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Research Article

SANATIVE EFFECT OF A LOW-COST NOVEL GREEN FORMULATION – IM-SSS20 TO MINIMIZE THE INFLAMMATORY AND CYTOKINE STORM AGAINST RESPIRATORY DISEASES

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ABSTRACT

Objective: To develop a herbal formulation combining four ingredients namely *Ocimum sanctum, Green tea, Nyctanthes arbor-tristis* and *Hygrophila auriculata* to combat infections which affects the respiratory system leading to inflammation, stress and other complications.
Method: The management of respiratory conditions was tried with the low cost multi herbal preparation IM-SSS 20.We have developed a herbal formulation combining four ingredients namely Ocimum sanctum, Green tea, Nyctanthes arbor-tristis and Hygrophila auriculata. Plant secondary metabolites in the developed multi-herbal formulation (MHF) (IM-SSS-20) were detected through quantitative analysis. Anti-bacterial and anti-fungal activity of the IM-SSS-20 were also determined. *In-vitro* clinical study was done to measure the haematological parameters like Haemoglobin (Hb), Haematocrit (PCV), Total RBC (RBC) and Total WBC (TC) and estimate the expression of several biomarkers like IL-6, IL-8, IL-10 and TNF-α.
Results: The phtyochemicals like triterpenes, flavonoids, saponins, and tannins were abundantly found in the IMSSS-20 extract. The inhibitory action of IM-SSS20 was dose dependent and significant anti-bacterial activity was seen in the case of *Escherichia coli* followed by *Streptococcus aureus* and *Klebsiella pneumonia* respectively. The IM-SSS 20 also showed antifungal activity, the activity was maximum in case of *C. albicans* and was least for *A.niger*. In this study we observed a lower value of hemoglobin, hematocrit, total RBC and total WBC in respiratory infected patients. IM-SSS20 was effective in normalizing the concentration of Hb, PCV, RBC and TC.
Conclusion: The multi-herbal formulation IM-SSS20 showed promising results *in-vitro* studies and further studies are required to explore its efficacy in humans. This low-cost formulation can improve health as well as economy of the developing countries.

Keywords: Respiratory diseases, *Ocimum sanctum*, Green tea, *Nyctanthes arbor-tristis* and *Hygrophila auriculata*.

INTRODUCTION

Respiratory complaints like cough, cold, and catarrh are commonly encountered in our daily life and have a significant impact on health all over the world. Respiratory diseases remain a big public health challenge for both the advanced and emergent nations because of their frequency and economic impacts. Viruses, especially rhino viruses are the root cause of most of the catarrhal disorders but individuals suffering from temporary or permanent asthenia of the immune system can also develop a secondary bacterial infection. Symptomatic treatment of such respiratory diseases include measures which improve the normal functioning of the mucous membranes of the upper respiratory tract, lessen symptoms of cold, and boosts the immune response.

According to the report published by the Forum of International Respiratory Societies, the main contributors to the respiratory disease burden are acute respiratory infections, asthma, tuberculosis (TB), chronic obstructive pulmonary disease (COPD), and lung cancer [1]. The respiratory diseases namely, infections of lower respiratory tract, COPD, TB, and lung cancer, are one of prime reasons of morbidity and mortality all over the world [2-6]. In the year 2016, 92.5 million disability-adjusted life years (DALYs) were lost as a result of chronic respiratory diseases worldwide [7].

Acute uncomplicated bronchitis, pharyngitis, rhino sinusitis, and the common cold are some of the symptoms associated with acute respiratory tract infection,which are mostly viral and antibiotics are used in treatment [8-9]. Antibiotics resistance can happen due to over usage causing adverse effects, including anaphylaxis and cost escalation [8-9]. The multi- herbal preparation has herbs which can have immune-stimulating and inflammation-modulating effects. This, in turn, can help prevent immune overreaction ("cytokine storm") and still helping the immune system cope better with the infections.

The standard treatment protocol for COPD is to relieve associated symptoms, prevent recurrent exacerbations, to preserve optimal lung function and improve the quality of life [10]. Complementary and alternative medicine (CAM) as well as herbal medicines have gained popularity because of adverse effects and disappointing treatment results of conventional drugs [11]. It has also been observed that a low concentration of Haemoglobin is observed in patients suffering from COPD [12-13].

As documented by the survey results of the National Asthma Campaign, 60% - 70% of people suffering from asthma had used CAM, where plant based drugs occupied third position in terms of popularity amongst adults (11%) and children (6%) to get relieve [14].

TB is one of the most prevalent disease in the world and is a severely pathogenic in HIV/AIDS patients. It has been reported by some of the researchers that half of TB survivors develop persistent pulmonary dysfunction despite microbiologic cure, [15-18] which puts the survivors at an increased risk to death [19-22]. Multidrug resistance and Extensively drug resistance TB calls for exploration for new anti-TB drugs. The search for novel anti-mycobacterial drugs leads to phyto-drugs which enhances safety and minimizes infection and side effects. Treatment of Lung cancer is very difficult because of multi-drug resistance and side effects so herbal formulation can bring a ray of hope with few side effects and high treatment outcomes by enhancing the quality of life and increasing the survival time of such patients.

The health benefits of these herbs namely *Ocimum sanctum, Nyctanthes arbor-tristis* and *Hygrophila auriculata* and many more are not only well documented in our Indian ancient scriptures but also in present-day literature [23-26].

Ocimum sanctum an aromatic shrub, commonly known as holy basil or Tulsi is belonging to the family Lamiaceae (tribe ocimeae). In Ayurveda which is the world's oldest medical system, Tulsi is known as "Queen of Herbs" because of its all-round medicinal properties including psychological and physiological benefits[27-33].The extracts of Tulsi leaf are documented in the treating bronchitis, rheumatism and pyrexia in Indian Materia Medica[34]. Studies have highlighted the anti-bacterial, anti-viral, anti-cancer [35] and antifungal activity of tulsi [36] and it also revealed that Tulsi enhanced immune responses [37] as well can reduce stress, anxiety and depression in humans[38]. Rama and Krishna Tulsi are two botanically and phytochemically distinct cultivars of *Ocimum sanctum* L.[39-40]. Many of the physiological benefits of Tulsi are often attributed to its high content of phenolic compounds and antioxidant properties, with Krishna Tulsi showing higher phenolic content and anti-oxidant [41]. 49 components were found in dried leaf powder , out of which, the major components were 1-Methyl eugenol, 2-Eugenol, 1-Stigmast-5-en-3-ol, 2-Stigmast-5, 22-dien-3-ol, 2-Neophytadiene, 2-Octadecane, 3-β-caryophylene, 3-Methyl eugenol, etc [42]. Alkaloids, flavonoids, tannins and carbohydrates are present in aqueous extract of Ocimum sanctum leaves[31,43].

Camellia sinensis L. commonly known as tea is a cultivated evergreen plant, which spread to India from China. Tea is only second to water in popularity as a drink [44]. It also boasts of having potential health benefits which can have important implications on human health [45-50]. Tea leaves are characterized based on the levels of antioxidant and degree of fermentation and thus three major forms namely the green tea, oolong tea, and black tea are available on this basis. [45,51]. Polyphenols , alkaloids, polysaccharides, volatile oils, amino acids, lipids, vitamins (e.g., vitamin C) and inorganic elements like F,Mn,Cr, Se, Ca, Mg, and Zn) are present in tea leaves. The flavonoids impart the antioxidant, anti-inflammatory, antiallergic and anti-microbial properties, which are characteristics of tea[52]. Epigallocatechin gallate (EGCG) is the most active component out of the six primary catechins present in Green tea. The catechins are exerting the antimicrobial and antiviral activities against infections. The polyphenols impart health benefits tea. Green tea contains more polyphenol and less caffeine and is mild with no adverse side effects, we are using green tea as one of our ingredients. Green tea also helps in digestion, lowering of body temperature and boosting the immune system as well as lowers blood sugar levels. The anti-carcinogenic mechanism encompasses the cellular immune function and inhibition of tumor growth[53]. Micronutrients present in green tea helps in strengthening the body cells. A stronger immune system, enhances tissue and cell repair thus the body retains its healing capability. The presence of antioxidants,vitamins, mineral products,caffeine, detoxifying and antibacterial agents in tea help in strengthening immunity. Tea is also a rich source of fluoride which is responsible for antiviral activities[49].

Nyctanthes arbor-tristis also known as Coral jasmine or night jasmine is a shrub growing to a height of about 10 meters belonging to the Oleaceae family and their leaves has been used by physicians who are practitioners of Ayurveda for a number of ailments like liver disorders, obstinate sciatica, arthritis, intestinal worm, malaria, and in alleviating gynecological problems. It has also been used as a tonic, and laxative as well as an analgesic, anti-viral, antipyretic, anti-allergic and ulcerogenic potencies[54-64]. The high therapeutic efficacy of *Nyctanthes arbor-tristis* is because of the presence of the nyctanthic acid, B-Sitosterol, D-mannitol, tannic , lenoliec and oleic acids in the leaves [65]. It is a known fact that anti-inflammatory effect is due to lenoleic acid.

Hygrophila auriculata (K. Schum) Heine commonly known as Kulekhara belongs to the family Acanthaceae has been used in traditional practice for many years and it is a commonly found wild herb throughout India. *Hygrophila auriculata* has been also widely distributed and used as traditional medicine by our neighboring countries like Sri Lanka, Burma and Nepal. Modern pharmacological studies have generally confirmed the traditional uses of *Hygrophila auriculata* and their extracts for the treating number of diseased states [66-70]. The decoctions of the young leaves are taken orally for two consecutive weeks in an empty stomach to treat anemia. The leaves contain alkaloids, carbohydrates, proteins, steroids, glycosides, flavonoids (gallic acid and quercetin), tannins, phenolic compounds, fats, and oils[71,72]. The results of the study undertaken by A. Gomes *et. al.* documented that ethanolic extract of Kulekhara leaves caused a significant increase in hemoglobin, hematocrit, RBC, Total WBC count and Total Iron Binding Capacity in male albino anaemic rats [73]. The study undertaken by authors to quantitate micronutrients present in leaves of *Hygrophila auriculata*, confirmed the presence of vitamin-C, iron, potassium, sodium, copper , calcium and β-carotene [74]. Iron absorption is enhanced by the co-ingestion of vitamin-C because ascorbic acid helps to reduce the ferric (Fe^{3+}) to ferrous (Fe^{2+}) form of iron and it also binds or chelates the ferrous form, which allows iron to be absorbed in the intestinal brush border [75].

With this background, the study was undertaken to prepare a polyherbal preparation to combat the effect of multi-facet clinical outcomes of respiratory diseases and give relief to people at large who are tired of the adverse effects and high cost of available medications. We have prepared the formulation IM-SSS20 with the ingredients which are easily and abundantly available at a very low cost showing no adverse reactions in our studies. We have carried out the study with a set of four dilutions (25%, 50%, 75% and 100 %) of IM-SSS20.

MATERIALS AND METHOD

Collection and Authentication of the Herbs

All raw medicinal plants were collected from registered local herbal suppliers and authenticated by pharmacognosist. They were further identified by an expert taxonomist and kept as a voucher specimen. The identification was based on Ayurvedic parameters such as Varna (color), Gandha (odor), Ruchi (taste), Akriti (shape) and Parimana (size). The plants and plant parts used in the preparation of the extract are listed in Table 1.

Table 1: Composition of ingredient(s) present in IM-SSS20

Scientific Name	Family Name	Common Name	English Names	Part Used	Source	Amount*
Ocimum sanctum	Lamiaceae	Tulsi	Holy basil	Leaves & Stems	India	50 mg
Camellia sinensis	Theaceae	Green tea	Tea	Leaves	India	50 mg
Nyctanthes arbor-tristis	Oleaceae	Seuli	Harsingar	Leaves	India	25 mg
Hygrophila auriculata	Acanthaceae	*Kulekhara*	Swampweeds	Stems & Leaves	India	25 mg

*** Amount required for preparation of 5 ml extract.**

Table 2: Qualitative analysis of the phytochemical constituents of multi herbal formulation (IM-SSS20)

Phytochemicals	DM	EA	AQ	ET	ME	AQM	AQE
Sterols	(+)	(+)	(++)	(++)	(+)	(+)	(+)
Triterpenes	(+)	(-)	(+++)	(+)	(+)	(-)	(-)
Flavonoids	(++)	(++)	(+++)	(++)	(++)	(+)	(+)

Alkaloids	(++)	(+)	(++)	(+)	(+)	(+)	(+)
Saponins	(+)	(+)	(++)	(+)	(+)	(+)	(+)
Glycosides	(+)	(+)	(++)	(++)	(++)	(-)	(-)
Tannins	(+)	(+)	(+++)	(+)	(+)	(+)	(+)

(+) = traces, (++) = moderate, (+++) = abundant, (-) = absence of constituents
DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%).

Table 3: Routine Quality Control analysis of multi herbal formulation (IM-SSS20)

Sl. No.	Test	Results
1.	Description	A brown colour liquid
2.	Wt. per ml	1.189 g
3.	pH	6.88
4.	Order	Characteristic
5.	Homogeneity	Uniform
6.	Total ash	<5% w/w
7.	LOD	46
8.	Bacterial Limit Test	243 cfu/ml

Table 4: Total polyphenol and flavonoids content of multi herbal formulation (IM-SSS20)

Extract	Total Polyphenols content gGAE/100g RM	Total flavonoids content gQE/100g RM
DM	2.46 ± 0.73^{a}	0.89 ± 0.04^{b}
EA	2.32 ± 0.37^{a}	0.48 ± 0.02^{a}
AQ	8.64 ± 0.47^{d}	1.21 ± 0.03^{d}
ET	4.51 ± 0.51^{b}	1.52 ± 0.06^{e}
ME	5.70 ± 0.53^{c}	1.10 ± 0.04^{c}
AQM	4.11 ± 0.44^{c}	0.91 ± 0.05^{b}
AQE	5.89 ± 0.51^{c}	0.97 ± 0.04^{c}

DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%).

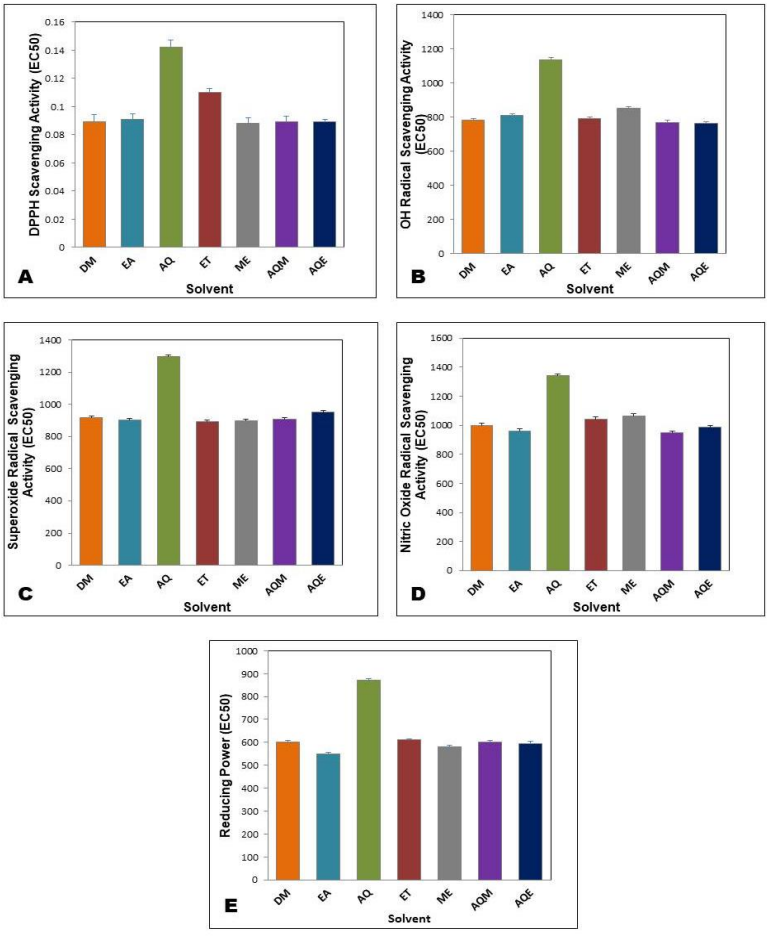

Fig.1: In-vitro antioxidant activity of IM-SSS20. A. DPPH scavenging activity, B. OH radical scavenging activity C. Superoxide radical scavenging activity D. Nitric oxide radical activity E. Reducing power. DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

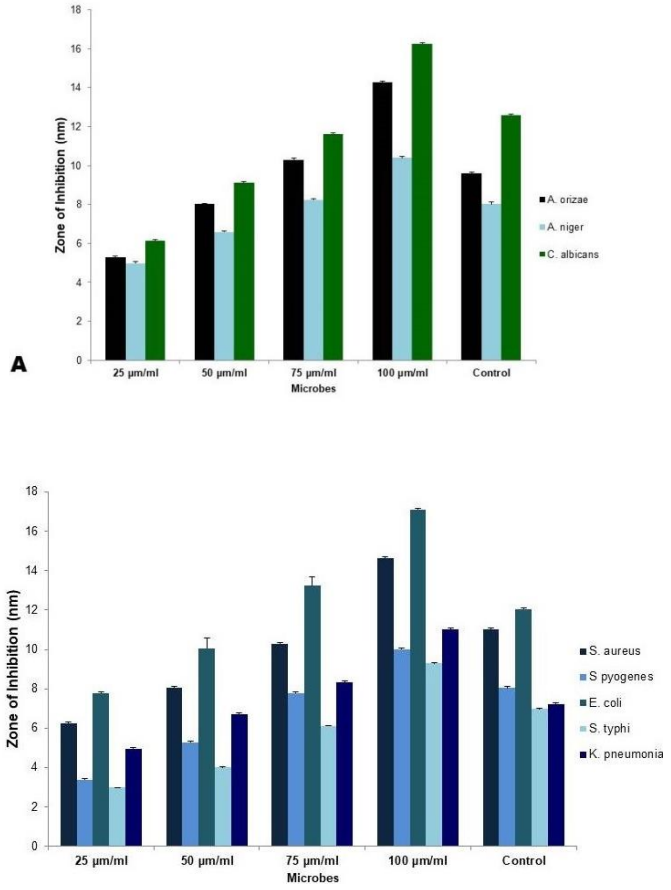

Fig.2: A. Antifungal activity of IM-SSS20 upon respiratory disease effected subjects. Data represent mean ± standard deviation. B. Antibacterial activity of IM-SSS20 upon respiratory disease effected subjects. Data represent mean ± standard deviation.

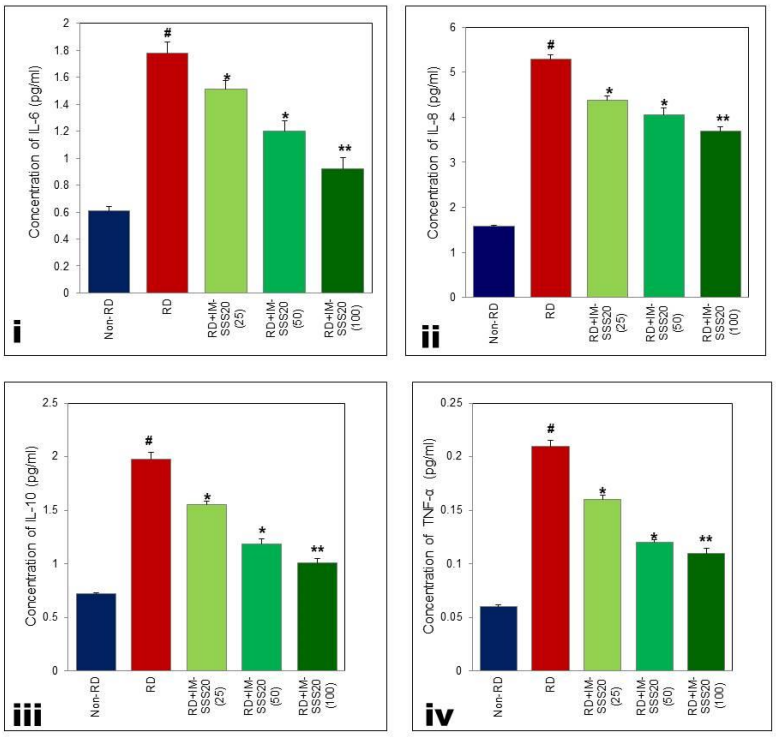

Fig.3: Immunological activity of IM-SSS20 upon respiratory disease effected subjects. Data represent mean ± standard deviation (n=5). i) Level of IL-6, ii) Level of IL-8, iii) Level of IL-10, iv) Level of TNF-α

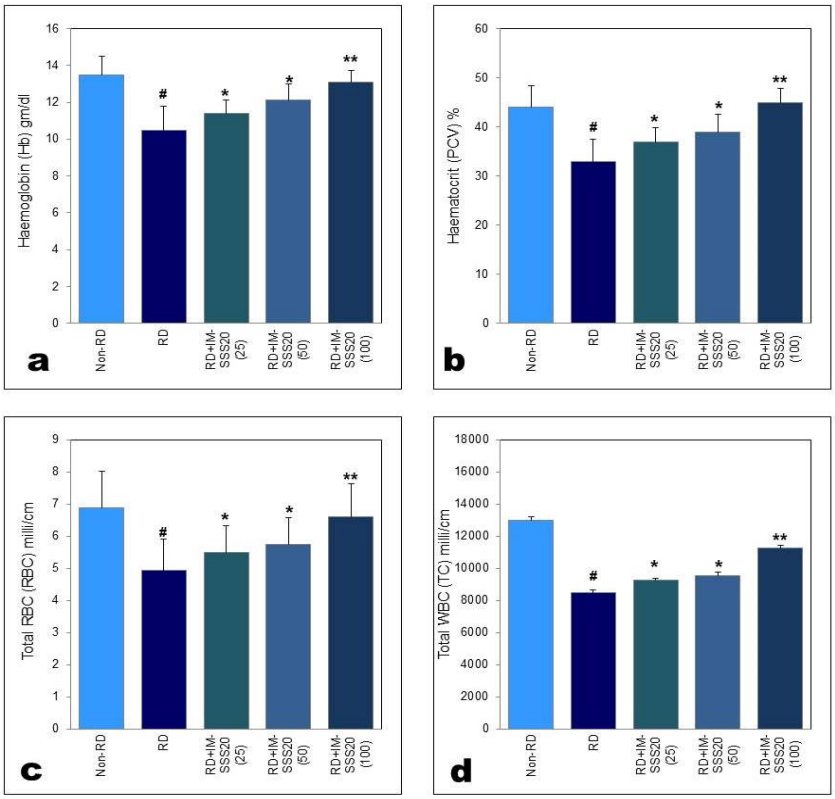

Fig.4: Determination of Haematological activity of IM-SSS20 upon respiratory disease effected subjects. Data represent mean ± standard deviation (n=5). (a) Haemoglobin (Hb) concentration, (b) Haematocrit value, (c) Total RBC count (d) Total WBC count.

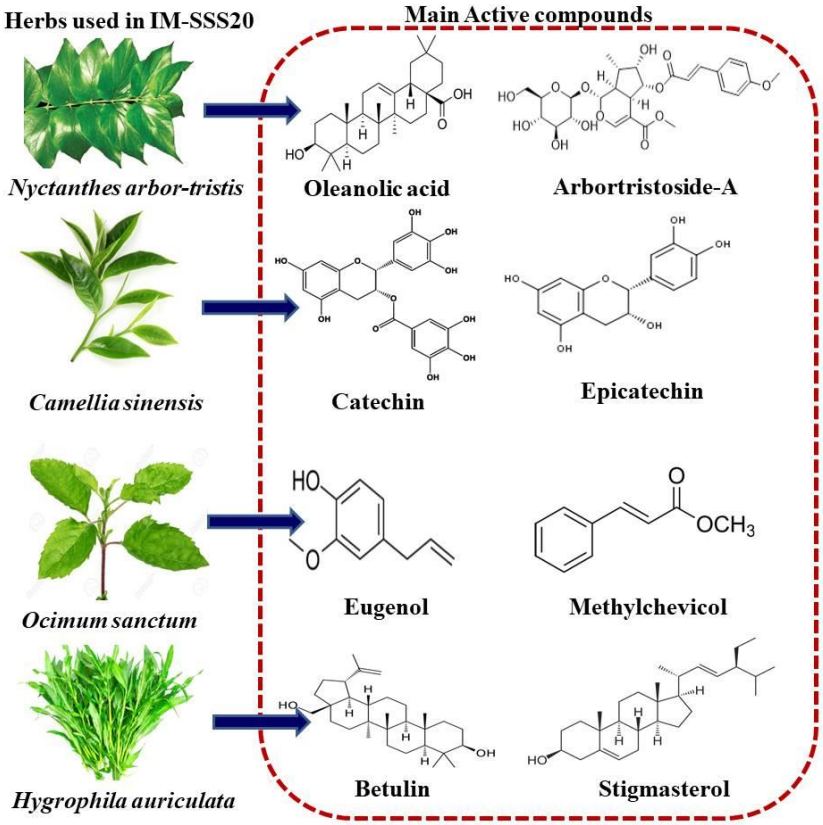

Fig.5: Main active components of the multi-herbal preparation IM-SSS20

Preparation of Extract

Fresh parts of the medicinal plants were first air-dried after cleaning with double distilled water and kept in an oven at 80ºC for 10 min and 60ºC for 30 min and grounded by a blade mill to a fine powder. After that the polar fraction was extracted by modified method of Taamalli et al. (2015) [76]. 10 ml of methanol was used to dissolve 5 gm of dry plant parts and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process repeated four times, finally supernatant was evaporated under reduced pressure at 35°C in a rotary evaporator. 3 ml of methanol was used to reconstitute the residue, filtered using Whatman filter paper (GE Healthcare and Life Sciences, MA, USA) and kept at 4ºC for further use.

Quality Control analysis

Wt. per ml, pH, homogeneity, total ash, LOD and bacterial limit tests were carried out according to the standard pharmaceutical protocol (IP-2014).

Phytochemical screening

Various essential plant secondary metabolites such as sterols and triterpenes, Mg^{2+} turning test of flavonoids, alkaloids, saponins, glycosides, tannins, phenolic content, total flavonoids content in the developed multi-herbal formulation (IM-SSS-20) were detected through quantitative analysis with slight modification as described by Evans and Gueverra [77,78].

In vitro Study

DPPH radical scavenging activity

To determine the free radical scavenging activity of IM-SSS20, DPPH method was used. 0.5 ml of IM-SSS20 extract was added to 3.0 ml of freshly prepared 0.1 mM DPPH (ethanolic) solution in different concentrations [81]. The reaction was carried out at 535 nm for 30 min. Antiradical power (ARP) and effective concentration fifty (EC50) were measured at different concentrations.

Percentage Inhibition= AbsControl-AbsTestAbsControl ×100%

Effective 50% of the concentration value that scavenged 50% of the DPPH radicals and antiradical power (ARP or AE) is the reciprocal of it (AE=1/EC50). The reference compounds used were ascorbic acid and quercetin.

Hydroxyl radical scavenging activity

The method of Erica *et. al.* was used to determine the hydroxyl radical scavenging activity [82]. The mixture containing 1.0 ml iron-EDTA solution, 0.5 ml 0.018% EDTA, 1.0 ml DMSO and 0.22% ascorbic acid was added to 1.0 ml of extract in different concentration, and heated in water bath (80˚-90˚C) for 15 min. The reaction was terminated by adding 1.0 ml ice cold TCA (17.5%) and 3.0 ml NASH reagent and incubated for 15 min at RT. The intensity of the yellow color formed was measured at 412 nm.

Superoxide free radical scavenging activity

The removal rate of xanthine/xanthine oxidase -generated from the substances, was used to determine Superoxide free radical scavenging activity [83]. 0.9 ml of tetrazolium blue solution was added to 0.1 ml plant extract of different concentrations. After which 1 mL of xanthine oxidase solution (0.05 units/ml PBE) was added very carefully and incubated for 20 min at 37˚C. Before termination of the reaction 2.0 ml of 2N HCl was added for developing the colour. Optical reading was taken at 560 nm against a blank.

Nitric oxide scavenging activity

The standard protocol was used to measure Nitric oxide scavenging activity [84]. 2.0 ml of 10 mM sodium nitroprusside was prepared in 0.1 M phosphate buffer, pH 7.4 and was taken in a conical flask and then 0.15 ml plant extract in varied concentrations was added. The solution was incubated at room temperature for 2h. After 2h, 5 ml Griess reagent was added. The absorbance of chromophore was measured at 546 nm.

Reducing power assay

Determination of reducing power of IM-SSS20 was done by the method of Abdullahi with slight modification [85]. In brief the initiation reaction was started by adding 2.5 ml extract, 2.5 ml phosphate buffer and 1% potassium ferricyanide and shaking gently, the mixture was then placed in a water bath at 50°C for 20 min.The solution was cooled and 2.5 ml of 10% trichloroacetic acid (TCA) was added to it. It was then centrifuged at 3,000 rpm for 10 min. 5 ml of distilled water was mixed with 5.0 ml fraction from the supernatant and 1ml of 1% ferric chloride was added and resulting solution was incubated at room temperature for 10 min. The absorbance was noted at 700nm.

Antibacterial activity of IM-SSS20

Five bacterial pathogens namely *Streptococcus aureus*, *Staphylococcus pyogenes*, *Staphylococcus typhi*, *Escherichia coli* and *Klebsiella pneumonia* were used to determine antimicrobial activity of developed formulation IM-SSS20 [86]. Tetracycline was used as a positive control. Nutrient broth was used for culturing the microbes and then placed for incubation at 37 ° C for 24hours and seeded in Mueller-Hinton sterile agar plates. The plates were left undisturbed for about 10 minutes to enhance the culture. A set of four dilutions (25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml of the herbal extract IM-SSS20 was used. The whole procedure was repeated for three times for obtaining the precise result.

Antifungal activity of IM-SSS20

To determine the in-vitro antifungal activity of IM-SSS20 we used three fungal pathogens namely A. niger, Aspergillus oryzae, and Candida albicans [86]. The plates were prepared with Potato dextrose agar (PDA) media and inoculated carefully with the fungal pathogens after the solidification of PDA. Five wells of size 5 mm were cut out on the agar plates. A set of four dilutions such as 25 µg/ml, 50 µg/ml , 75 µg/ml and 100 µg/ml of the plant extract IM-SSS20 and antifungal agent (positive control) ketoconazole (20 mg/ml) was introduced in well. At room temperature, the plates were incubated for 3–4 days. After 3 days, the zone of inhibition obtained was measured.

Clinical Chemistry

Analysis of selected Hematological parameters and biomarkers

The blood samples of patients having respiratory diseases were collected from the pathological laboratory of Government Hospital along with persons having no respiratory diseases. The following hematological parameters namely Haemoglobin (Hb), Haematocrit (PCV), Total RBC (RBC) and Total WBC (TC) were measured on a fully automated 3-part differential hematology analyzer (Abacus Junior Vet 5, Austria) [87]. The blood samples were treated with a set of three dilutions (25%, 50 and 100 %) of IM-SSS20 to determine its effect on the concentration of Hb, PCV, RBC and TC in vitro. ELISA test was used to measure biomarkers like Interleukin-6, Interleukin-8, Interleukin-10, and Tumor necrosis factor -α [88]. The effect of IM-SSS20 on the values of these biomarkers was also evaluated using the graded concentration of the herbal extract IM-SSS20.

Statistical Analysis

Analysis of Variance (ANOVA) was used as an exploratory tool to determine the significance of variation of hematological parameters after treatment with IM-SSS20 in vitro. Data are presented as mean ±SD.

RESULTS

The composition of the polyherbal preparation IM-SSS 20 is given in Table 1. We have extracted the plant materials in seven well-known

solvents and evaluated various properties including extraction efficiency, the quantity of various phytochemicals extracted and antioxidant activity to choose the best one. The highest yield was obtained with AQE while with EA the yield was the lowest.

Quality Control analysis

The developed multi-herbal formulation (IM-SSS20) was a clear brown color liquid extract with a characteristic odour (Table 2). Results depict that its Wt. per ml is 1.189 g and pH 6.88. The developed extract is uniform in nature. Total ash content is <5% w/w and LOD is 46 within the IP limit. 243 cfu/ml found in the bacterial Limit Test showed less bioburden and less pathological load. This formulation complies with the entire relevant quality control test as per Indian Pharmacopoeia limit.

Phytochemical constituents

From the qualitative analysis (Table 3) of the plant secondary metabolites of IM-SSS20 multi extract, it is observed that sterols are present in trace amounts, alkaloids and glycosides are in moderate amount, but triterpenes, flavonoids, saponins, and tannins are abundantly available. In the dichloromethane (DM) extract, sterols were abundant flavonoids, alkaloids, and glycosides were moderately present and saponins were detected in trace amount. in the ethyl alcohol (EA) extract triterpenes were not detected. In the aqueous extract (AQ) all the above phytochemical constituents were detected abundantly. We can conclude that more polar secondary metabolites were extracted with the solvents used as compared to non-polar metabolites.

Total polyphenol and flavonoids content

Spectrophotomeric method was used to determine the total phenolic compounds and flavonoids of the different solvent extracts. The concentrations of total phenolics was higher compared to that of total flavonoids. Table 4 represents the total phenolic and flavonoids content present in the different extracts of IM-SSS20. A significant variation ($P<0.05$) in the total content of phenolic and flavonoids for the five different extracts of IM-SSS20 was found. The total polyphenol content in EA (2.32±0.37) gGAE/100gRM, and in AQ was highest (8.64±0.47) gGAE/100gRM . The total flavonoids content was lowest in EA (0.48±0.02) gQE/100gRM but highest in AQ (1.52±0.06) gQE/100gRM .

In vitro Study

In vitro study was performed to confirm the high antioxidant content of the multi herbal extract. For this, we evaluated DPPH, hydroxyl, nitric oxide and superoxide free radical scavenging activity of the multi herbal extract. The results are given in Fig. 1A-D. In organic fractions of the extract, it was observed that increasing polarity of the solvent increased the DPPH radical scavenging capacities and higher DPPH scavenging activities was seen in all aqueous fractions which had a positive correlation with TPC. In case of AQE fraction, the observed EC50 and ARP (or AE) values (EC50=0.065 mg/mg DPPH; AE=15.4) found to be comparable to quercetin (EC50=0.06 mg/mg DPPH; AE=16.6) and even better than Trolox (EC50=0.096 mg/mg DPPH; AE=10.4), the two well-known standards frequently used to compare antioxidant efficacy. In aqueous fractions higher superoxide scavenging activity was observed in comparison to organic fractions. The neutralization of O^{2-} radicals are carried by various phenols present in the extract through hydrogen donation and inhibition of xanthine oxidase. AQE fractions showed significantly higher activity ($p<0.05$ compared to other solvents) in terms of hydroxyl free radical and nitric oxide scavenging.

The data from *in vitro* antibacterial study revealed that the inhibitory action of herbal extract IM-SSS20 was dependent on the dose, increasing with an increase in concentration. The inhibitory action on different pathogens was also variable. Significant activity was seen in the case of *Escherichia coli* which was followed by *Streptococcus aureus* and *Klebsiella pneumonia.*

Staphylococcus pyogenes and *Staphylococcus typhi* showed less inhibition as seen from Fig. 2 and the results reveal that the different concentrations of herbal extract IM-SSS20 showed efficient antifungal activity for three fungal pathogens taken. The antifungal activity was more for *C. albicans* and was least for *A.niger*. We can conclude that the herbal extract possesses the antimicrobial property.

The values of Interleukin-6, Interleukin-8,Interleukin-10 and Tumor necrosis factor -α were significantly elevated in patients with respiratory disease and the elevated levels of these biomarkers were found to decrease on treatment with the IM-SSS20 extract as seen in Fig.3.

It was observed that values of Hb, PCV, RBC and TC in blood of patients with respiratory diseases was less than that compared with a person who was not infected by respiratory diseases (Fig. 4)The prepared herbal extract IM-SSS20 was effective in increasing the concentration of Hb, PCV, RBC and TC in vitro. The maximum increase was found to be with 100% of IM-SSS20 extract. We can summarize that the increase was in a dose-dependent fashion.

DISCUSSION

Scientific reports [89, 90] confirmed that secondary metabolites of the plants were associated with various bio-activities and showed inhibitory action against microorganisms and pathogens. Upon these different metabolites, alkaloids has extensive antimicrobial and antiviral activities [91]. On the other hand research established that other metabolites like flavonoids, glycosides, saponins, triterpenes, tannins and sterols have potent anti-pathogenic activity [92-97]

In-vitro study showed that our newly developed multi herbal combination (IM-SSS20) have different plant secondary metabolites in seven solvents used, namely, DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%). In this experiment aqueous (AQ) extracts of the multi herbal extract have optimum secondary metabolites like flavonoids, glycosides, saponins, triterpenes, tannins and sterols. Other solvent extracts like DM, EA, ET and ME showed moderate secondary metabolites. Sterols and glycosides were completely absent in AQM (80%), and AQE(80%) solvent extract. The stated phyto constituents present in the aqueous (AQ) extracts have significant bio-activity against microorganisms and pathogens.

The structure-antioxidant activity relationships of flavonoids and phenolic acids in aqueous or lipophilic systems have been extensively reported. [98]. Antioxidants activity of phenolic compounds are due to the presence of hydroxyl substituents and their aromatic structure, enabling them to scavenge free radicals [99]. The content of flavonoids are considered as an important index for evaluating extract quality and are responsible for the biological activities [100]. It is evident from Fig. 5 the phenolic and flavonoids compounds are chief components of IM-SSS20 and are responsible for antioxidant properties. The aqueous extract (AQ) showed higher concentrations of total phenolics than total flavonoids compared all the different solvent extracts [101]. The routine Quality Control analysis of multi herbal formulation (IM-SSS20) indicate that the developed drug complies with all the relevant parameters as per IP 2014 specification. Medicines from plant sources have gained global importance because of medicinal and economical importance [102]. The widespread sale of adulterated products and misleading health claims of herbal products require stringent regulations. [103]. Generally most of the plants have more than one key ingredient which carries antimicrobial property. Herbs containing in IM-SSS20 also have some antimicrobial activity which shows its medicinal values against diseases. IM-SSS20 at concentration of 75 µg/ml and 100 µg/ml showed higher antimicrobial activity than the standard drug Tetracycline in the inhibiting the growth of *E. coli, S. aureus, K. pneumonia, S. typhi* and *S.pyogens*. Thus this superior antimicrobial activity was because the various active ingredients released form medicinal plants penetrated and disrupted the cell membrane of bacteria [104-105].

An important site of immune regulation is the respiratory tract and is responsible for protective immune responses, minimizes tissue

damage and avoids aberrant inflammatory responses. against inhaled allergens [106]. In lung tissues key regulators of immune response are the cytokines transforming growth factor β, IL- 10, IL-27, and IL-35. The role played by all cytokines (more than 50) in pathophysiology of asthma and COPD is often obscure [107-108]. T cells release cytokines predominantly, which are responsible for inflammation in asthma and COPD cases [109-12]. In-vitro clinical study clearly stated that during respiratory disease blood interleukins and cytokine levels as IL-6, IL-8, IL-10 and TNF-α significantly elevated which disrupt the normal cellular homeostasis and breakdown the immunological balance. Dose-dependent treatment with IM-SSS20 controls this alteration by normalizing the cytokines levels as compared with normal patients. Developed natural therapy (IM-SSS20) somehow prevent pathogenic respiratory infection and maintains the body's immunological integrity.

Erythropoiesis is regulated by number of factors and can manifest as either anemia or polycythemia in patients with COPD [113-14]. The various study stated that anemia in COPD patients lead to decreased hemoglobin levels, hematocrit values and decreased number of red blood cells (RBC) [115-16]. In this study we observed a lower value of hemoglobin, hematocrit, total RBC and total WBC in respiratory infected patients. This observation again confirms that patients effected by various respiratory problems have a lower level of hemoglobin (Hb) which suggests the patients are prone to anemia. Lower RBC and WBC count further confirm the respiratory diseases subjects might have lower immunity. *In-vitro* treatment with IM-SSS-20 normalized the condition. Further *in-vivo* clinical study needed for better clarification.

CONCLUSION

We can conclude from this preliminary study that the multi-herbal formulation IM-SSS20 showed promising results in-vitro studies and further studies are required to explore its efficacy in humans. We are hopeful that with extensive research the same effects could be reproduced in humans and can help humanity with a low cost, easily available ingredients with no adverse effects. This could be the next potential leap in the field of healthspan research.

In the present age coronavirus has become a household name and this deadly virus has brought the racing humans to a standstill position. The world's scientists, healthcare professionals and experts are all working day and night to give solution to this problem. Many countries were locked down for days to stop the community transmission of this disease, which is a precautionary measure but not a solution. It has been reported that Coronavirus manifests respiratory illness (as in flu) which shows preliminary symptoms of cough, fever, and in severe cases, breathing difficulty. It is a little premature but the authors are hopeful that this multi-herbal formulation can be useful in treating COVID-19 patients because of its ability to reduce the levels of biomarkers like IL-6, IL-8, IL-10 and TNF-α which get elevated during respiratory infections as well elevate the levels of hematological parameters like Haemoglobin (Hb), Haematocrit (PCV), Total RBC (RBC) and Total WBC (TC) which decreased during infection[117].

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Declaration of competing interests

The authors declare they have no competing interest

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All authors totally agreed for the publication of this research

Availability of data and material

Research data and materials can be provided on request.

Author Contributions

#S.A. and S.D. contributed equally.

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