

Evaluation of *in vitro* immunomodulatory and antimicrobial activity with safety study of granules of *Naque Nazla*

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Common cold and flu are frequently occurring in humans, if not managed, can cause secondary infections and other complications. It may be minimized by boosting immunity, which can be done by Unani Medicine. Objective of this study is to evaluate *in vitro* immunomodulatory and antimicrobial activity and safety of granules of *Naque Nazla*, a famous Unani infusion / decoction. *Naque Nazla* granules (NNG) were tested for its immunomodulatory activity by lymphocyte proliferation assay and macrophage phagocytosis and antimicrobial activity by well diffusion method. LC-MS and GC-MS was done for standardization. Acute toxicity study was carried as per OECD guidelines 423, to evaluate safety of granules. THP-1 cells study displayed EC50 value of 997.6 µg/mL with a proliferation of 53.95% at 1280 µg/mL. Phagocytosis assay displayed 67.85% phagocytosis at 1280 µg/mL, antimicrobial activity was tested against common respiratory tract bacteria which showed susceptibility against *Klebsiella pneumoniae*, *Salmonella* spp., *Staphylococcus aureus*, and *Streptococcus pyogenes*. LC-MS and GC-MS fingerprinting data was set in and revealed the presence of many phytochemicals. Acute toxicity study displayed the drug safe up to 2000 mg/kg. NNG displayed immunomodulatory and antimicrobial activity in *in vitro* study and was also found safe.

Keywords: Antimicrobial activity, Granules, Immunomodulation, *Naque Nazla*, Unani medicine

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Common cold and flu are most frequently occurring viral infectious diseases of humans, affecting the respiratory tract. Immune deficiency may be a risk factor for the disease. The conventional treatment primarily involves symptomatic relief from pain and fever medications and antimicrobials for prevention of secondary bacterial infection usually following the primary infection¹.

Respiratory viral infections may be asymptomatic or can cause lethal infections. Secondary bacterial infections can cause otitis media, sinusitis, or *pneumoniae*. This respiratory virus infection can impair the defense mechanisms that keep these anatomic sites free from infection with bacteria². Bacterial co / secondary infection can increase morbidity and mortality of influenza infection, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* are reported as the most common causes³. According to World Health Organization, widespread emergence of

multidrug-resistant (MDR) bacterial pathogens is an important public health challenge worldwide⁴. Infections with MDR pathogen are associated with increased mortality, *Klebsiella pneumoniae* is one of the important causes of MDR infections worldwide and these strains can even display resistance to last-line antibiotic such as colistin⁵. Use of antimicrobials indiscriminately can result in dissemination of these resistant bacteria making the situation worse⁶.

Robust immune response can protect from these conditions. Many natural products and Biological Response Modifiers (BRMs) are now popular in supplementing immune response. Many medicinal plants have long been known for their immunomodulatory prospective giving motivation in the search for investigating natural resources⁷. Several Unani single drugs and formulations can be useful in providing robust immunomodulatory response and protection from secondary bacterial infections.

Formulations used in the form of decoction and infusion whose availability and specific preparation method becomes difficult in certain conditions if

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modified into soluble granules, then their usage can become easy. Granulation is the process used extensively in the pharmaceutical industry⁸. One such Unani decoction / infusion formulation *Naque Nazla* (NN) containing Gule Banafsha (*Viola odorata* L.), Unnab (*Zizyphus jujuba* L.), Sapistan (*Cordia latifolia* Roxb.), Khatmi (*Althea officinalis* L.), Khubbazi (*Malva Sylvestris* L.), Gaozaban (*Borago officinalis* L.), Aslussoos (*Glycyrrhiza glabra* L.) was modified to granules by Zaigam *et. al.*⁹ and this modified *Naque Nazl* granules (NNG) was selected for this work, owing to immunomodulatory and other supportive activity in its ingredients for cold and flu, it was utilized for *in vitro* immunomodulatory, antimicrobial and toxicity evaluation with standardization test. Cell lines immunomodulatory studies such as macrophage phagocytosis are some common *in vitro* immunoassay models and antimicrobial study was done to check its antimicrobial profile particularly in organism pathogenic on respiratory tract, beside this acute toxicity study was also performed. Standardization of herbal drugs is of paramount importance because of many problems related to herbal drugs such as mis-identification and adulteration. Routine physicochemical studies can be employed for standardization but fingerprinting profiles like LCMS, GC-MS etc. are employed for this work.

Material and Methods

Procurement and preparation of drug

Prepared granule formulation contains excipient *viz.*, maltodextrine powder with formulation extract and powder, Starch (soluble), Sodium starch glycolate, Aerosil 200 excipients, as per method adopted by Zaigam *et. al.*⁹ was procured from the local market of Bengaluru. Ingredients of NN in each dose namely, Gule Banafsha, *Viola odorata* L. (Flower) 7 g, Unnab, *Zizyphus jujuba* L. (Fruit) 5 no., Sapistan *Cordia latifolia* Roxb. (Fruit) 7 no., Khatmi *Althea officinalis* L. (Seed) 7 g, Khubbazi *Malva sylvestris* L. (Seed) 7 g, Gaozaban, *Borago officinalis* L. (Leaf) 5 g, Aslussoos *Glycyrrhiza glabra* L. (Root) 5 g¹⁰ were authenticated by Trans-Disciplinary University (TDU), Foundation for revitalization of Local Health Tradition (FRLHT), Bengaluru. Voucher specimen of all the drug of NN was obtained by submitting in NIUM repository museum with No. 76/IA/Res/2020. Granules were prepared as per the method developed by Zaigam *et. al.*⁹ All the drugs were cleaned of all foreign matters,

fine powder of all seven drugs of formulation of *Naque Nazla* (NN) was prepared and sieved by no 100 #. Starch (Soluble), Sodium starch glycolate (SSG), fumed silica (Aerosil 200), *Satte pudina* (Mint), were mixed with dried extract and maltodextrin mixture (prepared from NN) with the help of mixer jar^{9,11}. Previously to prepare the infusion NN, all the drugs were soaked in 360 mL of boiling water overnight (drug water ratio 1:9)¹⁰. All the drugs were meshed manually & drained with the help of dried and cleaned muslin cloth. After that non-native extract of NN granules were obtained by mixing the excipient and drying on water-bath at 50 to 60°C¹². Fine powder of ingredients of NN was used as filler, and sterilized for one hour at 160°C before mixing with the dried extract^{11,13}.

Excipient mixed with dried extract was combined with 32 mL distilled water with the help of spray bottle then blended in the mixture until damp mass was obtained. After the process of wetting of mixture and getting the damp mass, it was passed through sieve No. 16 in the granulator (Cemach granulator, oscillating type 8 inch, GMP Model, M/C No 1417) and wet granules were collected in stainless steel tray and dried in Hot air oven (Labline Model. No. HO 6.7) for 1 h at 60°C. After cooling it is stored in an airtight jar made of glass with a pouch of silica gel in it for evaluation. Then *in vitro* immunomodulation test was performed at Skanda Life Sciences Pvt. Ltd Bangalore.

In vitro immunomodulation test

Cytotoxicity studies for THP-1 cell line

Conventionally, determination of toxic effects of unknown compounds by *in vitro* method has been done by staining with a vital dye and counting viable cells. Alternate methods can be measurement of radioisotope incorporation as a measure of DNA synthesis, and counting is done by automated counters and also other methods which are based on dyes and cellular activity. The MTT or (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) system is a method for measuring the activity of living cells *via* mitochondrial dehydrogenases. The MTT method is accurate, simple and it also gives reproducible results. MTT is a water soluble tetrazolium salt, it yield yellowish solution in media or salt solutions. MTT is converted to a water insoluble purple formazan due to cleavage of the tetrazolium ring through mitochondrial dehydrogenase enzymes of metabolically viable cells.

This water insoluble formazan can be solubilized by using DMSO (dimethyl sulfoxide), acidified isopropanol, propanol or ethanol. This purple solution can be measured spectrophotometrically. A decrease or increase in number of cells results in an associated change in the quantity of the formazan formed from MTT. This conversion indicates the degree of effects caused by the test drug / material.

The purpose of this SOP is to get concise information on cytotoxicity assay by MTT method. Materials used are: 1. MTT Powder (the solution made is filtered through a 0.2 µm filter and is stored at 2 - 8°C for frequent use or can be frozen for extended periods), 2. CO₂ incubator, 3. DMSO, and 4. Spectramax I3X. For the cytotoxicity studies, 2 mg/mL of stocks were prepared using RPMI plain media. Serial two-fold dilutions was prepared from 1280 µg/mL to 10 µg/mL by using RPMI plain media for procedure / treatment.

Cell lines and culture medium

THP-1 cells were obtained from ATCC, stock cells were cultured in RPMI supplemented with 10% of inactivated FBS (fetal bovine serum), penicillin (100 IU/mL), streptomycin (100 µg/mL) in humidified atmosphere of 5% CO₂ at 37°C till confluent. The cell was dissociated with the help of cell dissociating solution containing 0.2% trypsin, 0.02% EDTA and 0.05% glucose in PBS. The viability of the cells is then checked and is centrifuged. Further, 50,000 cells / well were seeded in a 96 well plate and is incubated for 24 h at 37°C, 5% CO₂ incubator. Source of reagents: RPMI, FBS, Pen., Strep., Trypsin was procured from Invitrogen.

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/mL using respective media, containing 10% FBS. To each well of 96 well microliter plate, 100 µL diluted cell suspension (50,000 cells / well) was then added. Then after 24 h, when a partial monolayer was formed, the supernatant was then flicked off, monolayer was then washed, once with medium and then 100 µL of different test concentrations of the test drugs were added on to partial monolayer in microtiter plates. The plates were then incubated at 37°C for 24 h in 5% CO₂ atmosphere. After incubation, the test solutions in the wells were discarded and then 100 µL of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were then incubated for 4 h. at 37°C in 5% CO₂ atmosphere. The supernatant was then removed and 100 µL of DMSO was added and the

plates were gently shaken to solubilize the formed formazan. The absorbance was then measured at a wavelength of 590 nm, using a microplate reader. The percentage growth proliferation was calculated using the formula mentioned below and concentration of test drug needed for proliferating cell growth by 50% (EC₅₀) values was generated from the dose-response curves for each cell line. Calculation was done using the following formula:

$$\text{Inhibition \% proliferation} = [(\text{OD of sample}/\text{OD of Control}) \times 100] - 100$$

Statistical evaluation

The EC₅₀ (half-maximal proliferative concentration) value was evaluated which is a measure for the effectiveness of a compound / test drug in proliferating biological or biochemical function. This quantitative measure indicates, how much, particular drug or substance / proliferator is needed to proliferate a given biological process or component of a process: *i. e.*, an enzyme, cell receptor, cell, or microorganism by half. EC₅₀ of a test drug can be determined by adose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity. EC₅₀ values can be calculated for a given antagonist by determining the concentration needed to proliferate half of the maximum biological response of the agonist. EC₅₀ values for cytotoxicity tests were derived from a nonlinear regression analysis / curve fit, based on sigmoid dose-response curve (variable) and is computed by Graph Pad Prism version 6, San Diego, CA, USA software. Nonlinear regression applied to it, is a form of regression analysis in which observational data are modelled by a function which is a non-linear combination of the model parameters and depends on one or more independent variables. The data was fitted by a method of successive approximations^{14,15}.

In vitro phagocytosis assay of macrophages using Murine macrophages model

Macrophages are quiescent cells and are activated when they are stimulated. Different forms of agents such as antimetabolites, antibiotics and cytokines may exert an immunomodulating action which is expressed in the augmentation and / or inhibition of diverse immune responses. This assay is performed to determine whether a compound or a natural product (drug) can increase in the rate of phagocytotic uptake by macrophages. Macrophages / neutrophils play a main role in phagocytosis of microorganisms and for

other foreign entities that enter the body. A drug that can increase the phagocytic capacity of these cells can be termed a potent immunostimulatory. These experiments require macrophage cell line, treatment with test/ samples followed by incubation with yeast. The phagocytosis is measured at a pre-determined time point using the fluorescent reader. The efficacy of the compound as an immune stimulant is determined by comparison of the phagocytotic index with the drug untreated macrophage set^{16,17}.

The term phagocytosis means phage: engulfment and cytolysis: cell process. Phagocytosis is the cellular process of engulfing solid particles by the cell membrane to form an internal phagosome (food vacuole). The phagosome is then delivered to the lysosome, which is an organelle involved in the breakdown of cellular components, lysosome then fuses with the phagosome. The contents then subsequently degraded and released extracellularly *via* exocytosis, or either intracellularly to undergo further processing. Phagocytosis is also involved in the acquisition of nutrients for some cells, in the immune system it is a main mechanism used to remove cell debris and pathogens. Bacteria, dead tissue cells, and also small mineral particles may be phagocytosed. The measurement of phagocytic activity of the immune cells indicates the strength of innate immune system in the host.

Principle of the phagocytosis assay is simple, briefly antigenic cells (yeast in this study) are incubated with the macrophages. The cells were stained with acridine orange, phagocytic cells are scored with the help of microscope. Reagent used are Potato dextrose broth (PDB), DMEM (Dulbecco's Modified Eagle Medium) without phenol red, 3.7% formaldehyde, acridine orange.

Protocol for preparation of yeast cells: 1. Yeast cells were cultured in PDB and cell count was adjusted to 108 cells/mL and heat inactivated for 45 min at 50°C., 2. Cells were centrifuged and the pellet was resuspended in DMEM and yeast cells of density 108 cells / dish was inoculated.

Phagocytosis assay: Cell lines and culture medium used- RAW264.7 cells line (macrophage cell line) was procured from ATCC, stock cells were cultured in DMEM supplemented with 10% inactivated FBS (Fetal Bovine Serum), penicillin (100 IU/mL) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated using cell scraper at 80% confluence. The viability of the cells

was checked and centrifuged. The cells were then counted and seeded at appropriate density.

Procedure of test: The cells were aspirated from the culture flask and then centrifuged at 1000 rpm for 5 min. The cell pellet was then re-suspended in 1 mL of DMEM, complete media and cell count was then adjusted using hemocytometer at a density of 1x10⁶ cells/mL. To each P 35 dish, 1 mL of cell suspension containing 1X10⁶ cells was added. After 24 h for determining the effect of samples on phagocytic activity, RAW264.7 cells were treated with various concentrations of test samples at 37°C in 5% CO₂ atmosphere. Post incubation, the cells were inoculated with heat inactivated yeast cells at a density of 108 cells / dish and the cells were further incubated for additional 4 h to assess the phagocytic activity. After incubation, test solutions were removed and the cell monolayer was gently washed with 1X PBS. The cells were then fixed with 3.7% formaldehyde for 2 min and phagocytic cells were stained using Acridine Orange stain (10 µg/mL), and scored by observing the cells under inverted UV microscope¹⁸⁻²⁰.

Antimicrobial activity study of NNG

In vitro antimicrobial activity was performed for the modified formulation. The samples of NNG were assessed for their MIC property against selected organisms involving the respiratory tract

To study the antimicrobial efficacy of the NNG sample on *Staphylococcus aureus*, *Streptococcus pyogenes*, *Mycoplasma pneumoniae*, *Klebsiella pneumoniae* and *Salmonella* spp., the bacterial samples were preserved at Dextrose Technologies Pvt. Ltd. Bacterial samples were sub-cultured on Luria-Bertani broth 24 h before the study. Test samples were dissolved in distilled water and a concentration of 20, 30, 40 and 50 mg were used for the study. The antimicrobial efficacy of the test sample was tested by the well -diffusion method. Wells of 6 mm diameter were punched on specific agar media. About 200 µL of 24 h old broth culture was spread onto the agar plates. The wells were then loaded with varying concentrations of the test samples. Erythromycin was used as a positive control (20 mg and 50 mg). Bacterial plates were incubated at 37°C for 24 h. The diameter of the zone of inhibition for extract was also measured.

MIC: 2 mL of LB broth was added to all tubes and 100 µL of 24 h culture (1x10⁷ CFU/mL) was inoculated. Samples of varying concentration (20 mg/mL – 50 mg/mL) were taken and added to the

tubes and incubated at room temperature for 24 h. OD was measured at 600 nm^{21,22}.

Acute toxicity study

Acute toxicity study was carried out as per OECD Guidelines (423) at animal house of National Institute of Unani Medicine. Essential ethical approval (IAEC-NIUM) was taken before the study with No. IAEC/6/16/1A/6. 18 young adult, nulliparous and non-pregnant female Wistar rats weighing 150-200 g, 8-12 week old were procured for the study. Each animal was planned to inoculated with initial limit dose of 2000 mg/kg. For toxicity study, animals were selected randomly, for acclimatization with the environment and kept in their cages for 7 days prior to dosing to. The animals were made to fast for 18 h but water was withheld only for 4 h. After fasting, the animals were weighed and the test drugs (NN granules) in the form of suspension were administered 2000 mg/kg as a single dose to group of six animals by oral route for a group of six animals. After administration of test drug food was withheld further for 4 h²³.

Observations

After the period of acclimatization and quarantine before giving the dose, physical and general behavioural examinations of animals were done to rule out infection for first 4 h. The animals were also observed individually for toxic sign specially onset of any toxic behaviour, changes of skin, fur, eyes and also respiratory & circulatory, and attention were also directed to observations for salivation, tremors, convulsions, lethargy, diarrhoea, sleep and coma. Up to 24 - h toxic behaviour and number of mortalities was recorded up to 14 days, as no mortality was noted, weights of the animal and food intake was recorded weekly once. General behaviour of animals, skin pigmentation, skin colour, palpable mass, body hair loss, tremor, convulsions & motility, were observed for 14 days.

Gas chromatography-mass spectroscopy (GCMS)

GCMS is a hyphenated system which is a very compatible technique and the most commonly used for the identification of unknown organic compounds in a complex mixture which can be determined by interpretation and also by matching the spectra with reference spectra (NIST). In this work fingerprinting data is generated with unknown peak detail.

The GC-MS test was performed by using Simadzu QP2010 Ultra Model and software Turbomassver 5.5. The fused silica column was packed with Elite - 5

MS. The temperature of oven in it was set up from 50°C with an increase of 8°C / min to 220°C for 5 min and 7°C / min to 280°C for 15 min. Helium gas (99.999%) was used as a carrier gas at constant flow rate of 1 mL / min. An aliquot of 10 µL of sample was injected into the column with the injector at 280°C of the split ratio of 10:1. The ionizing energy of 70eV was used and the electron ionization was involved. The mass range is 40-600 amu. The inlet line temperature was 200°C and source temperature was 150°C. Total GC running time was 60 min.

Liquid chromatography-mass spectrometry (LC-MS)

Instrument Details: HPLC- Shimadzu; Column- Phenomenox, Luna C18, 4.6*100 mm, 5 µm; Mobile phase - Acetonitrile: 0.1% formic acid in H₂O, 60:40v/v Flow rate: 0.5 mL/min; Column temperature - 35°C, Detector - UV 254 nm, Mass spectrometer - TSQ Quantum Access MAX Triple Quadruples LC-MS; Mass spectrometer parameters: curtain gas 10, gas-1 20 and gas-2 0, needle voltage 5000 V, and de clustering potential 100 V.; Mode - Positive ion and negative ion mode with precursor ion mass scan from 50-1050 Daltons.

Results

Immunomodulatory test, Proliferation / Cytotoxicity studies for THP-1 cell line is depicted in Table 1, Figure 1. Test sample (NNG) displayed an EC50 value of 997.6 µg/mL in THP-1 cells with a proliferation of 53.95% at 1280 µg/mL. *In-vitro* phagocytosis assay of macrophages is depicted in Table 2, and Figure 2. Raw 264.7 cells were treated with various samples for macrophage activation and were inoculated with heat inactivated yeast cells. The results suggest that the cells treated with test sample 1280 µg/mL have shown phagocytic activity of 67.85% whereas, the cells treated with 640 µg/mL showed 54.65% activity. Hence, it could be concluded

Table 1 — Immunomodulatory test, proliferation / cytotoxicity studies for THP-1 cell line

Compound	(Conc. µg/mL)	OD at 590 nm	% Proliferation	EC50 µg/mL
Control	0	0.724	0.00	
Sample	10	0.740	2.27	
	20	0.754	4.20	
	40	0.766	5.91	
	80	0.797	10.14	997.6
	160	0.881	21.77	
	320	0.906	25.21	
	640	0.961	32.81	
	1280	1.114	53.95	

that the test sample (NNG) displayed significant phagocytic activity.

Antimicrobial activity studies such as MIC and diameter of zone of inhibition is depicted in Table 3, Table 4 and Figure 3. 50 mg concentration of the extract is found to be effective against *Klebsiella pneumoniae*, *Salmonella* spp., *Staphylococcus aureus*, and *Streptococcus pyogenes*. The extract is found to be not effective against *Mycoplasma pneumoniae*.

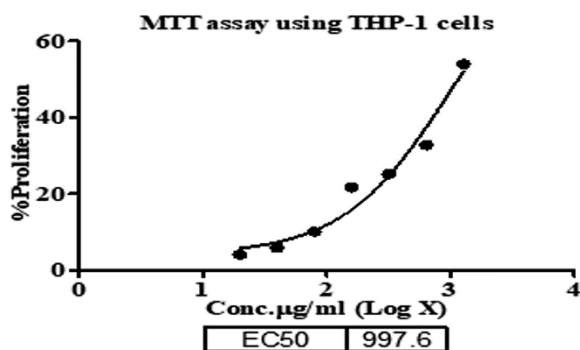


Fig. 1 — MTT Assay of NNG

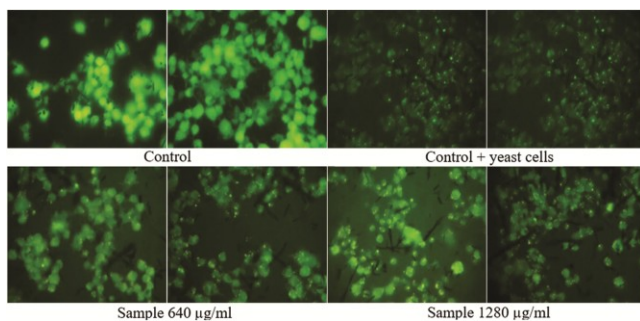


Fig. 2 — *In vitro* phagocytosis assay of macrophages (NNG)

MIC for *K. pneumoniae* is 35 mg/mL, for *M. pneumoniae* is 0 (No activity), MIC for *S. Typhi* is 25 mg/mL, for *S. aureus* is 25 mg/mL, for *S. pyogenes* is 35 mg/mL

In acute toxicity study, no death and abnormal signs was observed in any of the test animal administered with NN granules suspended in 1 mL of distilled water. Body weight of animals on 0, 8th, and 15th was found to be 155 ± 5.23 , 160 ± 6.34 and 165 ± 7.98 g. Statically non-significant effect was observed for food intake, hair loss, skin pigmentation, color change, palpable mass and Keratosis. Motility did not alter in any animal, nor convulsion and tremor occurred in any animal.

GCMS result of NNG: Detected peaks, Retention time, Area, Height and Area % are depicted in Table 5. LCMS analysis of NNG: LCMS extracted ion chromatogram in positive and negative mode of crude extraction are depicted in Figure 4. In fraction test sample (NNG), the positive mode mass spectrums of compounds showed abundant $(M+H)^+$ 104, 155, 381 Da, The negative mode mass spectrums of compounds show abundant $(M-H)^-$ mass of 366, 430, 528 Da. Details of LCMS chromatogram is depicted in Figure 4, Table 6.

Discussion

The common cold and flu are one of the most commonly occurring medical conditions all over the world caused due to viral infection. Another important aspect of such infection is chances of secondary bacterial infection. Though most viral infections are self-limiting, control of associated

Table 2 — *In vitro* phagocytosis assay of macrophages for NNG

Samples	Concentration (µg/mL)	Total no. of yeast cells (Mean of 10 fields)	No. of Phagocytes (Mean of 10 fields)	SEM for 24 h	% of Phagocytes
Control	0	0	0.00	0.00	0.00
Control+Yeast	0	178	63	1.01	91.57
Sample	640	161	88	0.79	54.65
Sample	1280	168	114	0.85	67.85

Table 3 — Zone of inhibition

Concentration (mg/mL)	Diameter of zone of inhibition in mm				
	<i>K. pneumoniae</i>	<i>M. pneumoinia</i>	<i>S. typhy</i>	<i>S. aureus</i>	<i>S. pyogenes</i>
20	0	0	11	0	0
30	0	0	18	16	0
40	16	0	25	18	10
50	20	0	29	26	22
Positive control	20	20	40	25	45
Erythromycin 20					
Positive control	30	26	55	28	50
Erythromycin 50					

medical conditions and prevention of secondary infections is important. The treatment in conventional medicine is mainly focused on relieving the associated symptom. Since conventional approach are target orientated and relieve the associated symptoms. There is no concept of immunomodulation with other

associated beneficial action. Unani medicine has different approach towards treatment of diseases including viral infection by its holistic approach. It uses drugs which have overall effects on the body, such as correction of the temperament of the body which has been deranged, stop flowing of morbid

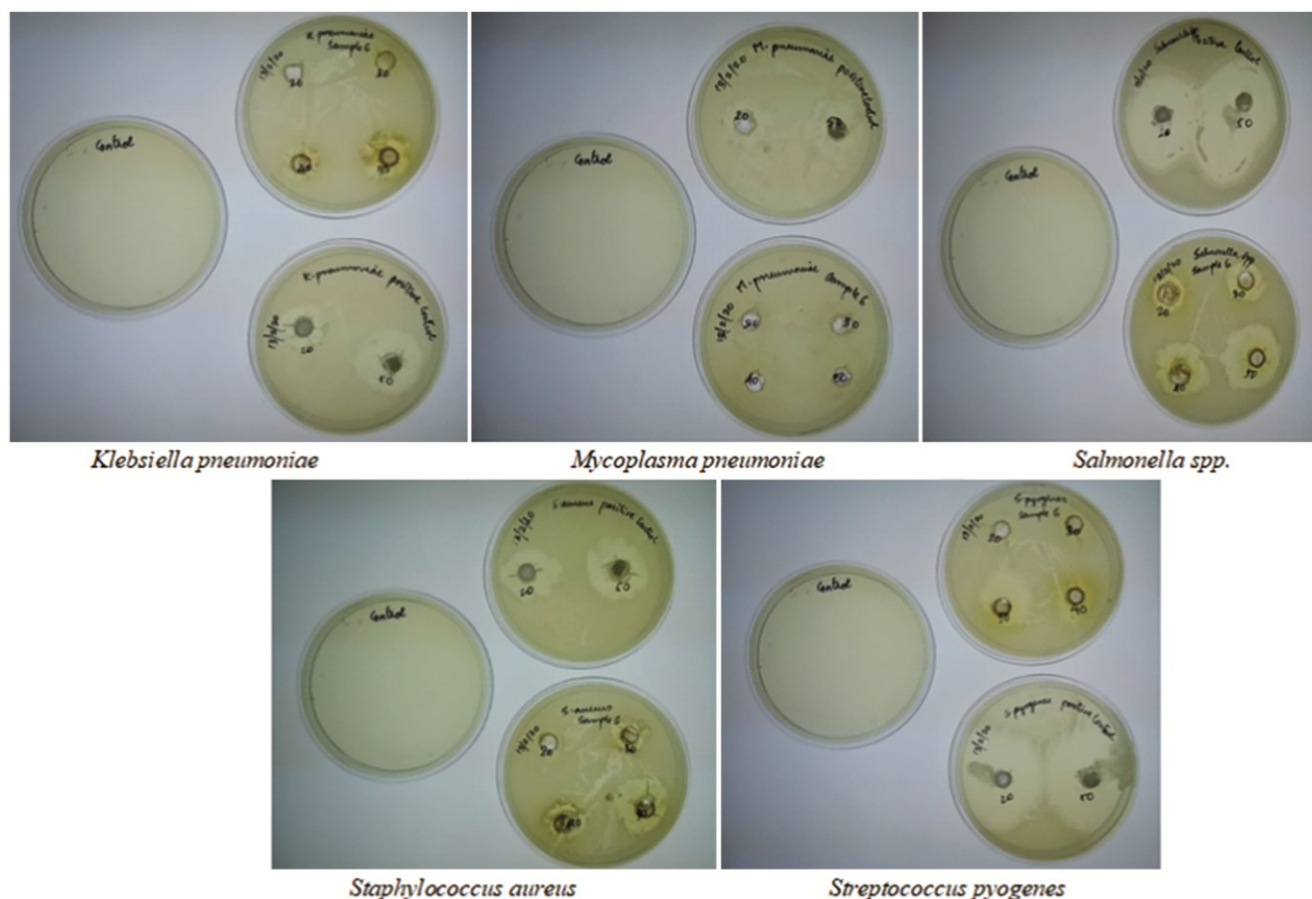


Fig. 3 — Antimicrobial effect shown by the test sample (NNG) in various concentrations

Table 4 — Minimal inhibitory concentration (MIC)

Concentration (mg/mL)	Minimal inhibitory concentration (MIC) OD600 nm				
	<i>K. pneumoniae</i>	<i>M. pneumoiniae</i>	<i>S. typhy</i>	<i>S. aureus</i>	<i>S. pyogenes</i>
20	0.126	0.398	0.011	0.045	0.634
25	0.112	0.411	0.000	0.000	0.452
30	0.098	0.387	0.000	0.000	0.089
35	0.000	0.399	0.000	0.000	0.000
40	0.000	0.387	0.000	0.000	0.000
45	0.000	0.393	0.000	0.000	0.000
50	0.000	0.393	0.000	0.000	0.000
Positive control (Organism)	0.967	0.432	0.665	0.972	0.945
Positive control 20 mg/mL (Erythromycin)	0.014	0.010	0.024	0.005	0.002
Negative control. (media)	0.000	0.000	0.000	0.000	0.000

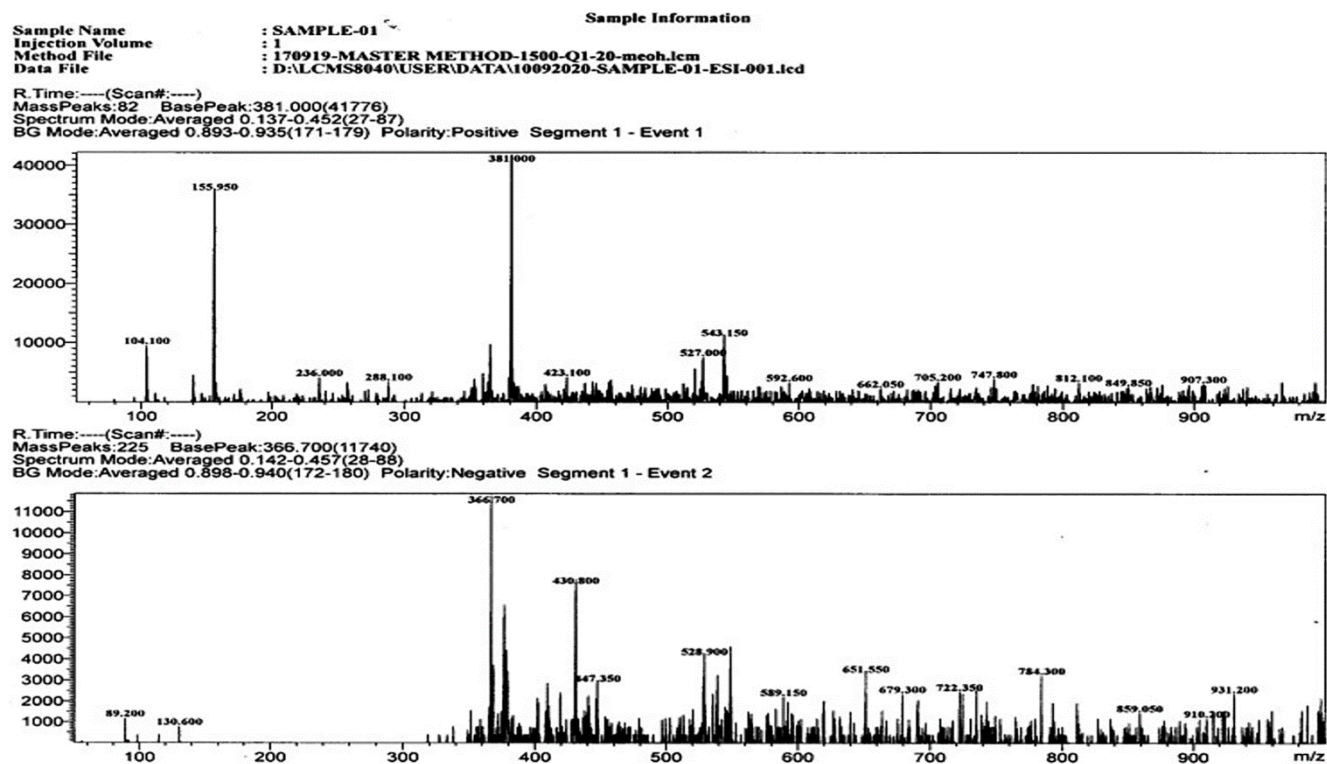


Fig. 4 —LCMS extracted ion chromatogram in positive mode and negative mode

Table 5 — GC-MS analysis of NNG

Retention time	Area	Height	Area %
6.225	817145	34673900	94.3
13.948	27072	668700	3.1
15.013	2735	137690	0.3
16.184	6430	255592	0.7
16.329	4171	244027	0.5
17.399	4969	246369	0.6
18.465	1069	47750	0.1
19.100	3189	133176	0.4

Table 6 — Summary results of MS analysis of Sample

Mode	Mass Spectrum	
	Retention Time (min)	Abundant mass recorded (M + H) ⁺
Positive	0.137-0.452	104,155, 381,
	Retention Time (min)	Abundant mass recorded (M - H) ⁻
Negative	0.142-0.457	366,430,528

matters, modify the consistency of morbid matters, minimizing complications boosting the immunity perspective of suitable diet, fomentation, steam inhalation with drugs etc. Most Unani physician favour use of single drugs but practically most medical conditions require a combination of drugs. The Nazla / Zukaam (Common cold / coryza and flu) is a condition in which combinations of drugs are required to alleviate associated symptoms, therefore is

to be formulated in such a way that it could address all the necessities with immunomodulation.

NN is one such formulation which is a combination of 7 drugs possessing different actions. *In vitro* test was applied for testing immunomodulatory activity on the NN granules (NNG) formulation modified by Zaigham *et. al.*⁹ from decoction / infusion. In terms of EC₅₀ value of 997.6 µg/mL THP-1 cells with the proliferation of macrophage by 53.95% at 1280 µg/mL, this test showed that the NN Granules can be effective as an immunomodulator. The immunomodulatory effect of the granules displayed 67.85% phagocytosis (Table 1, Table 2; (Fig. 1 & Fig. 2). A strong immune system helps in prompt neutralization and elimination of pathogenic micro-organism such as viruses and bacteria, and many kinds of toxic products form in the body due to them. Modulation of immune response by herbal / traditional formulations can provide a substitute / adjuvant for anti-viral and anti-microbial drugs. Several herbal drugs indicated in traditional medicine including Unani medicine possess immunomodulatory properties; they can produce both, specific and nonspecific immune responses. As new challenges faced by the humanity like recently prevailed SARS-CoV-2 virus (Covid-19), it is important that people

should have strong immune system to fight against such infections and stay safe from the complications.

The review of literature on reported immunomodulatory activity with other activity in drugs used in formulation support the activity of the NNG formulation, *Cordia latifolia* is reported to be active as an immunomodulator against cell-mediated immune response²⁴. Hydroalcoholic extract of *Z. jujuba* is also reported to be anti-inflammatory and immunomodulator^{25,26}. Similarly, *A. officinalis* is reported to be antitussive, demulcent, soothing and several works reported its immunomodulatory activity²⁷⁻²⁹. *Borago officinalis* is reported to be immunomodulator³⁰. *Glycyrrhiza glabra* and its type have also shown effect as immunostimulatory and immunomodulatory^{31,32} as well as reported to be antitussive and expectorant^{33,34}.

Unani physicians have developed many formulations keeping in view its multidimensional activities. In common cold / flu there are chances of secondary bacterial infections and as per the classical claim, this formulation can prevent secondary infection. Activity against common pathogenic microbes on respiratory system was studied in this work, keeping in view the hypothesis of antimicrobial activity of the drugs in NNG. It was tested by microtitre plate method (96 well) against 5 common bacteria. Interestingly, the test drug was found active against all the tested bacteria except *M. pneumoniae*. (Table 4, Table 3 & Fig. 3) The review of reported antimicrobial and other pharmacological activity of drug used in the study further justify the activity in formulation, *Viola odorata* and *Cordia latifolia* leaf extract are reported for its antibacterial activity against *S. aureus*, *Pseudomonas aeruginosa*, *E. coli* etc.^{35-37,38}. Antimicrobial activity is also reported in *Althea officinalis*, *Borago officinalis*^{39,40}. *Glycyrrhiza glabra* is reported as antimicrobial and antifungal^{41,42}. Bnasal *et. al.*⁴³ studied antimicrobial and antioxidant activities on the lozenges prepared from decoction / Joshanda of same ingredients present in NN, and checked this aqueous extract (Joshanda) for antibacterial activities against two organism and found susceptible against *Staphylococcus aureus* whereas no zone of inhibition was observed for *Pseudomonas aeruginosa*⁴³ but in this work NNG displayed activity against *Klebsiella pneumoniae*, *Salmonella* spp., *Staphylococcus aureus*, and *Streptococcus pyogenes* but observed no effect against *Mycoplasma pneumoniae* in respect of zone of inhibition (Table 3, Table 4 & Fig. 3).

Whenever an already existing dosage form is converted into a new dosage form, as a rule, its safety profile becomes necessary, in acute toxicity study of NNG no death and abnormal signs were observed in any animal fed with NNG which clearly indicates the safety of modified formulation in the animal study. Chromatography study of NNG which contains seven drugs displayed many peaks in GCMC and LCMS, fingerprinting data for the NNG formulation was also set in (Table 5, Table 6 & Fig. 4). These data may help in identification of the modified formulation. LC-MS is selected because it is more specific and sensitive than standard HPLC, and GCMS is done to evaluate the volatile organic content if any in NNG⁴⁴. Further detailed chromatography studies with both qualitative and quantitative estimation of constituent is needed for this modified data however HPTL and HPLC data with quantitative estimation of Glycerhizin was developed by Zaigham *et al.*⁹.

Based on the above findings, it can be concluded that NN can be effective against common cold and coryza, and apart from symptomatic relieving activity, it also possesses antimicrobial activity and immunomodulatory activity; these findings are in confirmation with Unani claims as well as reported pharmacological activities as antimicrobial and immunomodulator. THP-1, a human leukemia monocytic cell line, has become a common model to estimate modulation of monocyte and macrophage activities. THP-1 response can hint to potential responses that might occur *ex vivo* or *in vivo*⁴⁵. Similarly, phagocytosis assay is a tool in characterizing the magnitude of monocytic cell activation⁴⁶, However, more detailed and sophisticated studies especially toxicity studies, antimicrobial, *in vivo* immunomodulatory and clinical studies are needed to strengthen the claim. Further study is also needed to conduct a comparative study of NN as well as its converted dosage form NNG to prove the superiority of drug because in Unani medicine all dosage form has been designed based on extensive observations. Any change in the existing dosage form shall not be allowed only for innovation unless the converted form is proved to be more effective than existing dosage form, which should be based on optimization rather than simply on innovation.

Conclusion

From the findings of the study, it can be concluded that NNG displayed immunomodulatory and

antimicrobial activities in *in-vitro* study and NNG is safe as per acute toxicity study in animal model up to 2000 mg/kg. GCMS and LCMS data was also set in. Further sophisticated study using various other immunomodulatory parameters and effect of test drug on interferons levels can also be done for further validation of NNG.

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Conflict of Interest

The authors do not have any conflicts of interest to declare.

Author Contributions

KNBNA: Writing, Data curation, Investigation, AW: Writing, Editing, Investigation, Supervision, H: Methodology, Investigation, Editing, MZ: Methodology, Investigation, UJ: Investigation.

References

- Eccles R. Common cold. *Front Allergy*, Jun 22; 4: 1224988, (2023). doi: 10.3389/falgy.2023.1224988. PMID: 37426629; PMCID: PMC10324571.
- Couch R B, Englund J A & Whimbey E, Respiratory viral infections in immunocompetent and immunocompromised persons, *Am J Med*, (1997) Mar 17, 102 (3A) 2-9, discussion 25-6.
- Morris D E, Cleary D W & Clarke S C, Secondary Bacterial Infections Associated with Influenza Pandemics, *Front Microbiol*, 23 (8) (2017) 1041.
- World Health Organization (2014), Antimicrobial resistance: Global report on surveillance, Geneva: World Health Organization, <http://www.who.int/drugresistance/documents/surveillancereport/en/>
- Kidd T J, Mills G, Sá-Pessoa J, Dumigan A, Frank C G, *et al.*, A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence, *EMBO Mol Med*, 9 (4) (2017) 430-447
- Guyue Cheng, Menghong Dai & Saeed Ahmed, Antimicrobial drugs in fighting against antimicrobial resistance, *Front Microbiol*, 7 (2016) 470.
- Sapkota B, Makandar S N & Acharya S, Biologic Response Modifiers (BRMs), In: StatPearls. Publishing, Treasure Island (FL); (2021), PMID: 31194357, Available from: <https://www.ncbi.nlm.nih.gov/books/NBK542200>.
- Srinivasan Shanmugam, Granulation techniques and technologies: recent progresses, *Bioimpacts*, 5 (1) (2015) 55-63.
- Mohammad Zaigham & Hamiduddin, Formulation and evaluation of Naque Nazla as Granules, Dissertation submitted to the Rajiv Gandhi University of Health Sciences, Karnataka, Bengaluru, 2017.
- Kabeeruddin M, Bayaz-e-kabeer, Part III (Dawasazi), New Delhi, YNM: p. 23, 51, 52.
- Paul Beringer, *et al.*, Remington, 'The science and practice of pharmacy', Lippincott, 21st Edition, Vol I, (2005) p. 717, 758, 786, 896-899.
- Augsberger L L & Hoag S W, Pharmaceutical Dosage Forms Tablets, Third Edition, Vol II Design and Formulation, Informa health care, USA, (2008) p. 33,338.
- Shiv Narayan Sahu, The Technology of Preparation and Distribution of Drugs and Cosmetics, Kislav Book House, Muzaffarpur India, (1990) p. 123.
- Crouch S P, Kozlowski R, Slater K J & Fletcher J, The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity, *J Immunol Meth*, 160 (1993) 81-8.
- Gonzalez R J & Tarloff J B, Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity, *Toxicology in vitro*, (2001) Jun 1, 15 (3) 257-9.
- Valentin-Weigand P, Benkel P, Rohde M & Chhatwal G S, Entry and intracellular survival of group B streptococci in J774 macrophages, *Infect Immun*, 64 (1996) 2467-2473.
- More P & Pai K, Immunomodulatory effects of *Tinospora cordifolia* (Guduchi) on macrophage activation, *Biol Med*, 3 (2) Special Issue (2011) 134-140.
- Marcil A, Harcus D, Thomas D Y & Whiteway M, Candida albicans killing by RAW 264.7 mouse macrophage cells: Effects of Candida genotype, infection ratios, and gamma interferon treatment, *Infect Immun*, 70 (11) (2002) 6319-6329.
- Lewis L E, Bain J M, Okai B, Gow N A & Erwig L P, Live-cell video microscopy of fungal pathogen phagocytosis, *J Vis Exp*, (2013) 71.
- <http://www.protocol-online.org/prot/Protocols/In-vitro-Phagocytosis-Assay-of-Macrophages-3460.html>, Accessed on 24 /04 /2022
- Nalawade T M, Bhat K G & Sogi S, Antimicrobial activity of endodontic medicaments and vehicles using agar well diffusion method on facultative and obligate anaerobes, *Int J Clin Pediatr Dent*, 9 (4) (2016) 335.
- Jain I, Jain P, Bisht D, Sharma A, Srivastava B, *et al.*, Comparative evaluation of antibacterial efficacy of six Indian plant extracts against *Streptococcus mutans*, *J Clin Diagn Res*, 9 (2) (2015) ZC50.
- OECD Guidelines for testing of chemicals (423) (2001).
- Ali W R, Al-Asady Z T & Ibrahim A A, Immunomodulatory of *Cordiamyxa* (L.) aqueous extract fruit in immunized micewith hydatid cyst fluid, *J Nat Sci Res*, 5 (10) (2015) 75-83.
- Goyal R, Sharma P L & Singh M, Possible attenuation of nitric oxide expression in anti-inflammatory effect of *Ziziphus jujuba* in rat, *J Nat Med*, (2011) Jul, 65 (3-4) 514-518.
- Ghanchari M S, Kumar S & Bhat K G, Effect of *Zizyphus jujube* leaves extract on phagocytosis by human neutrophils, *J Nat Remedies*, 4 (1) (2004) 47-51.
- Fahamiya N, Shiffa M Aslam M & MUZN F, Unani perspective of Khatmi (*Althaea officinalis*), *J Pharmacogn Phytochem*, 5 (6) (2016) 357-360.
- Husain M, Zaigham M, Hamiduddin, Wadud A & Ali M A, A review on pharmacological and phytochemical profile of

- Khatmi (*Althaea officinalis* Linn.): An important mucilaginous plant and its utilization in Unani system of medicine, *Int J Res Ayurveda Pharm*, 12 (3) (2012) 63-72.
- 29 Modaresi M, Effect of marshmallow (*Althaea officinalis*) root extract on immune system in mice, *Res J Biol Sci*, 9 (1) (2014) 16-18.
- 30 Kadri Z H M, Immunomodulatory effects of *Crocus sativus* L. (petals) and *Borago officinalis* L. (whole plant) aqueous extracts in albino mice, *Al-Mustansiriyah J Sci*, 25 (1) (2014) 7-16.
- 31 Ayeka P A, Bian Y, Githaiga P M & Zhao Y, The immunomodulatory activities of licorice polysaccharides (*Glycyrrhiza uralensis* Fisch.) in CT 26 tumor-bearing mice, *BMC Complement Altern Med*, 17 (1) (2017) 536.
- 32 Wagner H & Jurcik K, Immunological studies of Revitonil: A phytopharmaceutical containing *Echinacea purpurea* and *Glycyrrhiza glabra* root extract, *Phytomed*, 9 (5) (2002) 390-397.
- 33 Gupta Y K, Katyal J, Kumar G, Mehla J, Katiyar C K, *et al.*, Evaluation of antitussive activity of formulations with herbal extracts in sulphur dioxide (SO₂) induced cough model in mice, *Indian J Physiol Pharmacol*, 53 (1) (2009) 61-66.
- 34 Shitole M & Pawar V T, Study of potential antitussive activity of *Glycyrrhiza glabra* granules using a cough model induced by sulphur dioxide gas in mice, *Asian J Pharm Clin Res*, 12 (10) (2019) 262-267.
- 35 Khatibi A, Shah A H, Ageel A M, Ahmad M S, Al-Yahya M A, *et al.*, Saudi Folk Medicine: Phytochemical and Antimicrobial Screening, *Pak J Pharm Sci*, 2 (1) (1989) 29-34.
- 36 Zarrabi M, Dalirfardouei R, Sepehrizade Z & Kermanshahi R K, Comparison of the antimicrobial effects of semipurified cyclotides from Iranian *Viola odorata* against some of plant and human oathogenic bacteria, *J Appl Microbiol*, 115 (2) (2013) 367-375.
- 37 Gautam S, Navneet & Kumar S, The Antibacterial and phytochemical aspects of *Viola odorata* Linn. extracts against respiratory tract pathogens, *Proc Natl Acad Sci India Sect B Biol Sci*, 82 (4) (2012) 567-72.
- 38 Pandey B, Deshpand B, Singh S & Chandrakar V, Estimation of elemental contents of *Cordia myxa* and its antimicrobial activity against various pathogenic microorganisms, *Indian J Sci Res*, 4 (1) (2014) 39-44.
- 39 Rashidi A, Mousavi B, Rahmani M R, Rezaee M A, Hosaini W, *et al.*, Evaluation of antifungal effect of *Lavandula officinalis*, *Salvia officinalis* L., *Sumac*, *Glycyrrhiza glabra*, and *Althaea officinalis* extracts on *Aspergillus niger*, *Aspergillus fumigatus*, and *Aspergillus flavus* species, *J Med Plant Res*, 6 (2) (2011) 309-313.
- 40 Abdullah R M, Hameed H Q & Ali A, Antimicrobial activity of *Borago officinalis* leaves extract against *Staphylococcus* Spp. and *Streptococcus pyogenes*, *Diyala J Pure Sci*, 13 (1) (2017).
- 41 Sharma V, Katiyar A, & Agrawal R C, *Glycyrrhiza glabra*: Chemistry and Pharmacological Activity, Sweeteners. Jul 31: (2017) 87-100. doi: 10.1007/978-3-319-27027-2_21. PMID: PMC7124151.
- 42 Fatima A, Gupta V K, Luqman S, Negi A S, Kumar J K, *et al.*, Antifungal activity of *Glycyrrhiza glabra* extracts and its active constituent glabridin, *Phytother Res*, 23 (2009) 1190-1193.
- 43 Bansal M, Gulati M, Singh S K & Duggal S, Antibacterial antitussive, antioxidant and toxicological evaluation of Joshanda lozenges, *J Appl Pharm Sci*, 5 (07) (2015) 64-07.
- 44 Kännaste A, Copolovici L & Niinemets Ü, Gas chromatography-mass spectrometry method for determination of biogenic volatile organic compounds emitted by plants, *Methods Mol Biol*, 1153 (2014) 161-9.
- 45 Chanput W, Mes J J & Wichers H J, THP-1 cell line: An *in vitro* cell model for immune modulation approach, *Int Immunopharmacol*, 23 (1) (2014) 37-45. doi: 10.1016 / j.intimp.2014.08.002. Epub 2014 Aug 14.
- 46 Caponegro M D, Thompson K K, Tayyab M & Tsirka S E, A rigorous quantitative approach to analyzing Phagocytosis assays, *Bio Protoc*, 10 (15) (2020) e3698.