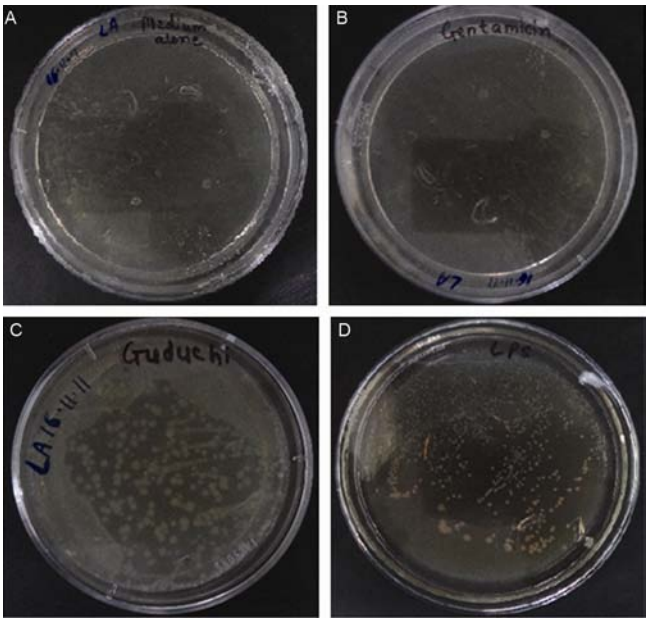


Fig. 2—Phagocytosed *E. coli* colonies grown on agar plates seeded with or (a) untreated macrophage cell lysates; (b) Gentamicin (negative control); (c) guduchi treated; and (d) LPS (positive control).

Table 2—Pinocytic rate [nl/$10^3$/h/2 $\times10^6$ cells] of drug treated and untreated macrophages [J774A.1]

| Treatment [µg/mL] | Pinocytic rate [nl/$10^3$/h/2 $\times10^6$ cells] | |
|---|---|---|
| | 24h | 48h |
| Guduchi [80] | $1.48\times10^3\pm0.01$ | $5.1\times10^3\pm0.05$* |
| LPS [10] | $1.7\times10^3\pm0.01$ | $4.6\times10^3\pm0.01$* |
| Medium alone | $1.43\times10^3\pm0.01$ | $3.4\times10^3\pm0.01$ |

[Values given in the table are mean± standard deviation and represent three independent experiments done in triplicate. *significantly increased in guduchi over medium alone, at 48 h. *$P$ <0.01].

Table 3—RBC haemolysis as a function of time and concentration of *Tinospora cordifolia* [guduchi].

| Concentration[x$IC_{10}$]↓ | | % Haemolysis | | | | |
|---|---|---|---|---|---|---|
| Time [Hours]→ | | 1 | 3 | 6 | 12 | 24 |
| Positive control [0.2%Triton X-100] | | 100 | 100 | 100 | 100 | 100 |
| *Tinospora cordifolia* [Guduchi] | 1 | 0.102±0.02 | 0.215±0.02 | 0.651±0.01 | 0.845±0.02 | 2.406±0.12 |
| | 2 | 0.481±0.15 | 0.548±0.05 | 1.257±0.09 | 1.758±0.02 | 4.476±0.13 |
| | 4 | 0.809±0.18 | 0.943±0.02 | 2.94±0.12 | 3.824±0.03 | 6.426±0.09 |
| | 8 | 2.519±0.11 | 2.781±0.22 | 4.665±0.08 | 8.776±0.05 | 11.236±0.02 |
| | 16 | 2.703±0.13 | 3.205±0.11 | 4.589±0.12 | 6.029±0.08 | 11.756±0.11 |
| | 32 | 5.053±0.12 | 5.488±0.12 | 6.861±0.12 | 8.075±0.11 | 14.746±0.10 |

[Values given in the table are mean± standard deviation and represent three independent experiments done in triplicate. Guduchi treated RBC showed significantly decreased haemolysis *vs.* positive control ($P$ <0.00001)]

## Discussion

Macrophages are major cells of innate immune response critical in defense and constitute important participant in the bi-directional interaction between innate and specific immunity. Our environment contains variety of microbes that may be potentially infectious and threaten our survival. As soon as microbes try to establish a site of infection, the host launches a complex defense mechanism. Innate immunity is a non-specific response and serves as the first-line of defence where phagocytes, such as neutrophils, macrophages, and NK cells play central roles in neutralizing and clearing microorganisms. Thus, migration of cells into infectious foci and subsequent activation of these cells appear to be a critical step, enabling the host to achieve effective and efficient removal of microbes[23]. The present study focused on the effect of guduchi on macrophage activation *in vitro* with respect to phagocytic and pinocytic activity.

The macrophages are usually in a quiescent state but in the presence of a pathogen or when treated with drugs become activated[24]. Indian system of medicine of ayurveda has a rich tradition in the treatment of many diseases by therapy with *'Rasayans'* or preparations from plant or herbal source including immunomodulatory properties[13,25-27]. Investigations on plants used in traditional and modern medicine serve as a source of inspiration and as models for the synthesis of new drugs with better therapeutic, chemical or physical properties than the original compounds[28]. The World Health Organization (WHO) also has recognized the importance of traditional medicine and has been active in creating strategies, guidelines and standards for botanical medicines[29]. In this line *Tinospora cordifolia* (guduchi) used widely in Indian traditional medicine ayurveda has been implicated as a good immunomodulator[15]. In Ayurveda, *T. cordifolia* and its preparations have been routinely used to boost the immune system and the resistance against infections[30].

In our previous studies, dose response curves were standardized[8] wherein it was found that the aqueous preparation of guduchi at 80 µg/mL[10] concentration did not show any inhibition in the macrophage cell viability. To rule out the macrophage cytotoxicity after guduchi treatment if any, 5 fold concentration was analyzed. Even subjecting macrophages to 5 fold concentration of optimal drug dose (80 µg/mL), low cytotoxicity (10%) and maximum viability (90%), after 24 h treatment was observed. Guduchi was found not to be toxic to human red blood cells. Our RBC haemolysis assay suggested this compound to be fairly safe as no significant haemolysis was found even at higher concentration and with prolonged incubation time. Similarly, we had reported enhanced reactive nitrogen intermediates (RNI) and lysozyme levels by macrophages treated with guduchi[7]. We presume that the guduchi mediated enhancement in the levels of these mediators may account for enhanced microbicidal activity of macrophages. The present study showed significantly enhanced phagocytic index after guduchi treatment as compared to medium alone. Guduchi (80 µg/mL) or LPS (10 µg/mL) showed increased phagocytic index and enhancement in percent phagocytosis by activated macrophages (Table 1). Macrophages also showed high pinocytic rate when treated with guduchi (80 µg/mL) or LPS (10 µg/mL) for 24 and 48 h (Table 2). Also guduchi (80 µg/mL) showed enhanced phagocytosis of non-infective heat-killed yeast and live infective *E. coli* (Fig. 1 and 2).

## Conclusion

Guduchi-mediated enhancement of phagocytosis and pinocytosis by macrophages suggests activation through the phagocytic pathway. To-date, several potent biological response modifiers, which are able to activate macrophages, has been extensively studied. Guduchi, a biological response modifier, proved to be a good macrophage activator.

The authors declare that they have no conflict of interest.

## Acknowledgements

This work was supported by a grant from Board of College and University Development (BCUD), University of Pune; UGC-Centre of Advanced Study (CAS), Department of Zoology, Savitribai Phule Pune University (Formerly University of Pune); Department of Atomic Energy-Board of Research and Nuclear Science (DAE-BRNS), Govt. of India; Indian Council of Medical Research, Govt. of India, UGC, DST-PURSE & DRDP-SPPU, Pune.

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