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Research Article

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DEVELOPMENT, STANDARDIZATION OF POLYHERBAL FORMULATION OF ANALGESIC OINTMENT OF PLANT CARUM COPTICUM, MENTHA PIPERITA, CEDRUS DEODARA

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Analgesic, formulation, standardization, evaluation, ointment.

ABSTRACT

Ayurveda is one of the world's oldest systems of medicine. It originated in India and has evolved there over thousands of years. The term "Ayurveda" combines then Sanskrit words ayur (life) andVeda (science or knowledge). Ayurveda means "the science of life. Medicinal plants and herbal drugs have played a key role in world health. According to world health organization (WHO), about 80% of the world population currently utilizes the herbal drugs. People are using herbal medicines from centuries for safety, efficacy, cultural acceptability, non-toxic, lesser side effects and easily available at affordable prices. In recent times, there has been a move in universal trend from synthetic to herbal medicine due to side effects of synthetic products. Herbal products may contain a single herb or combinations of several different herbs believed to have complementary and /synergistic effects. Some herbal products, including many traditional medicine formulations, also include animal products and minerals. Herbal products are sold as either raw plants or extracts of portions of the plant or in the form formulation i.e. tablet, capsule, syrup, cream and ointment etc. The different parts of plants with analgesic were taken up for the present study and investigated for the phytochemical screening and used for the formulation of analgesic ointment. Present study deals with formulation, Standardization, evaluation of ointment made from alcoholic extract and essential oil of different plants.

INTRODUCTION

Herbal medicine sometimes referred to as botanical medicine or herbalism it involves the use of plants or parts of plants to treat injuries or illnesses. Seeds, leaves, stems, bark, roots, flowers, and extracts of all of these have been used in herbal medicine over the millennia of their use. Some of the

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pharmaceutical medications on the market are extracts of some of these traditional herbs. The lower cost, and often safer use, has attracted many medical professionals. Plants had been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants. Indigenous cultures (such as African and Native American) used herbs in their healing rituals, while others developed traditional medical systems (such as Ayurveda and traditional Chinese medicine) in which herbal therapies were used.

Advantages of herbal medicine [1]

Better patient tolerance as well as acceptance and more affordable than conventional medicine.

- Medicinal plants have a renewable source, which is our only hope for sustainable supplies of cheaper medicines for the world growing population so easy to obtain than prescription medicine.
- Stabilizes hormones and metabolism.
- Natural healing.
- Prolong and apparently uneventful use of herbal medicines may offer testimony of their safety and efficacy and show strengthing in immune system.

Limitations of herbal medicines [2]

The prominent limitations of herbal medicines can be summarized as follow

- Ineffective in acute medical care.
- Inadequate standardization and lack of quality specifications.
- Herbal medicines take a longer time to work compared to pharmaceutical drugs.
- Half administered, as a result, there is no dosage or warnings specified.
- Herbal medicine may poison rather than cure someone when certain part of a plant may be edible and another part may be poisonous. Take rhubarb for example. The root of rhubarb is used as a laxative and the stem is edible. However, its leaves are poisonous.

Standardization of Herbal Medicine

Herbal medicines are being manufactured on the large scale in pharmaceutical units, where manufacturers come across many problems such as availability of good quality raw material, authentication of raw material, availability of standards, proper standardization methodology of single drugs and formulation, quality control parameters. The use of herbal medicine due to toxicity and side effects of allopathic medicines has led to sudden increase in the number of herbal drug manufactures.

Standardization and quality control of herbal crude drugs

According to WHO it is the process involving the physicochemical evaluation of crude drug covering the aspects, as selection and handling of crude material, safety, efficacy and stability assessment of finished product like Macro and Microscopic Examination, Foreign Organic, Ash Values, Moisture Content, Extractive Values, Crude Fiber, Qualitative Chemical Evaluation, Chromatographic Examination, Quantitative Chemical Evaluation and Toxicological Studies.

Analytical methods [3]

Chromatographic characterization

Chromatographic separations can be carried out using a variety of supports, including immobilized silica on glass plates (Thin layer chromatography), very sensitive High Performance Thin Layer Chromatography (HPTLC), volatile gases (Gas chromatography), paper (Paper chromatography), and liquids which may incorporate hydrophilic, insoluble molecules (Liquid chromatography).

Purity determination

Foreign matter, ash, acid-insoluble ash, moisture content, loss of moisture on drying, and extractives and High performance thin layer chromatography (HPTLC) is valuable quality assessment tool for the evaluation of botanical materials. It allows for the analysis of a broad number of compounds both efficiently and cost effectively and more complete profile of the plant than is typically observed with more specific types of analyses.

Quantitative analysis

The most appropriate quantitative analytical method with accompanying chromatograms shall be provided. The primary goal of the method(s) is to provide validated methods to be used for the quantization of the compound(s) most correlated with pharmacological activityand a survey of experts.

Mentha piperita

Scientific classification

Kingdom	Plantae
Order	Lamiales
Family	Lamiaceae
Genus	Mentha
Species	*Mentha piperita*

Vernacular names

Sanskrit -Putiha ,Hindi -Paparaminta, Pudina, Telgu-Pudina,Malyalam- Putina

Distribution

Originally the plant is a native of Europe and has been naturalized in many parts of India. The drug is almost entirely derived from cultivated source.

Part used: Leaves and stem

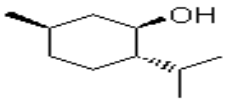
Description

Macroscopic: Leaves ovate- oblong to oblong-lanceolate , opposite, petiolate, slightly pubescent, apex acute, base rounded or narrow, margin sharply serrate. Upper surface slightly glabrous, lower surface with a few hairs on the midrib and veins along with many glandular hairs which are amber coloured .Odour characteristically aromatic, taste aromatic followed by a cooling sensation on drawing breath.

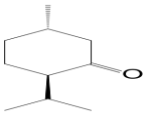
Microscopic: Amphistomatic lamina shows diacytic type of stomata. Cell walls are more wavy and the frequency of stomata is high in lower epidermis than that of upper epidermis. Glandular trachoma possesses 1-8 celled glandular head. Both vessels and tracheids show helical thickening.

Chemical constituent

Major: Volatile oil (1-3%), the principal components of which are menthol(30-55%) and menthone (14-32%).



Menthol



Menthone

Uses: Menthol is one of the main components of the essential oil of M. piperita that produce anti-cancer activity inducing cell death, either by necrosis or apoptosis. *M. piperita* relaxes the lower esophageal sphincter, which is useful as an antispasmodic agent by taking double contrast barium and in patients with dyspepsia. The constituents of the essential oil of *M. piperita* have different modes of action in bacteria and eukaryotic cells. They exhibit strong bactericidal properties. Menthol and menthone present in the essential oil components of *M. Piperita* is responsible for the antimicrobial activity. *Mentha piperita* is a promising plant thatmay offer low-cost alternative strategy for the use in Medicine and in food industry. [4]

Cedrus deodara

Scientific classification

Kingdom	Plantae
Order	Pinales
Family	Pinaceae
Genus	Cedrus
Species	*Cedrus deodara*

Vernacular names: Sanskrit-Devakastha, Daru,Hindi -Devdar, Devdaroo,Telgu-Devdaree,English–Deodar.

Distribution: A very large and tall ever green tree found in North Western Himalayas from Kashmir to Garhwal, between 1200 to 3000m and also cultivated in Kumaon.

Part used: Heart wood

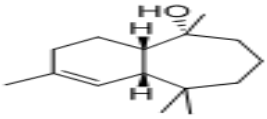
Description

Macroscopic: Wood moderately hard, light yellowish- brown to brown, wood splits readily longitudinally, annual rings well marked, medullary rays appear as whitish lines, resin canals, if present, arranged in long tangential rows, showing up as dark, narrow line on the radial surface of the wood pieces , odour, aromatic, taste, not distinct.

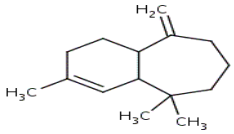
Microscopic: Mature wood almost entirely of narrow, quadrangular or rarely five or six sided tracheids, having very thick-wall with pits and a narrow lumen, xylem rays very fine, numerous and run straight throughout the region, uniseriate and 2 to 16 cells high in tangential section, vessels absent.

Chemical constituents

Mono- and sesquiterpenes of which α- and β- himachalene, himachalol, allo- himachalol, centdarol, isocentdarol and epoxyhimachalene



Himachalol



α- himachalene

Uses: As a sedative, cedarwood oil has relaxing and calming effects. Cedarwood oil soothes inflammation, cures itching skin and relieves tension and anxiety. For aromatherapy, the oil is used in a vaporizer, in a therapeutic bath, or through inhalation of the steam. Cedarwood oil also helps strengthen gums, cures toothaches and having expectorant properties.The fungicidal and antiseptic properties of cedarwood oil will help cure microbial infections both internally and externally.

Carum copticum

Scientific classification

Kingdom	Plantae
Order	Apiales
Family	Apiaceae

Genus Trachyspermum

Species ***Carum copticum***

Vernacular names:

Hindi – Ajowan, English - Caraway, Bishop's weed,

Distribution

The plant grown in Iran, Egypt, Afganistan and chiefly in India (U.P., Bihar, M.P., Panjab, Rajasthan, West Bengal)

Part used: Grayish brown fruit (seed)

Description

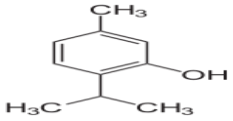
Macroscopic

Ajowan is an erect, glabrous or minutely pubescent, branched annual that grows upto 90 cm.The fruits are ovoid, greyish brown, aromatic cremocarps with single seed.

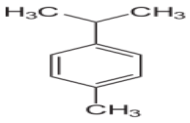
Microscopic

Chemical constituents

Thymol (41.34%), α-terpinolene (17.46%) and ρ-cymene (11.76%).



Thymol



Cymene

Uses: It is used as flavouring agent in food items and spices. It works as anti –oxidant. It is also work as preservatives.It also used in very medicinal preparation and its essential oil also used in perfumery.It is used in surgery as antiseptic and also found to be great value in the treatment of hookworm disease.

MATERIALS AND METHODOLOGY

Collection of Plant Material

Leaves of *Mentha piperita* , heartwood of *Cedrus deodara*, seeds of *Carum copticum*, these all were received from a reputed supplier Herbal auto mission (*Cedrus deodara*), and Global Herbs (*Carum copticum*) from Delhi and authenticated by Taxonomic Division of Maharishi Ayurveda Products Pvt. Ltd. NSEZ Noida.

Pharmacognostic Studies [2]

Macroscopical evaluation:

Organoleptic evaluations can be done by means of organs of special sense. The following Organoleptic investigations were done.

Colour: The untreated sample was properly examined under diffused sunlight or artificial light source with wavelengths similar to that of daylight.

Shape and size: A graduated ruler with basic unit in millimetre is adequate for the measurement. Seeds were measured by aligning ten of them on a sheet of a calibrated paper approx. 1mm apart between the lines and the result was divided by 10. Average length, breadth and thickness were determined.

Odour and taste: The sample was crushed in a mortar by applying pressure by pestle, and the strength of the odour like weak, distinct, strong was first noted and then the odour sensation like rancid, fruit, aromatic etc was determined.

Microscopical evaluation

Microscopical evaluation was done for qualitative and quantitative parameters. The parameters observed were: Arrangement of tissues in a transverse section, type of epidermal cells, testa and endosperm, Presence and type of crystalline structures e.g. Calcium oxalate, starch etc, presence of oil globules, aleurone grains and trichomes.

Powder microscopy:

Leaves of *Mentha piperita* , seeds of *Carum copticum* were powdered and sieved, fine powder thus obtained was taken up for microscopical evaluation as follows (W.H.O. guidelines): A small quantity was kept on a slide and after mounting on glycerin, 10 minutes were provided as spread out time. Finally, it was observed for microscopicalcharacters.Another small quantity was stained with phloroglucinol and HCl, ruthenium red, safranin, sudan red III, iodine and acetic acid respectively, mounted with glycerine on microscopical slide and observed for microscopic characters.

Physicochemical Studies

Physicochemical parameters help to determine the inorganic and moisture content from which dry weight of the drug can be calculated (w/w).

Determination of Ash values

Total Ash value

Weighed accurately 2 g of air dried roots powder in a tarred platinum or silica dish and incinerated at a temperature not exceeding 450°C until free from carbon, cooled and weighed. When a carbon free ash cannot be obtained in this way exhausted the charred mass with hot water, collected the residue on an ash less filter paper, incinerated the residue and filter paper until the ash was white or nearly so, added the filtrate, evaporated to dryness and ignited at a temperature not

exceeding 450°C. Calculated the percentage of ash with reference to the air dried drug

Acid insoluble ash

Boiled the ash with 25 ml of 2M hydrochloric acid for 5 min, collected the insoluble matter in a Gooch crucible or on an ash less filter paper, washed with hot water, ignited to constant weight, cooled in desiccators and weighed. Calculated the percentage of acid insoluble ash with reference to the air dried drug.

Loss on drying

Take 2 or 5 or 10g of sample (coarse powder) in a dry and evaporating dish. Place in a hot air oven at 105±5°C for 5 hrs. Cool to room temperature in a desiccator and weigh. Continue drying and weighing at half an hour intervals till difference between two successive weighing corresponds to not more than 0.1% of weight of sample.

Determination of extractive values.

The water-soluble, alcohol soluble and ether soluble extractive values of air-dried sample were evaluated using the procedure given below.

Water soluble extractives

Macerate 5 g of air dried drug, coarsely powdered, with 100 ml of water in a closed flask for 24 hour, shaking frequently for 6 hour and allow standing for 18 hours after then shaking the flask and filter. Take 25 ml filtrate in porcelain dish and evaporated at 100^0C on water both to dryness and dry in oven at 105^0C, to constant weight, cool in a desiccator and weighed. Calculated the percentage of water soluble extractive value with reference to the air dried drug.

Preliminary Phytochemical Screening

Chemical tests

Presence of types of constituents was determined by using following phytochemical tests as follows. The inference of which are summarized in result and discussion section.

Determination of pH

1g of drug was taken in a 100 ml volumetric flask and added in 100 ml of distilled water. The solution was put for about 4 hours and filtered. pH of filtrate was checked with the calibrated pH meter with standard of pH 4, 7, 9 (standard glass electrode).

Determination of Swelling Index

1-1 gm of coarsely powdered drug was taken in 25ml of glass stoppered measuring cylinder. 25ml water was added and the mean value of initial height of drug in the cylinder was determined. Shaken the mixture thoroughly at interval of every 10 minutes for 1hour.Allowed to stand for 3 hours at room temperature. The mean value of final height of drug in the cylinder was determined.

Calculation: Swelling index =Final mean - Initial mean

Determination of Foaming Index

Reduced 1gm of the plant material to a coarse powder weighed accurately and transferred to a 500 ml conical flask containing 100ml boiling water. Maintained at moderate boiling for 30 minutes. Cooled and filtered in to a 100 ml volumetric flask and add a sufficient water through the filter to dilute the volume to 100 ml. Placed the above decoction in to 10 stoppered test tubes in a series of successive portions of 1, 2, 3, 4 up to 10 ml and adjusted the volume of the liquid in each tube with water to 10 ml. Stoppered the tubes and shake them in a lengthwise motion for 15 seconds, 2 frequencies per seconds. Allowed to stand for 15 minutes and measure the height of the foam.

If the height of foam was found in every tube is less than 1cm, the foaming index is less than 100.

If in any tubes a height of foam was of 1 cm is measured, the dilution of the plant material in this tube (a) is the index sought.

If the height of the foam is more than 1 cm. in every tube, the foaming index is over 1000. In this case the determination was needs to be made on a new series of dilution of the decoction in order to obtained.

$$\text{Foaming Index} = 1000/a$$

Where a is the volume in ml. of the decoction used for preparing the dilution in the tube where foaming is observed

Fluroscence Analysis

Fluorescence analysis is done to determine the effect of the different reagent on the colour of the powdered drug in the normal day light, short wavelength(254nm) and long wavelength(366nm).Reagents used are Distill water,1N NaOH in water, 1N NaOH in Methanol, 50% Nitric acid, 50% Hydrochloric acid, Sulphuric acid, Acetone, concentrate hydrochloric acid, Chloroform.

Extraction of Essential Oil and Analysis of Oil by Gas Chromatography

Essential oil is extracted by distilling the drug with a mixture of water and glycerin (175:75) collecting the distillate in a graduated tube in which the aqueous portion of the distillate is

automatically separated ad returned to the distilling flask and measuring the volume of the oil the content of oil is expressed as a percentage v/w. Analysis of oil is done by the Gas chromatography under following

GC condition

Instrument	GC NUCON -5700
Column	Stainless steel
Length	10 feet
Inner diameter	2 mm
Outer diameter	3.175 mm
Mesh size	100-120 mesh
Column temperature	90-230°C
Injector	230°C
Detector	240°C
Starting voltage	0.05mv
Injection volume	0.2µl
Sensitivity	100
Attenuator	4
Height reject	0
Area reject	0
Threshold	0
End time	60 minutes
End value	70
Programming rate	4°C /minute
Initial oven temperature	90°C

Microbial Load Determination [5]

Microbial contamination. Aerobic bacteria and fungi are normally present in plants material and may be increased due to faulty growth, harvesting, storage or processing. Herbal ingredients particularly those with high starch content may be prone to increase microbial growth. It is not uncommon for herbal ingredients to have aerobic bacteria present at 10^2-10^8 colony forming unit per grams. Pathogenic organisms including *Enterobactor, Enterococcous, Clostridium, Pseudomonas, Shigella and Streptococcus* have been shown to contaminate herbal ingredients. The Indian Pharmacopoeia gives guidance to acceptable microbial limit.

Procedure

Total aerobic count

1gm of the sample was taken in case of powder in to flask containing 99 ml of the Sodium creolite phosphate buffer solution (SCPS), shake the flask & keep in water bath at 37 – 45°C for 5 to10 minutes for dissolving & incubation (100 dilution). After this 1ml was taken from the flask and transferred it to tubes containing 9ml of the Sodium creolite phosphate buffer solution (SCPS) (1000 dilution). Transferred 1 ml dilution to the sterile petridish with the help of sterile pipette under laminar air flow. Poured 15 to 20 ml of cooled (45°C) Soyabean casein digest agar to plated & mix dilution with medium by rotating of plates. Allowed the plates to solidify. After solidification incubate the plate at 37° C for 2 days in an inverted position in incubator. After the incubation period count the number of bacterial colonies in each plate using colony counter, and calculate the number of bacteria in the sample.

$$\text{TAC Count} = \frac{\text{Number of colonies on plates}}{\text{Amount plated x Dilution factor}}$$

Coliform

Add 1gm of the sample in case of powder in to flask containing 99 ml of the Sodium creolite phosphate buffer solution (SCPS), Shake the flask & keep in water bath at 37°C. – 45°C for 5 to10 minutes for dissolving & incubation.(100 dilution). Transfer 1 ml. dilution to the sterile petri plate with the help of sterile pipet under laminar air flow. Now pouring 15 to 20 ml of cooled (45°C) Mac konkey agar (MCA) to plate & mix dilution with medium by rotating of plates. Allow the plates to solidify. After solidification incubate the plate at 37°C for 2 days (48 hours) in an inverted position in incubator. After the incubation period count the number of bacterial colonies in each plate using colony counter and calculate the number of coli forms in the sample.

Bacillus:

Add 1gm of the sample in case of powder in to flask containing 99 ml of the Sodium creolite phosphate buffer solution(SCPS), shake the flask & keep in water bath at 37°C – 45°C for 5 to10 minutes for dissolving & incubation(100 dilution). Transfer 1 ml. dilution to the sterile petri plate with the help of sterile pipet under laminar air flow. Now pouring 15 to 20 ml of cooled (45°C) MYPA media to plate & mix dilution with medium by rotating of plates. Allow the plates to solidify. After solidification incubate the plate at 30°C for 2 days (48 hours) in an inverted position in incubator. After the incubation period count the number of Bacillus colonies in plate using colony counter and calculate the number of Bacillus in the sample.

Calculation:

$$\text{TAC Count} = \frac{\text{Number of colonies on plates}}{\text{Amount plated x dilution factor}}$$

Yeasts and Molds

1 gm of the sample was added in case of powder in to flask containing 99 ml of the Sodium creolite phosphate buffer solution (SCPS), shaked the flask & keep in water bath at 37–45°C for 5 to10 minutes for dissolving & incubation (100 dilution). Transferred 1 ml dilution to the sterile petri plate with the help of sterile pipette under laminar air flow. Now pouring 15 to 20 ml of cooled (45°C) Potato dextrose agar (PDA) Media to plate & mix dilution with medium by rotating of plates. Allowed the plates to solidify. After solidification Incubate the plate at 25°C for 3 days (Yeasts) to 5 days (Moulds) in an inverted position in BOD. After the incubation period count the number of Yeasts and Moulds colonies in plate using colony counter, and calculate the number of Yeasts and Moulds in the sample.

$$\text{TACCount} = \frac{\text{Number of colonies on plates}}{\text{Amount plated x Dilution factor}}$$

Primary test: Aseptically 10 gm of sample was added into 100 ml of nutrient broth. Incubate the Nutrient broth at 37°C for 24 hours, Transferred 1ml of aliquots of enriched culture to 5ml of Selenite cystine broth (SCB) and incubated it at 37°C for 48 hours, After incubation transfer a loopful of culture on Bismuth sulphite agar (BSA) and incubate at 37°C for 24 hours, pick up the suspected colonies and prepare grams stain slide.

Confirmatory test: Transfered suspected colonies on Tripple sugar iron (TSI) slants by inoculating the surface of the slope and then making a stab culture with the same incubating needle. Incubate at 37^0 C and observed daily for up to 7 days. The absence of acidity from the surface and blackening in the butt of TSI slant indicate presence of Salmonella.

Pseudomonas aeruginosa

It is slender, grams negative bacillus, non-capsulated, non-sparing and actively motile by a polar flagellum most staring possess pill.

Primary test: Aseptically added 10 gm of sample into 100 ml. of Cetrimide broth (CB), Incubated it at the37°C. For 72 hours. After incubation transferred the loopful of culture on cetrimide agar plate and incubated at 37°C for 72 hours. Picked up of the suspected colonies and prepare grams stain slide.

Confirmatory test: Oxidase test

Placed 2-3 drops of freshly prepared 1 % (w/v) solution of N, N, N, NTetramethyl-p- Phenylenediaminedihydro chloride on piece of filter paper (whatman No 1) and smeared with suspected colony. If purple colour is produced within 5 to 10 seconds the test is positive.

Staphylococcus aureus

These are gram (+ve), facultative anaerobic, non motile cocci non spore forming, having both an oxidative & fermentative type of metabolism. The temperature & PH range for growth are 7-50°C & 4.5-9.3 respectively many specials are commensals, others pathogenic the major pathogenic species is Staphylococcus aurous which can cause boils, wound infection, toxic shock syndrome food poisoning acid thus forming yellow halo around colonies.

Primary test: Aseptically add 10 gm of sample into 100 ml of Nutrient broth. Incubate the nutrient broth at 37°C for 24 - 48hours. After incubation transfer the 1ml of enriched culture in a tube contain5 ml of Salt meat broth (SMB) and incubate it at the 37°C for 48 hours. After incubation streak a loopful of culture on Mannitol salt agar (MSA) medium and incubate it at 37°C for 48 hours, pick up the suspected colonies and prepare grams stain slide.

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Confirmatory test DNAase test

Pick up the suspected colonies from agar surface of Manital salt agar and streak on DNAase agar medium, and incubate it at 37^0 C for 24 hours. If growth is there DNA containing culture plates flooded with 3.6% HCL solution to pptunhydrolysed DNA. DNAase positive culture surrounded by clear zones

Determination of residual organ chlorine pesticides (Gc-Ms) [6]

Procedure: Crush the composite sample to coarse powder and homogenize. Mix 10 gm sample vigorously with 120 ml of acetonitrile-water mixture (2:1) using glass rod in a 250 ml

beaker and keep overnight. Filter via suction using non-absorbent cotton pad pre-rinsed with acetonitrile on Buchner funnel. Transfer the filtrate into a separating funnel. Add 120 ml sodium chloride solution, shake. Extract with 50 ml n-hexane thrice, shaking vigorously. Dry and combine organic phase over anhydrous sodium sulphate granules and concentrate it to 5ml using water bath at 50 to 60°C. Clean the extract with 20-25 gm preactivated florosil (at 50 to 55°C) and 5 gm anhydrous sodium sulphate column pre-rinsed with petroleum ether. Elute using 150 ml mixture comprising of n-hexane (141ml) and diethyl ether (9ml) at a flow rate of 1 drop per second. Concentrate the extract close to dryness on water bath and make up the volume up to 1 ml with n-hexane.

For ppb = C x V/W

Where: C= concentration of compound in ppb or microgram/kilogram.

V= final make up volume of the sample.

W= initial weight of sample which taken

Heavy metal content determination

As per the latest guidelines issued by World Health Organization (WHO) no plant analysis is complete without the evaluation of certain parameters mentioned therein, heavy metal analysis being one of them. Out of twenty one types of heavy metals reported in medicinal plants, four of the most commonly reported ones i.e. Arsenic (As), Lead (Pb), Mercury (Hg) and Cadmium (Cd) content was determined in the experimental plant material.

Heavy metal analysis (By Atomic Absorption Spectrometer)

Preparation of sample

Weigh required amount of sample in a silica crucible and burn it on hot Plate till organic matter is charred. Place in a muffle furnace at 500°C for 5 hr. and cool. Add 20 ml nitric acid (65%) to the Ash and heat on boiling water bath for an hr. Filter and wash the residue with water and make up volume to 100 ml with water.

Trace Metal (ppm) =C xD/Wx1000

Where: C=concentration (ppb), D=Dilution factor, W= weight of sample (gm)

HPTLC Analysis

Identification of menthol in *Mentha piperita* leaves

***Mentha piperita* TLC identity test:**

Test solution: 0.1 ml of powdered drug was distilled in 2 ml acetone for 10 min with slight warming. Filtered and used the filtrate.

Reference solution: 1 mg menthol dissolves in 2 ml of Dichloromethane.

Stationary phase: Precoated silica gel 60 F_{254} TLC Aluminum sheets plates of uniform thickness (0.2mm) is used as stationary phase**.**

Solvent system: Toluene: ethyl acetate (9 : 1)

Procedure: 5µl each of test solution and reference solution were applied on two different tracks on a pre-coated silica gel 60 plate (2.5 x 10 cm) of uniform thickness (0.2 mm). The plate was developed in the solvent system

Visualization of spots (Post scanning): TLC plate spray with vanillin sulphuric acid reagent and in day light blue spot seen at Rf0.29. There are other spots seen of defferentcolour like violet at Rf 0.54, green at Rf 0.69, blue at Rf 0.77, reddish pink at Rf 0.94 in uv chamber at wavelength 254 nm .

Development of polyherbal formulation for topical pain analgesic ointment

A cream is a topical preparation usually for application to the skin. Creams are semi-solid emulsion that is mixtures of oil and water. They are divided into two types: oil-in-water (O/W) creams which are composed of small droplets of oil dispersed in a continuous phase, and water-in-oil (W/O) creams which are composed of small droplets of water dispersed in a continuous oily phase. Oil-in-water creams are more comfortable and cosmetically acceptable as they are less greasy and more easily washed off using water. Water-in-oil creams are more difficult to handle but many drugs which are incorporated into creams are hydrophobic and will be released more readily from a water-in-oil cream than an oil-in-water cream. Water-in- oil creams are also more moisturising as they provide an oily barrier which reduces water loss from the stratum corneum, the outermost layer of the skin. The cream containing medicinal substance is called medicated cream. This formulation should be stored in well dried completely filled and well stoppered tubes or other dispensing items in a cool dark place. The syrup should be stored at a temperature not exceeding 25°C.

Preparation of ointment [7]

Water phase was prepared at temperature of 75°C to 80°C. Oil phase was prepared by heating hard paraffin and petroleum jelly in a stainless steel vessel till temperature of oil phase attains 75°C to 80°C. Both water phase and oil phase were mixed by passing them through 40# and 150# double cone

sandwich stainless steel filter respectively into ointment manufacturing vessel under vacuum. The mass was stirred and cooled for 1.5 hours. Active ingredients like thymol, menthol, were made into homogenous slurry by stirring it for 30 minutes. The slurry was transferred to ointment manufacturing vessel and homogenization was continued for 1.5 hours. Then it was cooled and again stirred till ointment is obtained.

Evaluation of ointment

Determination of pH value:

Two methods for measuring pH i.e. colorimetric methods using indicator solutions or papers, and other electrochemical methods using electrodes and a mili voltmeter (pH meter)

Determination of TFM:

The emulsion is taken with dilute mineral acid and the fatty matter is extracted with petroleum ether and then weighed after removal of the solvent.TFM can measure with reagent Dilute HCL– 1:1 (v / v), Petroleum ether– B.P 40°C to 60°C, Methyl orange indicator solution by Dissolving 0.1 g of methyl orange in 100 ml of water and Sodium sulphate.TFM can calculated by percent by mass = 100(M1/M2) ,Where M1 = mass in g of residue, and M2 = mass in g material taken for test.

Determination of moisture content (LOD) [8]

In this sample is weighed and then subsequently heated to allow moisture loss, then cooled in desiccator. Moisture content is results in by difference in wet and dry weight.

Microbial analysis:

The test having a plating a known dilution of the preparation or any digest agar medium that is suitable for the total count of aerobic bacteria and fungi after incubating them for certain period to allow the formation of shown colonies.

RESULTS AND DISCUSSION

Authentication

Leaves of *Mentha piperita*, heartwood of *Cedrus deodara*, , seed of *Carum copticum* were authenticated by Taxonomic Division of Maharishi Ayurveda Products Pvt Ltd, NSEZ Noida.

Pharmacognostic Studies

Morphological evaluation: Leaves of *Mentha piperita*, heartwood *of Cedrus deodara*, seed of *Carum copticum* showed the following characteristic on morphological examination.

Table: 1 Morphological characters of *Mentha piperita* leave

Characters	Description
Color	Green
Odour	Aromatic
Taste	Aromatic with cooling sensation
Size	1-2 cm wide, 1-4 cm length
Shape	Ovate, lanceolate

Table 2 Morphological characters of *Cedrus deodara* heartwood

Characters	Description
Color	yellowish –brown
Odour	Aromatic
Taste	Astringent and febrifuge
Size	Not specific
Shape	Heavy

Table: 3 Morphological characters of *Carum copticum* seed

Characters	Description
Color	Green
Odour	Aromatic
Taste	Aromatic with cooling sensation
Size	1-2 cm wide, 1-4 cm length
Shape	Ovate, lanceolate

Microscopical evaluation

Transverse section characters: Leaves of *Mentha piperita*, heartwood *of Cedrus deodara*, seed of *Carum copticum* on transverse section showed following characters.

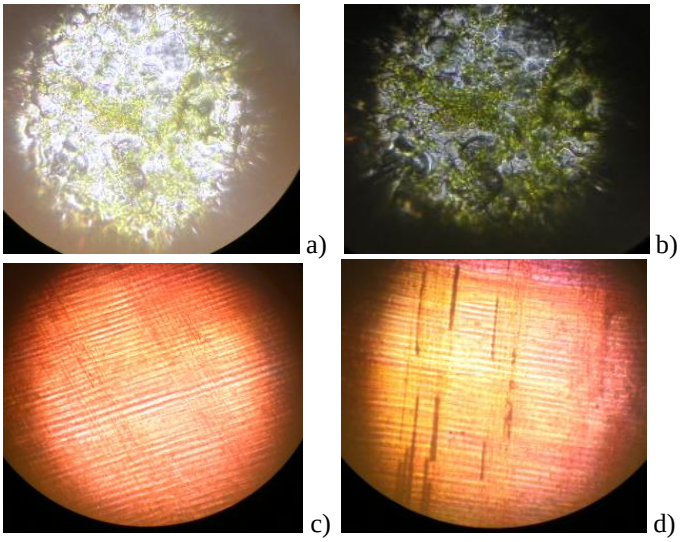


Fig. 2 T.S. of seed of a) *Mentha piperita* showing diacytic type of stomata; b) *Mentha piperita* showing diacytic type of stomata; c) *Cedrus deodara* showing presence of very fine xylem rays; d) *Cedrus deodara* showing presence of very fine xylem rays, vessels absent

Powder microscopy

The powder was observed microscopically after staining with different reagents and then glycerin mounting:

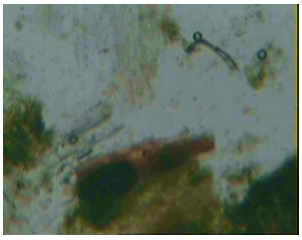
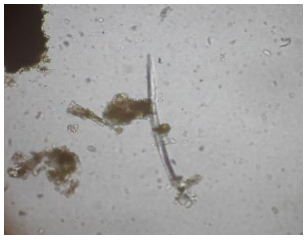
 a)  b)

Fig. 3 Powder microscopy of a) *Mentha piperita* showing non glandular trichomes b) *Mentha piperita* showing glandular trichomes

Quality control parameter & analysis of raw materials

Physicochemical studies

Leaves of *Mentha piperita*, heartwood *of Cedrus deodara*, seed of *Carum copticum* were evaluated for determination of physiochemical parameters, namely ash value, moisture content and extractive values.

Table 4 Result of physicochemical parameters of *Mentha piperita* leaves

Parameter	Specification	Result (%w/w)
Loss on drying at 105° C	NMT 12 %w/w	7.99
Water soluble extractives	NLT 22 %w/w	23.01
Total ash	NMT 11%w/w	10.72
Acid insoluble ash	NMT 1.5 %w/w	1.23

Table: 5 Result of physicochemical parameters of*Cedrus deodara* wood

Parameter	Specification	Result (% w/w)
Loss on drying at 105°C	NMT 9 % w/w	7.97
Water soluble extractives	NLT 2 % w/w	4.75
Total ash	NMT 1 % w/w	0.84
Acid insoluble ash	NMT 0.5 % w/w	0.39

Table 6 Result of physicochemical parameters of *Carum copticum* seed

Parameter	Specification	Result (%w/w)
Loss on drying at 105° C	NMT 11 % w/w	5.37
Water soluble extractives	NLT 15 % w/w	25.33
Total ash	NMT 10 % w/w	7.66
Acid insoluble ash	NMT 2 % w/w	0.96

Heavy Metal Analysis

Seed powder of ***Carum copticum,*** **heart wood powder of** ***Cedrus deodara*** and leaves powder of ***Mentha piperita*** were evaluated for determination of heavy metal content by using Atomic Absorption Spectroscopy (AAS).

Table 7 Result of heavy metal analysis of *Mentha piperita* leaves

Parameter	Specification	Result
Lead	NMT 3.0 mg/kg	0.74 mg/kg
Arsenic	NMT 1.0 mg/kg	0.14 mg/kg
Cadmium	NMT 0.5 mg/kg	0.02mg/kg
Mercury	NMT 0.1 mg/kg	Nil

Table 8 Result of heavy metal analysis of *Cedrus deodara* heartwood

Parameter	Specification	Result
Lead	NMT 3 mg/kg	0.077mg/kg
Arsenic	NMT 1 mg/kg	0.074 mg/kg
Cadmium	NMT 0.5 mg/kg	0.009 mg/kg
Mercury	NMT 0.1 mg/kg	Nil

Table 9 Result of heavy metal analysis of *Carum copticum* seed

Parameter	Specification	Result
Lead	NMT 3.0 mg/kg	0.112 mg/kg
Arsenic	NMT 1.0 mg/kg	0.09 mg/kg
Cadmium	NMT 0.5 mg/kg	0.48 mg/kg
Mercury	NMT 0.1 mg/kg	Nil

Pesticide Analysis

Seed powder of ***Carum copticum***, **heart wood powder of** ***Cedrus deodara,*** & leaves powder of ***Mentha piperita*** were evaluated for determination of pesticide content using GC-MS.

Table 10 Result of pesticide analysis of *Mentha piperita* leaves

Parameter	Specification	Result
Alpha and Beta HCH	NMT 10mcg/kg	0.006 mcg/kg
Gama HCH	NMT 10mcg/kg	1.77mcg/kg
DDT &Metabolites	NMT 50mcg/kg	0.748 mcg/kg

Table 11 Result of pesticide analysis of *Cedrus deodara* heartwood

Parameter	Specification	Result
Alpha and Beta HCH	NMT 10 mcg/kg	2.2 mcg/kg
Gama HCH	NMT 10 mcg/kg	2.487 mcg/kg
DDT & Metabolites	NMT 50 mcg/kg	4.049mcg/kg

Table 12 Result of pesticide analysis of *Carum coticum* seed

Parameter	Specification	Result
Alpha and Beta HCH	NMT 10mcg/kg	0.003 mcg/kg
Gama HCH	NMT 10mcg/kg	0.091 mcg/kg
DDT &Metabolites	NMT 50mcg/kg	1.045 mcg/kg

Phytochemical Screening

Aqueous extracts over screened for various classes of the phytoconstituents

Table 13 Result of phytochemical screening of aqueous extract of *Mentha piperita* leaves

Phyto-constituents	Aqueous extract
Volatile oils	+ve
Protein/Amino acid	-ve
Steroids/ Terpenoids	+ve
Tannin and Phenolic compounds	+ve
Alkaloids	+ve
Glycosides	-ve
Saponins	+ve
Flavonoids	+ve

Table: 14 Result of phytochemical screening of aqueous extract of *Cedrus deodara* heartwood

Phyto-constituents	Aqueous extract
Volatile oils	+ve
Protein/Amino acid	-ve
Steroids/ Terpenoids	-ve
Tannin and Phenolic compounds	+ve
Alkaloids	+ve
Glycosides	+ve
Saponins	-ve
Flavonoids	+ve

Table: 15 Result of phytochemical screening of aqueous extract of *Carum copticum* seed

Phytoconstituents	Aqueous extract
Volatile oils	+ve
Protein/Amino acid	-ve
Steroids/ Terpenoids	-ve
Tannin and Phenolic compounds	+ve
Alkaloids	+ve
Glycosides	+ve
Saponins	+ve
Flavonoids	+ve

(+) = Present; (-) = Absent

Determination of Swelling Index

Leaves powder of *Mentha piperita, heartwood powder of Cedrus deodara*, seed powder of *Carum copticum* were evaluated for determination of swelling index.

Table 16 Result of determination swelling index

Drug	Swelling index
Mentha piperita	1.5
Cedrus deodara	1.0
Carum copticum	0.5

Determination of Foaming Index

Leaves powder of *Mentha piperita, heartwood powder of Cedrus deodara*, seed powder of *Carum copticum* were evaluated for determination of foaming index.

Table 17 Result of foaming index of *Mentha piperita* leaves

Decoction	1	2	3	4	5	6	7	8	9	10
Water	9	8	7	6	5	4	3	2	1	0
Height	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	1cm	1.1cm
Foaming index	<100	<100	<100	<100	<100	<100	<100	<100	>1000	>1000

Table 18 Result of foaming index of *Cedrus deodara* heartwood

Decoction	1	2	3	4	5	6	7	8	9	10
Water	9	8	7	6	5	4	3	2	1	0
Height	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	1.0cm	1.0cm	1.1cm	1.2cm
Foaming index	<100	<100	<100	<100	<100	<100	>1000	>1000	>1000	>1000

Table 19 Result of foaming index of *Carum copticum* seed

Decoction	1	2	3	4	5	6	7	8	9	10
Water	9	8	7	6	5	4	3	2	1	0
Height	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm	<1cm
Foaming index	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100

Fluroscence Analysis

Fluroscence analysis is done to determine the effect of the different reagent on the colour of the powdered drug in the normal day light, short wavelength (254nm) and long wavelength (366nm). Reagents used are distilled water, 1N NaOH in water, 1N NaOH in methanol, 50% nitric acid, 50% hydrochloric acid, sulphuric acid, acetone, concentrate hydrochloric acid, chloroform.

Table 20 Result of fluorescence analysis of *Mentha piperita* (Leaves)

S. No	Reagents	Day light	λ (254nm)	λ (366nm)
1.	Crude powder	Bottle green	Light green	Light green
2.	Distilled water	Light green	Colourless	Colourless
3.	1N NaOH in water	Dark green	Black	Greenish black
4.	1N NaOH in methanol	Dark green	Black	Greenish
5.	50% Nitric acid	Green	Black	Black
6.	50% HCl	Greenish black	Black	Green
7.	Sulphuric acid	Green	Black	Black
8.	Acetone	Blackish green	Reddish yellow	Green
9.	Conc. HCl	Black	Black	Black
10.	Chloroform	Dark green	Yellow	Black

Table 21 Result of fluroscence analysis of *Cedrus deodara* (Heart wood)

S. No.	Reagents	Day light	λ (254nm)	λ (366nm)
1.	Crude powder	Cream	Cream	White
2.	Distilled water	Light cream	Colourless	Colourless
3.	1N NaOH in water	Yellowish cream	Light yellow	Yellow
4.	1N NaOH in methanol	Dull yellow	Colourless	Colourless
5.	50% Nitric acid	Whitish	Colourless	Colourless
6.	50% HCl	Cream	Colourless	Colourless
7.	Sulphuric acid	Black	Black	Black
8.	Acetone	Yellowish	Yellow	White
9.	Conc. HCl	Cream white	Colourless	Colourless
10.	Chloroform	Whitish	White	Colourless

Table 22 Result of fluorescence analysis of *Carum copticum* (Fruit)

S. No	Reagents	Day light	λ (254nm)	λ (366nm)
1.	Crude powder	Brown	Light brown	Light green
2.	Distill water	Creamy	Colourless	Colourless
3.	1N NaOH in water	Yellowish	Yellow	Light yellow
4.	1N NaOH in methanol	Yellow	Yellow	Colourless
5.	50% Nitric acid	Brown	Blackish	Green
6.	50% HCl	Brown	Black	Light green
7.	Sulphuric acid	Reddish brown	Black	Green
8.	Acetone	Dull yellow	Yellow	White
9.	Conc. HCl	Black brown	Black	black
10.	Chloroform	Dull brown	Colourless	Colourless

Gas Chromatography Analysis of Essential Oils [9]

There are two essential oil used in formulations named like oil of *Cedrus deodar* wood, *Mentha piperita*. The most common method of separating essential oil is distillation. Essential oil is extracted by distilling the drug with a mixture of water and glycerin (175:75) collecting the distillate in a graduated tube in

which the aqueous portion of the distillate is automatically separated ad returned to the distillation flask and measuring the volume of the oil the content of oil is expressed as a percentage v/w. GC analysis of essential oil of *Cedrus deodara* wood gives 32 peak and total area is 2173.961 mv-secs and GC analysis of essential oil of *Mentha piperita* gives 14 peak and total area is 2237.855 mv-secs. The detailed GC curves were given in annexure.

Microbial load determination

Pathogenic organism including *Enterobactor, Enterococcous, Clostridium, Pseudomonas, Shigella and Streptococcus* has been shown to contaminate herbal ingredients. The Indian Pharmacopoeia provides guidance to the acceptable microbial limit.

Table 23 Result of microbial analysis of*Mentha piperita* leaves

S.No.	Parameters	Specification	Results
1.	Total aerobic count	NMT 1250000 CFU per gm	600000
2.	*Enterobacteriaceae* (Coliform)	NMT 1000 CFU per gm	NAD
3.	*E.coli*	NMT 10 CFU per gm	NAD
4.	*Salmonella sp.*	Absent	Absent
5.	*Staphylococcus aureus*	NMT 100 CFU per gm	NAD
6.	Yeasts	NMT 100 CFU per gm	NAD
7.	Moulds	NMT 10000 CFU per gm	1000
8.	*Bacillus cereus*	NMT 1000 CFU per gm	NAD
9.	*Pseudomonas aeruginosa*	NMT 100 CFU per gm	NAD

Table 24 Result of microbial analysis of*Cedrus deodara* heartwood

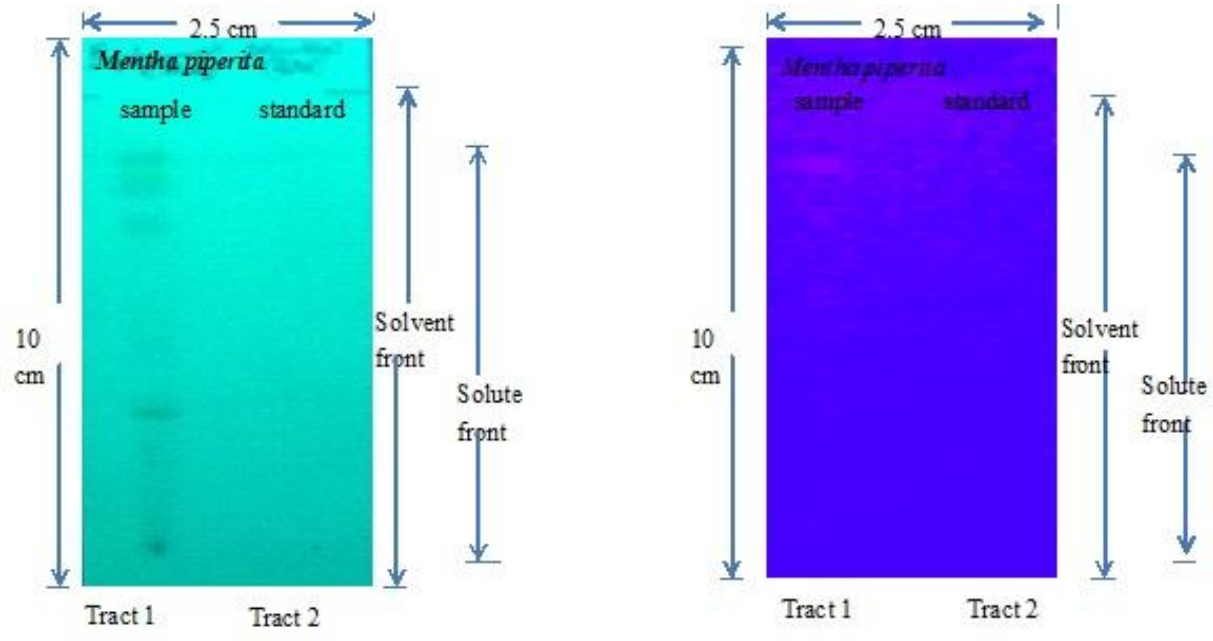
S. No.	Parameters	Specification	Results
1.	Total aerobic count	NMT 1250000 CFU per gm	130000
2.	*Enterobacteriaceae*(Coliform)	NMT 1000 CFU per gm	NAD
3.	*E.coli*	NMT 10 CFU per gm	NAD
4.	*Salmonella sp.*	Absent	Absent
5.	*Staphylococcus aureus*	NMT 100 CFU per gm	NAD
6.	Yeasts	NMT 100 CFU per gm	NAD
7.	Moulds	NMT 10000 CFU per gm	2000
8.	*Bacillus cereus*	NMT 1000 CFU per gm	NAD
9.	*Pseudomonas aeruginosa*	NMT 100 CFU per gm	NAD

Table 25 Result of microbial analysis of*Carum copticum* seed

S.No.	Parameters	Specification	Results
1.	Total aerobic count	NMT 1250000 CFU per gm	110000
2.	*Enterobacteriaceae* (Coliform)	NMT 1000 CFU per gm	NAD
3.	*E.coli*	NMT 10 CFU per gm	NAD
4.	*Salmonella sp.*	Absent	Absent
5.	*Staphylococcus aureus*	NMT 100 CFU per gm	NAD
6.	Yeasts	NMT 100 CFU per gm	NAD
7.	Moulds	NMT 10000 CFU per gm	1100
8.	*Bacillus cereus*	NMT 1000 CFU per gm	NAD
9.	*Pseudomonas aeruginosa*	NMT 100 CFU per gm	NAD

TLC and HPTLC Analysis

Identification of Menthol in *Mentha piperita* leaf



Track 1 = Test solution, Track 2 = Standard solution of Menthol

Thin layer chromatography of leaves extracts of *Mentha piperita*

For identification of menthol in rhizome extracts of*Mentha piperita* samples TLC analysis was performed by using standard solution of Menthol. The solvent systems consisting Toluene: ethyl acetate (9 : 1)Track 1 showed five spots and Track 2 showed one spots, Rf values of Track T_1 was 0.27, 0.33, 0.72, 0.83, 0.86 and Track T_2 was 0.83.

Evaluation of Cream and Ointment

Table 26 organoleptic evaluation of ointment

S. No.	Parameter	Ointment
1.	Colour	Light green
2.	Odour	Aromatic
3.	Appearance	Semi solid

Table 27 Physicochemical evaluation of ointment

S. No.	Parameter	Ointment
1.	Total fatty matter	22.85%w/w
2.	pH	4.74
3.	Total solid	24.38%w/w

CONCLUSION

A detailed pharmacognostic studies including morphology and microscopy (Fresh sample and powder) were carried out. Microscopic studies (T.S.) of ***Mentha piperita*** leaves showed the presence of diacytic type of stomata. Microscopic studies (T.S.) of ***Cedrus deodara*** powder showed the presence of very fine xylem rays, vessels absentPowder microscopy of ***Mentha piperita*** showed the presence of non- glandular trichomes, vessels. Leaves of ***Mentha piperita*** showed total Ash value 10.72%, acid insoluble ash 1.23%, loss on drying 7.99%, water soluble extractives 23.01%. Heartwood of Cedrus ***deodara*** showed total Ash value 0.84%, acid insoluble ash 0.39% , water soluble extractives 4.75% and loss on drying 7.97%. Seeds of ***Carum copticum*** showed total Ash value 7.66%,acid insoluble ash 0.96% , water soluble extractives 25.33% and loss on drying 5.37%. The water extraction was carried andwas subjected to preliminary phytochemical tests to detect the presence of various classes of chemical constituents. From leaves of ***Mentha piperita*** was found to contain steroids, terpenoids, alkaloids, saponin, flavonoids , oils and fat mainly , heartwood of ***Cedrus deodara*** was found to contain steroids, terpenoids, alkaloids, glycosides, tannins and phenolic compounds, flavonoids , oils and fat mainly and seeds of ***Carum copticum*** was found to contain alkaloids, saponin, flavonoids, glycosides mainly.

Leaves powder of *Mentha piperita,* heartwood powder of *Cedrus deodara,* seed powder of *Carum copticum* were

evaluated for determination of swelling index and leaves powder of *Mentha piperita* was found 1.5, heart wood powder of *Cedrus deodara* was found 1.0, and seed powder of *Carum copticum* was found 0.5. leaves powder of *Mentha piperita*, heartwood powder of *Cedrus deodara*, seed powder of *Carum copticum* were evaluated for determination of foaming index by using different dilution of water and decoction (1 - 10) and, leaves powder of *Mentha piperita* of dilution from 1 to 8 was found less than 100, the dilution 9 to 10 was found greater than 1000, heartwood powder of *Cedrus deodara* of dilution from 1 to 8 was found less than 100, the dilution 9 to 10 was found greater than 1000 , seed powder of *Carum copticum* was of dilution from 1 to 10 was found less than 100. The fluroscence analysis of leaves powder of *Mentha piperita*, heartwood powder of *Cedrus deodara*, seed powder of *Carum copticum* was done by using different reagent and different colour are observed in the day light, short wave length (254nm) and long wave length (366nm). Herbal ingredients particularly those with high starch content may be prone to increase microbial growth. It is not uncommon for herbal ingredients to have aerobic bacteria present at 10^2 - 10^8 colony forming unit per grams. Pathogenic organism including *Enterobactor, Enterococcous, Clostridium, Pseudomonas, Shigella and Streptococcous* has been shown to contaminate herbal ingredients. The WHO gives guidance regarding acceptable microbial limit. leaves powder of *Mentha piperita*, heartwood powder of *Cedrus deodara*, seed powder of *Carum copticum* was within the permissible limits mentioned by WHO guidelines. The levels of pesticide residues α, β and γ HCH, DDT and DDE were analyzed in leaves powder of *Mentha piperita*, heartwood powder of *Cedrus deodara*, seed powder of *Carum copticum* using GC-MS. The levels of pesticide residues in all the samples were within the permissible limits mentioned by WHO guidelines. The levels of heavy metals Pb, As, Cd and Hg were analyzed in leaves powder of *Mentha piperita*, heartwood powder of *Cedrus deodara*, seed powder of *Carum copticum* using Atomic absorption spectroscopy.

The levels of heavy metals in all the test samples were within the permissible limits as mentioned by WHO guidelines. Essential oil is extracted and GC analysis of essential oil is done, number of peak obtained from *Cedrus deodara* wood gives 32 peak and total area is 2173.961 mv-secs and *Mentha piperita* gives 14 peak and total area is 2237.855 mv-secs.

Analgesic ointment was prepared by using different ingredient like *Mentha piperita, Cedrus deodara, Carum copticum*. Quality analysis like physicochemical parameters, heavy metal, pesticide and microbial load analysis were carried out for all crude herbs used in the formulation and almost the all parameters were found to be within the permissible limits and the organoleptic evaluation like Colour appear light green, Odour Aromatic, Appearance Semi solid obtained and Total fatty matter (TFM)22.85%w/w, pH 4.74, Total solid 24.38% w/w presented in polyherbal formulation and were found to be within permissible limits.

FINANCIAL ASSISTANCE
Nil

CONFLICT OF INTEREST
The authors declare no conflict of interest

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