Original Research

PHARMACEUTICO-ANALYTICAL STUDY OF NIRGUNDIGHRITA AND EVALUATIONOF ITS ANTIOXIDANT AND ANTIMYCOBACTERIAL ACTIVITY W.R.T. TUBERCULOSIS: A STUDY PROTOCOL

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ABSTRACT

Introduction: In Ayurveda, Rajyakshma (Tuberculosis) is mainly caused due to Oja kshay (lack of immunity). Nirgundi ghrita is Sneha kalpa that contain two ingredients which are panchang of Nirgundi (*Vitex negundo* Linn.) and Ghrita. Nirgundi ghrita is a formulation mentioned in Chakradatta for the treatment of Yakshma (Tubeculosis). It is mentioned in the text that consumption of Nirgundi ghrita will leads in improvement in the immunity of the person.

Need of the Study: In view of the properties of Nirgundi and Ghrita, evaluation antioxidant effect and anti-mycobacterial activity of formulation Nirgundi Ghirta in Tuberculosis is needed.

Aim: Pharmaceutico-analytical study of Nirgundi ghrita, evaluation of its antioxidant and anti-mycobacterial activity in tuberculosis.

Methodology: This is a Pharmaceutico-analytical and Experimental study. Pharmaceutico-analytical study will be done at MGACH & RC, DMIHER Wardha and experimental work will be done at Central India Institute of Medical Sciences (CIIMS Nagpur).The study duration will be of 18 months. First, Nirgundi ghrita will be prepared as per Ayurvedic classical text and then will be subjected to analytical study as a part of standardization of formulation. Antioxidant study of Nirgundi ghrita will be done by using the DPPH free radical scavenging assay method. Evaluation of anti-mycobacterial activity of Nirgundi Ghrita will be done using BacT/ALERT 3D automated culture system.

Discussion & Conclusion: PTB is one of the ancient diseases of human being, in spite of newer modalities for treatment of tuberculosis unfortunately, people are still suffering and it is among the top 10 killer infectious diseases. Considering the multi drug resistance and Adversarial effect of ATD, there is a need to develop alternative treatment module along with ATD. Hence the Ayurveda management helps to find out better treatment. Hence, Nirgundi Ghrita will be prepared to study their anti-mycobacterial activity in Tuberculosis and its antioxidant effect.

Keywords: Ayurveda Pharmaceutics, DPPH Assay, Nirgundi Ghrita, Rajyakshma.

INTRODUCTION

Despite decades of effective therapy being available, tuberculosis continues to be the deadliest infectious diseases and ranks among the top ten leading causes of death in the world.

In Ayurveda, an ancient Indian system of medicine, herbs and herbo-mineral formulations play an important role in prevention as well as curing the diseased conditions. Yakshama, a disease of ancient origin described in Ayurveda, could be correlated with Tuberculosis.

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In rajayakshma, tissue emaciation is one of the reasons which initiate the pathogenesis. And there is vitiation in metabolic functions results in gradual loss of all dhatus which is in sequence like rasa, rakta, mamsa, meda, shukra.

Ultimately, it affects the immunity of the body, which is Oja as per Ayurveda (1).Pulmonary tuberculosis being a global emergency is at threat to the world medical community. The treatment module ATD's is giving appreciable success in treating the Tuberculosis. Increasing trend to multi drug resistance (MDR) among the patients is alarming (2). Certain studies were conducted in which along with ATD's ayurvedic treatment protocol was planned and the result was encouraging, but this practice was discontinued (3).Considering the multi drug resistance in TB,

there is a need to develop alternative treatment module along with ATD.

REVIEW OF LITERATURE

Sneha kalpana is one of the widely used and preferred dosage forms in Ayurveda (Ref. Sharangdhar Samhita). It is a group of products of medicated taila (oil) and ghrita (ghee). Based on the stages of paka, origin (yonies), nature of media and the types of utility, sneha kalpana is classified into different categories. Goghrita and Tila taila are considered as best snehas for sneha kalpana (Tubeculosis) and indicated to improve the strength of over exerted person. It is mentioned in the text that consumption of Nirgundi ghrita will leads in improvement of immunity and overall health of the person (4). Excessive envy, eagerness, fever, terror, anger, grief, excessive indulgence in sexual intercourse, and fasting leads to depletion of shukra and ojas. This, in turn, due to loss of unctuousness, provokes vata which further aggravates the other two doshas and causes eleven symptoms such as- coryza, fever, cough, body ache, headache, dyspnea, and diarrhea, anorexia, pain in flanks, feeble voice and feeling of warmth in shoulders. These eleven symptoms indicate the advent of the great disease rajayakshma due to wasting (5).

Nirgundi ghrita is prepared with two ingredients which are Panchang (whole plant) of Nirgundi (*Vitex negundo* Linn.) and Ghrita. Nirgundi leaves have anti-eosinophilic, mast cell stabilizing, anti-inflammatory and analgesic activity (6). Leaf extracts of *Vitex negundo* show antibacterial and cytotoxic activity against *Bacillius subtilis, Bacillius megaterium, salmonella typhi, Vibrio mimicus* and some fungal strain. Cow ghee is an important part in Nirgundi leaves have anti-eosinophilic, mast cell stabilizing, anti-inflammatory and analgesic activity (6). Leaf extracts of *Vitex negundo* show antibacterial and cytotoxic activity against *Bacillius subtilis*, *Bacillius megaterium*, *salmonella typhi*, *Vibrio mimicus* and some fungal strain. Cow ghee is an important part in Ayurvedic therapeutics.

In ayurveda, Ghee is considered as soft, soothing and cold in potency (7). It is used as a vehicle along with bhasmas and many medicines (8). Cow ghee is used as a base for many internal and topical applications (9). Cow ghee being lipophilic facilitates the transportation of the active principles through the cells. Thus, absorption of the active nutrients is enhanced with ghee (10, 11). Ghee is an important carrier of fat soluble vitamins, essential fatty acids and contain natural antioxidants (12). It is also a suitable carrier for medicinal properties of herbs which are to be absorbed by and transported to targeted areas of the body. Butyric acid from ghrita supports the production of killer t cells thereby maintaining a strong immunity. Considering all properties of Nirgundi and Ghrita, there is need to evaluate antioxidant effect and anti-mycobacterial activity of formulation Nirgundi Ghirtain Tuberculosis.

Aim- Pharmaceutico-analytical study of Nirgundi ghrita and evaluation of its antioxidant and anti-mycobacterial activity in tuberculosis.

Objective-

1. Pharmaceutical preparation of Nirgundi ghrita.
2. To analyze Nirgundi ghrita on different organoleptic and Physico-chemical parameters.
3. Analytical study of assessment for free radical scavenging activity.
4. Evaluation its anti-mycobacterial activity using BacT/ALERT3–D automated liquid culture in tuberculosis.

METHODOLOGY

Place of Study- Ingredients of Nirgundi Ghrita and other study materials will be procured and preparation and analytical study of formulation will be carried out MGACHRC, Wardha. Experimental work will be carried out at Central India Institute of Medical Sciences (CIIMS Nagpur).

Pharmaceutical Study-

Table No.1: Ingredients of Nirgundi Ghrita.

Drug Name	Latin Name	Part Used	Proportion	Form of Drug	Role
Nirgundi	Vitex negundo Linn.	Panchang (Whole plant)	16parts	Juice extract	Dravya dravya
Ghrita	---	---	4parts		Sneha dravya
Nirgundi	Vitex negundo Linn.	Leaves	1part	Bolus	Kalka dravya

Pharmaceutical Preparation of Nigundi Ghrita- Fresh Nirgundi leaves complete blend or kalka will be prepared. Nirgundi Panchang juice extract will be prepared. Further ghrita will be heated over moderate flame till complete evaporation of moisture. Then prepared kalpa will be added to ghrita. When kalpa became light brown in colour, juice extract will be added and will be heated on moderate flame (mandangi) with intermediate stirring to complete the snehapaka till snehasiddhi lakshana appeared. Final product will be store in well-closed airtight glass container. (13)

Analytical Study-

Organoleptic Study- Colour, odour, taste, appearance, consistency after cooling. Physico-chemical analysis- Refractive index, Specific gravity, acid value, Saponification, Iodine value, pH, Unsaponifiable matter (%) HPTLC. Assessment for free radical scavenging activity. (14)

A. Organoleptic Character-

1. **Colour**- The colour is used to indicate the drug's general origin. Stuff obtained from the aerial section of the plant, for example, is usually green, but material derived from the underground platies usually brown.
2. **Odour**- The aroma and taste of crude stuff, according to an expert, are particularly delicate parameters dependent on individual judgment. As a result, the description of this feature may occasionally cause problems. Aromatic indistinct, fruity flavour, or musty is a term used to describe something that smells mouldy or musty.
3. **Taste-** The following are the different sorts of tastes: True flavor Bitter Alkaline Acid (Sour) Saline (Salty) Saccharine (Sweet) Metallica. False sense of taste (Sensations to the tongue), Mucilaginous (soft slimy feeling) Oil is a kind of petroleum (Bland smooth feeling) having astringent properties (Contraction of mouth tissue) astringent (Warm biting sensation) acidic (Unpleasant, Irritating) a feeling of nausea (Induce vomiting).
4. **Touch**- The texture is best checked by rubbing a tiny amount of material between the thumb and fingers, which is normally done with a small amount of material. Described as smooth, rough, and gritty.
5. **Appearance**- The external appearance of the drug is a very important factor. It is used for checking the authenticity of herbs.

B. Physico-Chemical Parameters

1. **Refractive Index-** The refractive index of ghrita is measured at 40℃ temperature using the digital refractometer. The instrument is calibrated with the glass prism of known refractive index or by using distilled water. The light source such as sodium vapour lamp (589.3nm) issued. The refractive index is the ratio of velocity of light in vacuum to the velocity of light in medium. The pure ghee has the refractive index of 1.45. If any adulteration in the samples is present, the refractive index of ghee will either decrease or increase. The refractive index also very helpful in determination of unsaturation. Refractive index increases with increase in unsaturation.
2. **Specific Gravity-** The ghrita is generally present in solid or semi-solid state. The sample is first melt and is filtered. The sample should be free from moisture. The specific gravity is measured at 30˚.The instrument used for this purpose is pycnometer. The specific gravity of ghee is measured with respect to distilled water.
3. **Acid Value-** Free fatty acids present in ghrita are readily soluble in rectified spirits. The acid value is the number of milligrams of KOH required to neutralize free fatty acids in1g of ghee sample. The value is determined by titrating the with standard potassium hydroxide using phenolphthalein indicator, the mixture may be warmed to about 70°C and is swirled vigorously. It is measure of fatty acids which have been liberated by hydrolysis of triglycerides due to action of moisture, temperature or enzymes.
4. **Saponification-** The Saponification value is the number of milligrams of potassium hydroxide required to saponify 1 gm of ghee. The Saponification value is the index of mean molecular weight of fatty acids of glycerides comprising of fat. Lower is the Saponification value, large the molecular weight of fatty acids and triglycerides and vice-versa.
5. **Iodine Value-** The iodine value is used to determine the degree of unsaturation of constituent fatty acids thus relative measure of unsaturated bond present in sample. It is the measure of number of gramsofiodineconsumedby100 gm of sample.
6. **Unsaponifiable Matter-** It is defined as the substances soluble in a fat which after Saponification are insoluble in water but soluble in solvents. It includes lipids of natural origin such as sterols. Higher aliphatic alcohols, vitamins and hydrocarbons.
7. **High Performance Thin Layer Chromatography (HPTLC)-** It is applied for the identification of alkaloids of the drug and is helpful in determining the ingredients of the drug.

Analytical Study of Assessment for Free Radical Scavenging Activity- DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging is an accepted mechanism for screening the antioxidant activity of plant extracts. In the DPPH assay, violet colour DPPH solution is reduced to yellow coloured product, diphenyl picryl hydrazine, by the addition of the extract in a concentration dependent manner. This method has been used extensively to predict antioxidant activities because of the relatively short time required for analysis. A set of clean and dry test tubes will be prepared and added with 3 ml of methanol and 75 µl of DPPH reagent solution in each test tube and mixed thoroughly. The initial absorbance (Ac) of each test tube will be measure on UV-Visible spectrophotometer (Model uv-1, Merck

Thermo Spectronic) at516 nm. Methanolic solution of standard ascorbic acid (0.5mg/ml) will be prepared and added in range of 5-35ul in test tubes already containing methanol and DPPH reagent solution, as control. All these tubes will keep aside for 4 min at room temperature and measured the final absorbance (As at 516 nm). The % reduction in absorbance will be calculated from the initial and final absorbance at each level by using the following formula:

Ac = Control Absorbance As = Sample Absorbance

% Reduction = (Ac-As) / Ac X 100

By Constructing a plot between concentration vs % reduction in absorbance of DPPH by adding the ascorbic acid and calculated the IC50 (Concentration of Ascorbic acid required for 50%reductionin absorbance) from the equation,

$$y = 1.803x + 12.69$$

Similarly, IC50 of Methanolic solution of all three residues i.e. ethyl acetate (0.5mg/ml), alcohol (2mg/ml) and water (2mg/ml) will be determined by adding increased concentration i.e. ethyl acetate (5-35 pl), alcohol (25-150 pl) and water (25-150 L) in above prepared test tubes containing methanol and DPPH reagent solution.

Experimental Study- Experimental study will be carried out at Central India Institute of Medical Sciences (CIIMS), Nagpur. Evaluation of anti-mycobacterial activity of Nirghundi Ghrita using BacT /ALERT 3 –D automated liquid culture will be done as follows.

Preparation of stock solutions of the selected compounds: Stock solutions of the compounds Nirgundi Ghrita will be prepared by dissolving 500 mg/ml of each compound in 2% dimethyl sulphoxide or any other likely solvent (or any other given concentration).

1. **TB Drug Cocktail Preparation-** Standards Anti-Tb drug cocktail will be prepared by mixing the four drugs namely Isoniazid, Rifampin, Streptomycin, and Ethambutol at concentration of 1.25mg/25µl each drug.
2. **Standardization of the Growth Curve for MIC Study-** Prior to MIC studies with drugs and Herbal extract, standard growth curve of H37Rv strain (time taken to reach Log phase) will first be obtained by inoculating 0.2-0.5 µl (~107 CFU) suspension of culture into BACT / Alert 3D system (Biomeriux, France) along with Middle brook oleic acid, albumin, dextrose and catalase (OADC) enrichment (Biomeriux, France). The positive growth along with time taken will be used for standard curve generation.
3. **Determination of Minimum Inhibitory Concentration (MIC) of Standard Anti-TB Drugs Cocktail Using Automated Liquid Culture System-** MIC of anti-TB drugs against *M. tb* culture will be determined by inoculating *M. tb* culture along with different dilutions of TB-drugs cocktail in BacT / alert system at 37oC. Standard culture without any drugs will be used as positive control. MIC is defined as the lowest drug concentration of the drug that inhibits growth of more than 99.0% of a bacterial proportion of the tested *M. tb* strains in liquid culture. Cocktail of four drugs in the above-mentioned concentration was first tested for MIC.
4. **Evaluation of Anti-Mycobacterial Activity of Different Extract of Nirgundi Ghrita Using Advance Bact /ALERT 3D Automated Culture System-** MIC of an Ayurvedic formulation Nirgundi Ghrita against *M.tb* culture will be determined by inoculating *M.tb* culture along with different dilutions of extracts of Nirgundi Ghrita in BacT/alert system at 37oC. Standard culture without any drugs will be taken as positive control. MIC concentration of anti TB drugs will also be taken in experimental sets for comparative studies with herbal extract.
5. **Study of Synergistic Anti-Mycobacterial Activity of Nirgundi Ghrita Along With Anti-TB Drugs on *M. Tb* Using Bact / Alert-** This study will be carried out to evaluate whether there is any synergistic activity of TB drugs and Nirgundi Ghrita when used together against *M.tb* cells. To study synergistic activity, both drugs and extract of Nirgundi Ghrita will be inoculated together in MIC values and concentration below their MIC values. The concentration below MIC values will be used to study whether lower concentration of drugs in herbal formulation can inhibit *M.tb* growth.

Statistical analysis – All the statistical analyses will be performed using Med Calc (version 10) by t-test. P-value < 0.05 will be considered statistically significant. (15, 16).

Outcome Measures- The outcome measures of this study are refractive index, specific gravity, acid value, saponification, Iodine value, pH, Unsaponifiable matter (%), HPTLC, Organoleptic character like Colour, Odour, Taste, Appearance, Touch, DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay and anti-Mycobacterial activity through determination of minimum inhibitory concentration (MIC).This study will provide scientific evidence for the use of Nirgundi ghrit in the management of Tuberculosis as claimed by Ayurvedic classical text through antioxidant and anti-mycobacterial study.

Author's Contribution- The study is conceptualized by investigators and co-investigators and also will be conducted by the investigators and co-investigators.

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Ethical Approval- Not required as this is an analytical and experimental study.
Conflicts of Interest- The authors declare that they have no conflicts of interest.

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