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In-vitro cytotoxicity and phagocytic activity of AQUATURM®-water soluble extract of *Curcuma longa*, on mouse macrophage RAW 264.7 Cell line

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Abstract
Curcuma longa (Turmeric) is well known for its immunity boosting and antimicrobial activities. There is a need for water soluble/miscible *Curcuma longa* extract are gaining popularity for better bioavailability and therapeutic activity. The present study evaluated the cytotoxic and phagocytic activity of AQUATURM® (a water-soluble extract of *Curcuma longa*) in mouse macrophages RAW 264.7 cell lines. Phagocytic activity was evaluated by observing the amount of engulfed zymosans (absorbance at 405 nm) isolated from yeasts. The study demonstrated that AQUATURM® significantly enhanced the phagocytic activity of macrophages compared to vehicle treated/control group at non-toxic concentration of 1.5 µg/mL. The observations made in the present study confirms that *Curcuma longa* extract-AQUATURM® stimulates macrophages and enhances their phagocytic activity and therefore, may have potential enhancing effects on innate immunity. The results corroborate observations of previous studies on *Curcuma longa* species, which could help in augmenting the immune response against foreign antigens or disease-causing pathogens.

Keywords: Cytotoxic, phagocytic, zymosans, macrophages, AQUATURM®

1. Introduction
Infections and non-communicable illnesses have been time and again increasing and peaking inspite all the health care developments made across the globe. The recent and ongoing pandemic of COVID-19 is one such devastating experience. Strong immunity is one of the most sought after or desired health requirement that everyday individual are looking to develop. Various medical and non-medical interventions are being used across the world to combat infections and immunity related disorders [1].

The immune enhancing and immunomodulatory effects of herbs have been extensively researched and published literatures provide sufficient support to validate these effects. Development has also been made at the level of plant processing and extraction technologies to provide the most active moiety of the plant material to achieve high bio-availibility resulting in the desired therapeutic effect. *Curcuma longa*, commonly known as Turmeric is one of the most popular herbs used throughout the world, for both internal use and external application, for various therapeutic benefits ranging from wound healing to sugar management to immunity [2-7].

Many species of Turmeric (*C. longa, C. zanthorrhiza, C. amada, C. mangga, C. aeruginosa,* and *C. zedoaria*), are reported to modulate the immune functions and possess a variety of immunomodulatory effects [3]. The strong immunomodulatory activity of these plants is attributed to its bioactive compounds *viz.* curcuminoids, which has been reported as the major components of plants in Curcuma species, besides other compounds such as xanthorrhizol [4]. Curcumin (diferuloylmethane) is a principal yellow pigment present in the rhizome of turmeric *(Curcuma longa* L.) and related species [6]. Curcuma longa has also been studied for its anti-inflammatory and anti-oxidant activity [5]. The beneficial effect of curcumin in various types of cancer is also known [8]. The stimulatory effect of curcumin on antibody production from splenocytes and natural killer (NK) cell activity *in vivo* has been shown in some studies [9].

The objective of the present study is to investigate the dose dependent phagocytic activity and cytotoxic activity of a water-soluble *Curcuma longa* extract - AQUATURM® in mouse macrophages RAW 264.7 cell line. This study aims to explore immunomodulatory (phagocytic) and cytotoxicity of a proprietary water-soluble extract of *Curcuma longa* - AQUATURM® being developed by LODAAT Pharma. AQUATURM® contains water-soluble turmeric extract standardized to not less than 23% total Curcuminoids.

2. Materials and methods

Mouse Monocyte Macrophage (RAW 264.7) cell line was procured from National Centre for Cell Service, Pune and Sub cultured at Sri Dharmasthala Manjunatheshwara Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi as per standard procedure. The cell line was used forcytotoxicity and phagocytic activity studies in mouse macrophage cell line (RAW264.7) using coded test products.

2.1 Cell culture and MTT assay - cytotoxicity of test products against mouse macrophage cell line RAW 264.7

Cytotoxicity of test product AQUATURM® against Mouse Macrophage cell line (RAW 264.7) was carried out by MTT assay with modification (Mosmann, T. 1983). Mouse Monocyte Macrophage (RAW264.7) cell line was procured from NCCS Pune and subculture dusing Dulbecco's Modified Eagle Medium (DMEM) with fetal bovineserum (FBS). 70-80% confluent RAW 264.7 cell line was taken and medium from the culture flask was removed. The cells were washed twice with sterile phosphate buffer saline (PBS) without disturbing the cells. The wash solution from the culture flask was removed. The cells were detached from the flask by scraping on surface of flask using a sterile cell scraper. After the detachment of cells from the flask, around 1-2 ml of fresh medium (DMEM medium with 10% fetal bovine serum) was added to the flasks and cell suspension was transferred to 15 ml sterile centrifuge tube and cells were centrifuged at 800 rpm for 5 minutes. After centrifugation, the pellet was carefully washed twice with PBS and re-suspended with growth medium (DMEM medium with 10% FBS). 100µl of tryphan blue (0.04 %) was pipetted to a vial and equal volume of cell suspension was added. Both were mixed carefully and loaded to haemocytometer and counted under inverted microscope. After counting the cells, 50,000 cells/well in 100 µl of medium was added to 96 well plate and the plate was incubatedat CO_2 incubator for 24 hours. After 24 hours, the old medium from 96 well plate was carefully discarded and cells were carefully washed once with PBS using multichannel pipette. Different concentrations of coded test product AQUATURM® was dissolved in serum free DMEM medium and added to different wells respectively in 96 well plate and incubated for 24 hours. Control cells were supplemented with routine growth medium. After completion of incubation time, 20 µL of MTT dye (5mg/mL in PBS) was added to all wells and plate was covered with aluminium foil and incubated in CO_2 incubator at 37° C for 4 hours. After 4 hours, 100 µL of 0.4 NHCl and isopropanol (1:24) was added to all the wells and mixed carefully to dissolve the crystals. By using multiplate reader, the absorbance was recorded at 570 nm and 640 nm reference range. The percentage of viable cells was calculated using the formula - % of viable cells = [(Test sample-blank)/(Control-blank)] x 100.

2.2 Phagocytic activity of mouse macrophage cell line (RAW 264.7)

The phagocytic activity of RAW 264.7 cells was determined using CytoSelect™ 96-well phagocytosis assaykit (Zymosan Colorimetric format, Cell Biolabs Inc., San Diego, CA, USA) as per manufacturer's instruction. Cells were seeded in 96-well plate at 1 x 10^5 cells/well and allowed to attach to the plate for 24 h. Then, the cells were treated with the extract of AQUATURM® (1.5 µg/mL & 3 µg/mL). One group of cells was treated with 0.25% Dimethyl sulfoxide (DMSO) as control group. The second group of cells was treated with 50 µg/ml of Lipopolysaccharide (LPS) as positive control group. Subsequently, non-opsonized zymosan was added and the amount of engulfed zymosan (absorbance) was measured at 405 nm after 2h incubation at 37 °C by microplate reader (TECAN, Infinite M NANO· Austria).

2.3 Plant material and extracts

The present study was conducted on AQUATURM® a proprietary extract of Curcuma longa rhizome developed by LODAAT Pharma. AQUATURM® contains water-soluble Curcuma longa extract standardized to contain not less than 23% total Curcuminoids.

2.4 Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was done by two-tailed Student t-test using graph pad software and statistical significance between the treatment groups and control was then assessed. Significant differences were considered at $p<0.05$. Data has been presented as graphs and tables.

3. Results

3.1 Effect of AQUATURM® on cell viability

Mouse macrophages RAW 264.7 cell line showed increasing cytotoxicity with increasing doses/ concentration of AQUATURM®. Cell viability was >50% at concentration of AQUATURM® up to 20 µg/mL. Further, cell viability remained between 25-50% at doses between 40-80 µg/mL of AQUATURM®. The cell viability dropped below 25% at doses above 80 µg/mL. (Figure 1).

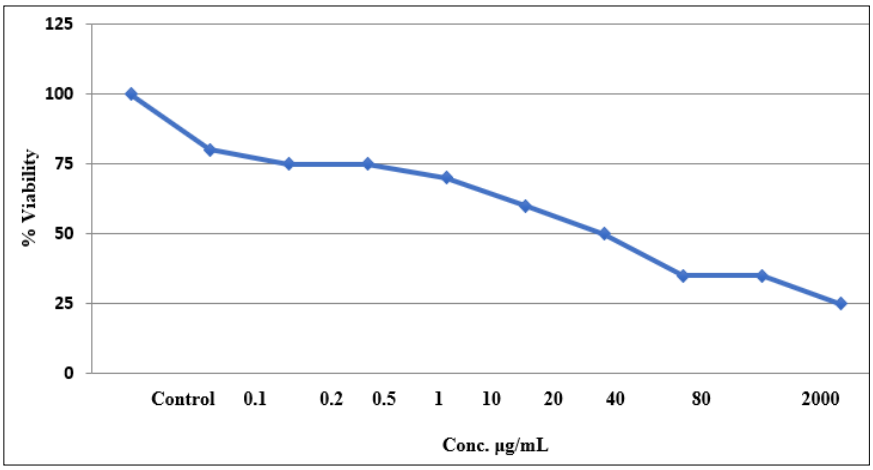

Fig 1: Cytotoxicity of AQUATURM® against (RAW 264.7) cell line.

3.2 Phagocytic activity of extract of AQUATURM®

Phagocytosis is a critical immune function of macrophages as a defense to protect the host from foreign antigens/pathogens. Phagocytic activity was evaluated by observing the amount of engulfed zymosans (absorbance at 405 nm) isolated from yeasts. The study demonstrated that the extract of Curcuma longa - AQUATURM® significantly enhanced the phagocytic activity of macrophages compared to vehicle treated/control group at non-toxic concentration of 1.5 µg/mL (Figure 2, Table 1) in mouse macrophage RAW 264.7 cell line. The study demonstrated that the absorbance with AQUATURM® at 1.5 µg/mL concentration (mean OD value 0.5174 ± 0.0117) was significantly higher (p<0.0046) than absorbance observed with the control group (mean OD value 0.393 ± 0.0002). Further the absorbance/mean OD values with extract of *Curcuma longa*-AQUATURM® at 1.5 µg/mL concentration were closer to the absorbance observed with positive control i.e. Lipopolysaccharide (mean OD value at 0.7486 ± 0.0003; p-0.5182). However, increasing dose of extract of AQUATURM® did not observe increase in absorbance as the absorbance at 3 µg/mL concentration (mean OD value 0.4408 ± 0.0759) was closer to the control group and significantly lower (p-0.0290) than both the positive control as well as AQUATURM® 1.5 µg/mL.

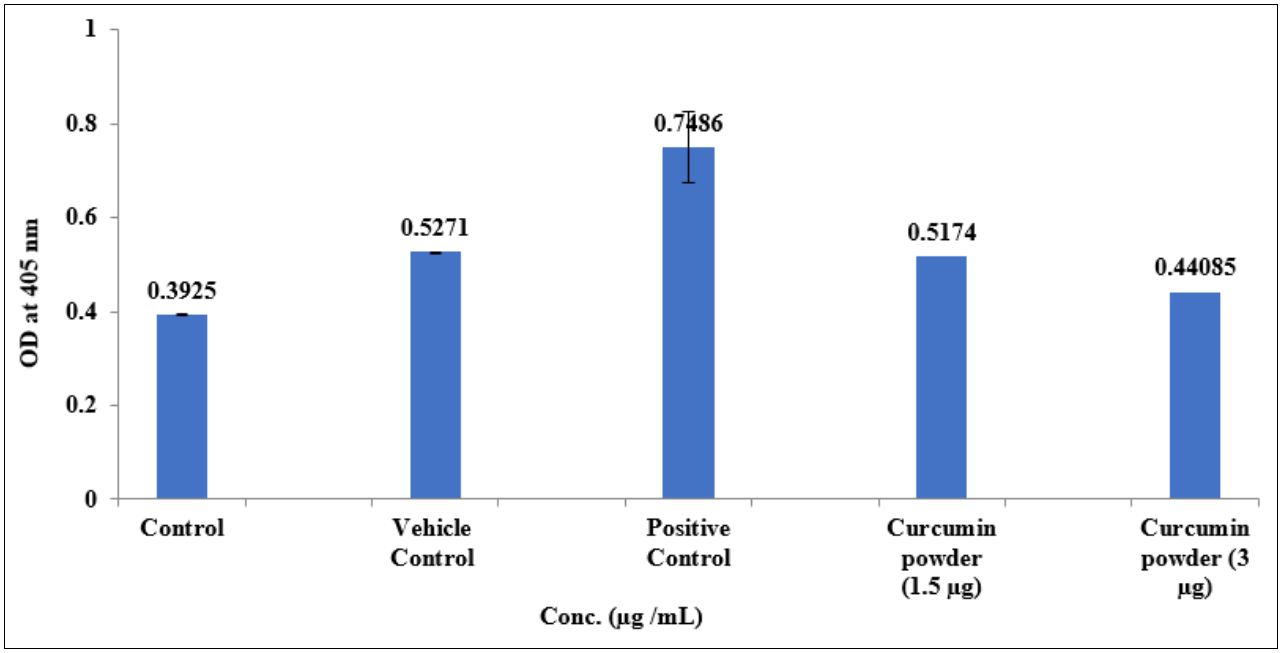

Fig 2: Phagocytic activity of mouse macrophage cell line (RAW 264.7) of AQUATURM®. The values are presented as Mean OD values ± SD. Control (Zymosan free group); vehicle control (without LPS); positive control (with LPS).

Table 1: OD mean and SD values of phagocytic activity of AQUATURM®.

Experimental groups	Mean OD values	SD	p value between the groups
Control	0.393	0.0020	p-0.0046 (vs. control) p-0.3503 (vs. vehicle control) p-0.0013 (vs. positive control)
Vehicle control	0.527	0.0002	
Positive control	0.749	0.0003	
AQUATURM (1.5 µg)	0.5174	0.0117	
AQUATURM (3 µg)	0.441	0.0759	p-0.4656 (vs. control) p-0.2502 (vs. vehicle control) p-0.0290 (vs. positive control)

4. Discussion

In this study we evaluated the cytotoxic and phagocytic activity AQUATURM® a water-soluble extract of Curcuma longa. After screening for cytotoxicity in mouse macrophages RAW 264.7 cell line, the non-cytotoxic doses/concentration of AQUATURM® (1.5 µg/mL and 3 µg/mL) were tested for the phagocytic activity. The experiment compared the activity of extract of Curcuma longa - AQUATURM® with a positive control (LPS) and the untreated/control group. The extract of Curcuma longa - AQUATURM® (1.5 µg/mL) demonstrated a similar phagocytic activity to positive control (LPS) and higher activity than the untreated/control group. However, a higher dose/concentration (3 µg/mL) of the extract of Curcuma longa - AQUATURM® showed a lower phagocytic activity compared to 1.5 µg/mL as well as positive control.

The immune potentiating effects of extract of the rhizome of *Curcuma longa* have been observed in various *in-vitro* and *in-vivo* studies, points toward nonspecific immune mechanisms. These mechanisms include activation of macrophages, phagocytic activity and NK cell activity. One of the foremost limitations of using Curcumin as a drug is its poor aqueous solubility, which eventually is responsible for its instability and poor bioavailability [10]. Attempts have been made to increase its aqueous solubility and bioavailability by the nanoparticle-based approach producing micelle and lipid-drug hybrid nanoparticle formulations with improved water/plasma solubility [11]. It appears AQUATURM® may have overcome the water solubility and bioavailability conundrum of curcuma long, however, further research is required to confirm this hypothesis.

The results of our *in vitro* study corroborate the observations of previous studies on *Curcuma Longa* species, and confirms that AQUATURM® activates macrophages and enhances their phagocytic activity, which could help in augmenting the

immune response against foreign antigensor disease causing pathogens.

5. Conclusion

The observations made in the present study confirms that extract of *Curcuma Longa* - AQUATURM® stimulates macrophages and enhances their phagocytic activity, and therefore, may have potential enhancing effects on innate immunity. Clinical studies are underway on AQUATURM® to further validate the effect as an immunity booster compound.

6. Acknowledgement

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7. Disclosure

Financial assistance for the study was provided by Target Institute of Medical Education & Research, Mumbai and LODAAT Pharma, 1415 West 22nd Street-Tower Floor, Oak Brook, Illinois 60523.

AQUATURM®-Curcumin extract was supplied by LODAAT Pharma.

8. Conflict of Interest - None

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