Evaluation of immunity enhancing potential of *Ocimum sanctum* L. (TulsiOdaat™) on mouse macrophage RAW 264.7 cells, network pharmacology and *in silico* studies

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Research Article

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Abstract

Tulsi (Ocimum sanctum Linn), commonly known as Holy Basil, has traditionally been used as a remedy for common infections of the respiratory tract, and as an immunity booster. The herb has potential anti-microbial, anti-bacterial, and anti-viral effects. The present study evaluated the cytotoxic and phagocytic activity of a standardized extract of Ocimum sanctum (TulsiOdaat™) in mouse macrophages RAW 264.7 cell lines. Phagocytic activity was evaluated by observing the amount of engulfed zymosans isolated from yeasts at 405 nm. The study demonstrated that TulsiOdaat™ significantly enhanced the phagocytic activity of macrophages compared to the vehicle-treated/control group at a non-toxic concentration of 3 µg/mL. The observations made in the present study confirm that TulsiOdaat™ stimulates macrophages and enhances their phagocytic activity and therefore, may have potential enhancing effects on innate immunity. Moreover, the elected target proteins showed strong correlation to each other including such as CASP3 and MAPK. Interestingly, in silico docking of ursolic acid have strong binding affinity to CASP3 and MAPK as well as SARS-CoV-2 proteins. The in-vitro and in-silico studies revealed that bioactive compounds of Ocimum sanctum may augment the immune response against foreign antigens or disease-causing pathogens.

1. Introduction

The emergence of the 2019 novel coronavirus SARS-CoV-2, the cause of the COVID-19 pandemic, is the pre-eminent public health issue of this decade (Wang et al., 2020). The symptoms of severe COVID-19 infection range from fever and coughing to pneumonia or acute and severe respiratory distress (shortness of breath), and multi-organ failure due to hyper-inflammation and a cytokine storm syndrome (Mamber et al., 2020). More than two billion people have been infected and millions of people has died (Ingraham et al., 2020). There are no treatments that have been definitively shown to be effective for COVID-19. Theoretical data suggests patients with COVID-19 lost homeostasis of cell mediators due to weak immunity (Mehta et al., 2020; Chen et al., 2020). Though, the COVID-19 pandemic has emphasized the importance of immunity, Co-morbid conditions associated with weak immunity have been considered factors for increased mortality and increased risk of serious complications from COVID-19 (Callender et al., 2020). In addition to COVID-19, other common viral and bacterial infections may have significant complications for individuals with lower or compromised immunity. Advisories from international government agencies have suggested medicinal and non-medicinal interventions for boosting immunity that may prevent the likelihood of infection as well as reduce its severity.

Herbal medications and their constituents provide long-term fitness benefits and can be utilized to treat human diseases and disorders well for primary health care (Ibrahim et al., 2021). Herbs in various forms have been traditionally recommended as well as scientifically studied for their immune-enhancing and immunomodulatory effects (Khanna et al., 2021). Bioactive compounds present in these herbs, including alkaloids, flavonoids, polyphenols, etc., have been attributed to possessing possess various pharmacological potential benefits including immunomodulatory activity.
O. sanctum Linn, popularly known as Holy Basil, is one of the oldest and most well-known herbs in traditional systems of medicine (Cohen et al., 2014). Bioactive compounds of O. sanctum have been investigated for their various medicinal properties and their effects at the molecular level (Vasudevan et al., 2014). Experimental evidences suggest that bioactive compounds of O. sanctum protects from toxic chemical-induced injury by increasing levels of antioxidants such as glutathione and enhancing the activity of antioxidant enzymes, such as superoxide dismutase, catalase, and other toxic agents (Shivananjappa et al., 2012; Manikandan et al., 2007). It is also ameliorates metabolic dysfuntion, inflamations, hepatic disease and most importantly modulate immunomodulatory activities. Traditionally, O. sanctum has been utilized for its specific action on the respiratory system in the prevention and treatment of infections and inflammation-related conditions (Almatroodi et al., 2020).

Nowadays, biomedical scientist are aware that the “one key, one lock“ hypothesis is insufficient to decipher the drug actions, especially in complex diseases (Zhou et al., 2019). Intriguingly, for fast screening bioactive compounds in the mixture of plant based products computer-aided techniques are useful to explain or predict the pharmacological effect of a drug. (Huang et al., 2020) (Li et al., 2014). Network pharmacology and insilico molecular docking studies, analyzing drugs and drug targets in a systemic manner may provide us with novel insights into drug actions. As a useful tool for systematically evaluating and demonstrating the rationality of drugs, it has now been widely accepted. As a useful tool for systematically evaluating and demonstrating the rationality of drugs, it has now been widely accepted. And it could provide us a comprehensive understanding for the management of various disease and disorders.

The objective of the present study was to investigate the dose-dependent phagocytic activity and cytotoxic activity of standardized O. sanctum extract (TulsiOdaat™) in mouse macrophages RAW 264.7 cell line. This study aimed to explore the immunomodulatory (phagocytic) and cytotoxicity effects of TulsiOdaat™ developed by LODAAT Pharma. TulsiOdaat™ is an O. sanctum extract standardized containing 2.5% ursolic acid.

2. Materials And Methods

2.1. Chemical and Reagents

All the chemicals and reagents were used as analytical grades. Dulbecco’s Modified Eagle Medium (DMEM) with fetal bovine serum (FBS), MTT was perched from Himedia and Sigma Aldrich.

2.2. Plant material and Extracts

The present study was conducted on TulsiOdaat™, a proprietary extract of O. sanctum developed by LODAAT Pharma. TulsiOdaat™ contains a standardized extract of O. sanctum having 2.5% ursolic acid.

2.3. Procurement of cell line
The mouse monocyte macrophage (RAW 264.7) cell line was procured from the 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 the standard procedure. The cell line was used for cytotoxicity and phagocytic activity studies in mouse macrophage cell lines (RAW264.7) using coded test products.

2.4. Cell culture and MTT assay (cytotoxicity of test products against mouse macrophage cell line RAW264.7)

Cytotoxicity of the test product, TulsiOdaat™, against Mouse Macrophage cell line (RAW 264.7) was carried out by MTT assay with modification (Chandra et al., 2021). Mouse Monocyte Macrophage (RAW 264.7) cell line was procured from NCCS Pune and subcultured using Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum (FBS). Between 70–80% confluent RAW 264.7 cell line was taken and the 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 then removed. The cells were detached from the flask by scraping the surface of the flask using a sterile cell scraper. After the cells were detached from the flask, between 1–2 ml of fresh medium (DMEM medium with 10% fetal bovine serum) was added to the flasks and the cell suspension was transferred to a 15 ml sterile centrifuge tube. Cells were centrifuged at 800 rpm for 5 minutes. After centrifugation, the pellet was washed twice with PBS and re-suspended with a growth medium (DMEM medium with 10% FBS). 100 µl of trypan blue (0.04%) was pipetted to a vial and an equal volume of cell suspension was added to the same vial. Both were mixed carefully, loaded to a hemocytometer, and counted under an inverted microscope. After counting the cells, 50,000 cells/well in 100 µl of the medium was added to a 96-well plate and the plate was incubated with a CO2 incubator for 24 hours. After 24 hours, the old medium from the 96-well plate was discarded, and cells were washed once with PBS using a multichannel pipette. Various concentrations of the coded test product, TulsiOdaat™, were dissolved in serum-free DMEM medium and added to different wells respectively in the 96-well plate and incubated for 24 hours. Control cells were supplemented with a routine growth medium. After completion of incubation time, 20 µL of MTT dye (5 mg/mL in PBS) was added to all wells and the plate was covered with aluminum foil and incubated in a CO2 incubator at 37°C for 4 hours. After 4 hours, 100 µL of 0.4 sodium chloride and isopropanol (1:24) was added to all the wells and mixed to dissolve the crystals. Using a multi-plate reader, the absorbance was recorded at 570 nm and 640 nm reference range. The percentage of viable cells was calculated using the formula mentioned below:

\[
\% \text{ of viable cells} = \frac{\text{(Test sample-blank)}}{\text{(Control-blank)}} \times 100
\]

2.5. Phagocytic activity of Mouse Macrophage cell line (RAW 264.7)

The phagocytic activity of RAW 264.7 cells was determined using a CytoSelectTM 96-well phagocytosis assay kit (Zymosan Colorimetric format, Cell Biolabs Inc., San Diego, CA, USA) as per the manufacturer's instructions. Cells were seeded in a 96-well plate at 1 x 105 cells/well and allowed to attach to the plate
for 24 hours. The cells were then treated with TulsiOdaat™ (3 µg/mL & 6 µg/mL) or with 0.25% dimethyl sulfoxide (DMSO) as a control group. The second group of cells was treated with 50 µg/ml of lipopolysaccharide (LPS) as the positive control group. Subsequently, non-opsonized zymosan was added and the amount of engulfed zymosan (absorbance) was measured at 405nm after 2 h incubation at 37°C by a microplate reader (TECAN, Infinite M NANO, Austria).

2.6. Toxicity analysis of the bioactive compounds

The toxicity property of the compounds were determined by ProTox-II. Subsequently, target information of ursolic acid, key constituent of *O. sanctum* was analyzed through numerous databases such as Traditional Chinese Medicine Systems Pharmacology database and Analysis Platform (TCMSP) (https://lsp.nwu.edu.cn/). The target names were imported into the UniProt database (http://www.uniprot.org/) with the species selected as “Homo sapiens,” and the gene names of the targets were obtained from the UniProt database.

2.7. Target genes related to selected compounds and immune disorders

Based on the SMILES, target genes linked to the compounds were selected through Swiss Target Prediction (STP) (http://www.swisstargetprediction.ch/) with “Homo Sapiens” mode. Immunity related genes were identified by DisGeNET (https://www.disgenet.org/search).

2.7.1. Protein-protein interaction (PPI) network construction

To explain the interaction between target proteins, the target proteins of related ursolic acid was uploaded to STRING (http://string-db.org) online website to obtain the information of protein-protein interaction (PPI). The higher the score represent higher degree of confidence in the interaction between target proteins. The obtained protein interaction data were imported into Cytoscape 3.2.1 software to construct a PPI protein interaction network. In addition, we have also constructed the network between disease and important genes involved in pathology of immune related disorders.

2.7.2. Gene ontology and KEGG enrichment analysis of target proteins

To elucidate the role of target proteins that interact with the ursolic acid, gene function and signaling pathway, Enrichr was used to analyze the Gene Ontology (GO) function and KEGG pathway enrichment of proteins involved in PPI network. The target proteins involved in the biological process (BP), and the pathways were also described.

2.8. *In silico* molecular docking

2.8.1. Target sequence retrieval
The protein sequence of CASP3, MAPK and SARS-CoV-2 virus was downloaded from the research collaborators for structural bioinformatics protein data bank (RCSB PDB).

2.8.2. Active site prediction

The active site was predicted by using the site finder option of using pymol software. The site finder option was used to calculate possible active sites in SARS-CoV-2 virus from the 3D atomic coordinates of the receptor. Calculations were made to determine potential sites for ligand binding and docking, and restriction sets for rendering partial molecular surfaces.

2.8.3. Preparation of bioactive leads, targeted enzyme and molecular docking

The ligands namely ursolic acid was downloaded as 3D structure SDF file from PubChem and optimized using Ligands Input in the AD 4.2. Further, target enzyme was prepared by addition of all polar hydrogen atoms and removal of water molecule and other heteroatoms to the target enzyme with the AutoDock. For the ligands, Gasteiger charges were added and all the rotatable bonds were set to be rotatable. The optimized ligand molecules were docked with refined immune related receptors using autodock 4.2. The docking results were analyzed using PyMOL molecular graphics visualization tool. After completion of docking searches, the best conformation was selected from the maximum populated cluster with the minimum binding energy.

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was done by ANOVA. Statistical significance between the treatment groups and control was assessed. Significant differences were considered at p < 0.05. Data has been presented as graphs and tables.

3. Results

3.1. Effect of extract of *Ocimum sanctum* (TulsiOdaat™) on cell viability

Mouse macrophages RAW 264.7 cell line showed increasing cytotoxicity with increasing doses/concentrations of TulsiOdaat™. Cell viability was > 60% at a concentration of TulsiOdaat™ up to 80 µg/mL. Further, cell viability remained between 25–50% at doses between 80-1000 µg/mL of TulsiOdaat™. The cell viability dropped below 25% at doses above 1000 µg/mL (Fig. 1).

3.2. Phagocytic activity of extract of *Ocimum sanctum* (TulsiOdaat™)

Phagocytosis is a critical immunological 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 standardized extract of *O. sanctum* (TulsiOdaat™) significantly enhanced the phagocytic activity of macrophages compared to vehicle-treated/control group at a non-toxic concentration of 3 µg/mL in mouse macrophage RAW 264.7 cell line (Fig. 2). The study demonstrated that the absorbance with TulsiOdaat™ at 3 µg/mL concentration (mean OD value 0.431 ± 0.0261) was significantly higher (p < 0.0034) than the absorbance observed with the control group (mean OD value 0.393 ± 0.0020). However, increasing the dosage of TulsiOdaat™ did not show any observable increase in absorbance as the absorbance at 6 µg/mL concentration (mean OD value 0.467 ± 0.0005) was closer to the absorbance at a dosage of 3 µg/mL.

### 3.3. Toxicity analysis

Toxicity class study revealed that ursolic acid is a safe molecule having value of 4 [Toxicity score ranging from [1 (toxic) to 6 (non-toxic)]]. Toxicity near to one is considered more toxic to human health and vice versa. LD50 value of ursolic acid was found 2000mg/kg. During the early stages of drug design, the Toxicity properties play an important role in the drug filtering in the early stage of drug developments (Yi et al., 2018).

### 3.2. Target genes and disease association network

To clarify the relationship between target proteins and disease association, we have constructed a target-disease interaction network using the NetworkAnlyst (Fig. 3), and the immune related disease and pathways involving ten target proteins were screened. The results showed that these target proteins were mainly involved in asthma, diabetes mellitus, Alzheimer, inflammation, etc. Network analysis revealed, there are multiple target proteins in one pathway, and the same target protein exists in multiple pathways. Essentially, a pathway involving multiple target proteins is more important than the interaction between one target protein and multiple pathways.

### 3.3. PPI network of targeted genes

The selected potential gene targets were analyzed using the STRING, and the PPI network was obtained (Fig. 4). The network diagram shows the close interactions between genes. Moreover, we have selected the bio potential genes (CASP3, and MAPK) in the same PPI network to further understand the molecular mechanism of ursolic acid through molecular docking studies. Network construction clearly revealed that CASP3 and MAPK are top targets are potentially involved in multiple pathways.

### 3.4. GO biological and KEGG enrichment analysis of target genes

The biological process results suggested that these targets participated in neutrophil activation involved in immune response, neutrophil degranulation, cellular protein metabolic process and many more. The KEGG pathway enrichment signaling pathways (p < .05, count > 10), which are involved in various signaling pathways, such pertusis, inflammation, diabetes, MAPK signaling pathways. From these finding
it may hypothesise that ursolic acid may regulate multiple signaling pathways and helping the body return to normal (Fig. 5).

**Molecular docking studies**

AutoDock is an extensively used platform for analyzing the interaction between a protein and its ligand/inhibitor. In order to get close to the mechanism of SARS-CoV-2 virus inhibition by ursolic acid, we performed molecular docking using AutoDock.

**Molecular docking analysis of ursolic acid with SARS-CoV-2 virus**

The molecular docking between ursolic acid to, CASP3, MAPK and SARS-CoV-2 virus protein has been carried out in order to examine interaction between those ligands and protein. The interaction of ligand-protein was shown by the types of chemistry bond formed and the binding sites of amino acid residues. The molecular docking results are presented in Table 1 and Figs. 6.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Protein</th>
<th>Binding energy</th>
<th>H-bonds, residual hydrophobic/Pi-Cation/Pi-Anion/Pi-Alkyl Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ursolic acid</td>
<td>CASP3</td>
<td>-10.1</td>
<td>Gly128(B), Arg167(B), Pro206(C), Glu127(B), Arg167(A), Pro206(D), Val271(C), Val271(D), Leu139(A), Lys140(A), Tyr200(C), Met273(C), Thr143(A), Tyr202(C)</td>
</tr>
<tr>
<td></td>
<td>MAPK</td>
<td>-10.8</td>
<td>Asp145(F), Gln202(F), Lys89(G), Phe82(G), Gln87(G), Pro322(F), Lys84(G), Val319(F), Asp321(F), Lys64(G), Ala320(F), Ser143(F)</td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>-11.2</td>
<td>Asn907(A), Arg1107(C), Gly1035(A), Gln1036(C), Val1040(C), Tyr1047(A), Trp886(A)</td>
<td></td>
</tr>
</tbody>
</table>

**4. Discussion**

Herbal medicine has traditionally played an essential role in the control of infectious diseases (Ahmad et al., 2019). Clinical evidence from a variety of herbal medicine research in the treatment of SARS coronavirus (SARS-CoV) has demonstrated considerable results, bolstering the hypothesis that herbal medicine might help treat and prevent pandemic diseases (Anget al., 2020). Herbal medication mixed with Western treatment may enhance symptoms and quality of life in SARS-CoV patients, according to a
Cochrane systematic review (Liu et al., 2020). Herbal medicine was also found to lessen the rate of H1N1 influenza infection in a recent meta-analysis (Luo et al., 2020). Herbal medication is regarded as one of the alternate ways the treatment of COVID-19, based on prior experience.

In this study, we evaluated the cytotoxic and phagocytic activity of a proprietary, standardized extract of *O. sanctum*, commercially known as TulsiOdaat™. After screening for cytotoxicity in the mouse macrophage RAW 264.7 cell line, the non-cytotoxic doses/concentration of TulsiOdaat™, (3 µg/mL and 6 µg/mL), were tested for the phagocytic activity. The experiment compared the activity of TulsiOdaat™ with positive control (LPS) and the untreated/control group. TulsiOdaat™, at a dose of 3 µg/mL, demonstrated similar phagocytic activity to a positive control (LPS) and higher activity than the untreated/control group. Increasing the dose to 6 µg/mL did not show any enhanced activity and was found to be similar to the dose of 3 µg/mL.

*O. sanctum* hydro-alcoholic extract reduced viral intracellular multiplication. In H9N2 viruses, it also prevents non-specific interference with virus-cell interactions (Ghokeet al., 2018). The immunomodulatory ability of alcoholic leaf extracts was demonstrated by a reduction in hepatic parasites and a skewing of the humoral response toward Th1 type at an IC$_{50}$ value of 73.3 g/ml (Bhalla et al., 2017). In cultivated HL-60 cells, O. sanctum suppresses the activities of leukotriene-C4-synthase, leukotriene-A4-hydrolase, and cyclooxygenase-2, resulting in a considerable reduction in OVA-induced lung inflammation (Soniet al., 2015).

Previous scientific research has revealed that the *O. sanctum* has anti-bacterial, anti-viral, and anti-fungal activities that include activity against many pathogens responsible for human infections. *O. sanctum* has also been shown to boost defenses against infective threats by enhancing immune responses in non-stressed and stressed animals and healthy humans. There is experimental evidence that *O. sanctum* may help in the treatment of various human bacterial infections including urinary tract infections, skin and wound infections, typhoid fever, cholera, tuberculosis, gonorrhea, acne, herpes simplex, leishmaniasis, various pneumonia and fungal infections, as well as mosquito-borne diseases such as dengue, malaria, and filariasis (Hemalatha et al., 2011; Tripathi et al., 2008; Goelet al., 2010; Mondal et al., 2011).

The results of our in-vitro study corroborate the observations of previous studies on *O. sanctum* and confirm that this proprietary, standardized extract of *O. sanctum*, commercially known as TulsiOdaat™, activates macrophages and enhances their phagocytic activity, which could help in augmenting the immune response against foreign antigens or disease-causing pathogens.

Molecular docking is a computer-aided model to predict the binding affinity of biomolecules toward a particular receptor. Although docking technology is of great help in pharmacology, these techniques are very helpful to predict the affinity of drugs/bioactive leads within the binding site of the target of interest. Interestingly, thousands of biomolecules can be evaluated for the potential efficacy with the help of docking study at a small cost in a short time span. In the process of searching novel molecules and pharmacological research, discovery of bioactive compounds has always been challenging. With the help
of molecular docking studies, number of bioactive compounds will be screened at a faster pace. We hope to accelerate the development cycle of future forecasting applications, to aid an increasing number of researchers to appropriately and reasonably use these technologies.

5. Conclusion

The observations made in the present study confirm that TulsiOdaat™ stimulates macrophages and enhances their phagocytic activity, and therefore, may have potential enhancing effects on innate immunity. Summarily, we can conclude that formulation of *O. sanctum* might be useful to treat immune related disorders due to enrichment of bioactive constituents including ursolic acid.

Declarations

Acknowledgement

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Conflict of Interest

None

References


**Figures**
Figure 1

Cytotoxicity of TulsiOdaat™ against (RAW264.7) cell line.
Figure 2

Phagocytic activity of mouse macrophage cell line (RAW264.7) of TulsiOdaat™. The values are presented as Mean OD values ± SD. Control (Zymosan free group); Vehicle control (without LPS); Positive Control (with LPS).

Figure 3
Targets-disease interaction analysis. The blue color represents the disease and rest shows the target genes.

Figure 4

Protein-protein interactions of immune related gene targets.
neutrophil activation involved in immune response (GO:0002283)
neutrophil degranulation (GO:0043312)
regulation of cellular macromolecule biosynthetic process (GO:2000112)
nervous system development (GO:0007399)
regulation of nucleic acid–templated transcription (GO:1903506)
cellular protein metabolic process (GO:0044267)
cellular response to DNA damage stimulus (GO:0006974)
post-translational protein modification (GO:0043687)
chemical synaptic transmission (GO:0007268)
proteolysis (GO:0006508)

**Biological process**

Pertussis
AGE–RAGE signaling pathway in diabetic complications
TNF signaling pathway
Lipid and atherosclerosis
IL-17 signaling pathway
Human cytomegalovirus infection
C-type lectin receptor signaling pathway
MAPK signaling pathway
Hepatitis B
Tuberculosis

**KEGG pathway**

Figure 5

GO biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of potential target genes of ursolic acid.
Figure 6

3D figure of ursolic acid ligand interaction with the target proteins CASP3, MAPK and SARS-CoV-2 virus.