PREPARATION AND CHARACTERIZATION OF MINT DISTILLATE AND ITS HEPATOPROTECTIVE EFFECT ON HEPG2 CELL LINE

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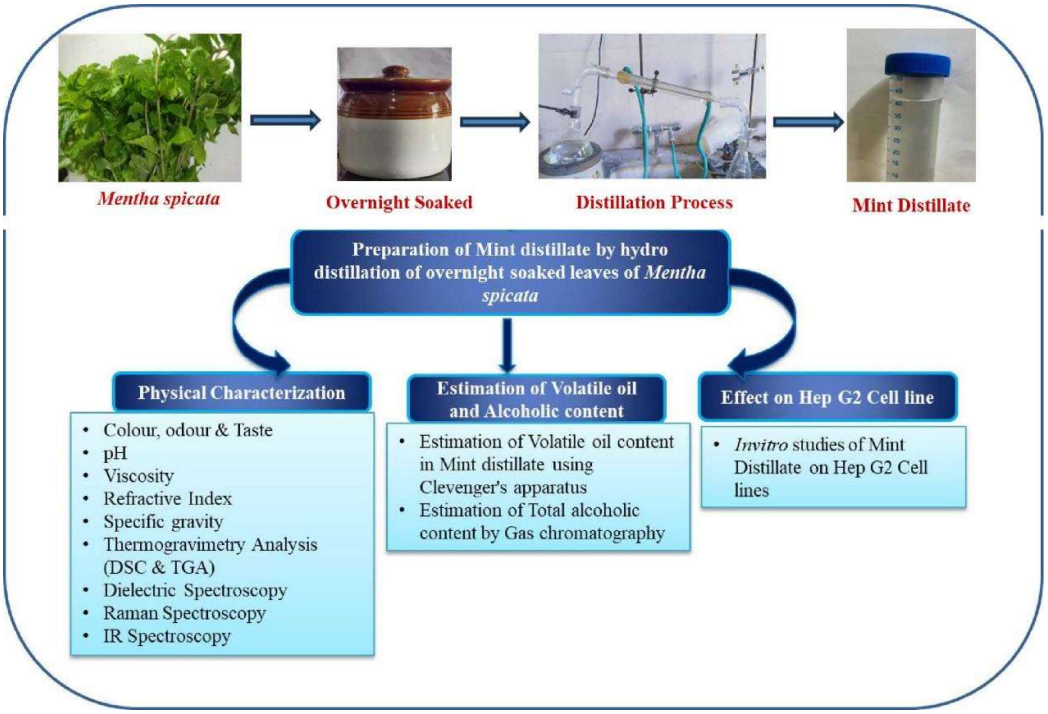
Abstract

Mint leaves are used to enhance the digestion process by stimulating the digestive enzymes and increasing bile flow in the liver. Mint distillate traditionally is being prescribed by Indian traditional practitioners to enhance digestion process. This research work focuses on preparation and physical characterization of Mint distillate and investigating its effect on HepG2 cell line. Mint distillate was prepared by distilling overnight soaked *Mentha spicata* leaves and subjected to physical characterization, thermal analysis using DSC and TGA, dielectric Spectroscopy, FTIR and Raman Spectroscopy were used for qualitative fingerprinting and functional group identification. Gas Chromatography (GC) was employed to estimate volatile oil content and to detect *in-situ* methanol production. The hepatoprotective effect of mint distillate was evaluated in HepG2 cells exposed to isoniazid-induced toxicity using MTT assay. Our research findings suggest that to prepare Mint distillate, the distillation temperature should not exceed 57ºC to avoid thermal decomposition of phytomolecules in Mint distillate. Further mint distillate was scientifically validated by MTT assay for its hepatoprotective action.

Keywords: Mint Distillate, *Mentha spicata*, HepG2, Thermal analysis, IR and Raman Spectroscopy, Dielectric Spectroscopy.

GRAPHICAL ABSTRACT



1. Introduction

India is known for its rich heritage of botanicals as ornamental plants and herbal medicines [1]. The herbal medicines are employed in Indian traditional systems of medicine - Ayurveda, Siddha, Unani and Homeopathy [2]. One such herbal medicine prepared in the Siddha system of medicine is herbal distillates practiced by siddha practitioners to ameliorate various diseases and disorders for the betterment of mankind [3].

Herbal distillates are used as therapeutics especially to enhance digestion and nutraceuticals as health supplements [4,5]. The herbal distillates are prepared by subjecting herbs for purification, followed by soaking in suitable media in the distillate vessel and subsequently subjected to the distillation process. In case of aromatic herbs essential oil may float as a supernatant layer in distillate. In India traditionally, herbal distillates are called Theeneer and the distillates containing volatile oil are called Theeneer ennai. The distillates obtained from distillation of salt, called as Dravagam [6,7].

Mint leaves are an important herb to enhance the digestion process by stimulating the digestive enzymes and increasing bile flow in the liver. In India, Mint distillate is one of the herbal medicines prescribed by traditional practitioners to enhance digestion, act as a good appetizer and used as liver tonic [8,9]. As yet, there are no extensive physical characterization studies on Mint distillate and lacuna in scientific investigation on its cytoprotective effect on liver cell line, this research work focused on preparing herbal distillates from the leaves of *Mentha spicata* (Lamiaceae) and its physical

characterization by analysing the thermal stability by Differential Scanning Calorimetry and Thermogravimetry Analysis, Raman Spectroscopy, Infra-Red spectroscopy and Dielectric spectroscopy, estimating volatile oil content and *in-situ* methanol production, since during overnight soaking of Mint leaves in water causes fermentation results in production of toxic metabolite methanol. Further the prepared mint distillate was evaluated for its effects on HepG2 cell line by MTT assay.

2. Materials and Methods

2.1. Collection and Authentication of *Mentha spicata*

The fresh leaves of *Mentha spicata* were purchased in a local market in Puducherry, India. The collected plant material was botanically authenticated by the taxonomist, Department of Ecology, French institute of Puducherry and the herbarium specimen voucher was deposited at Department of Pharmacognosy, College of Pharmacy, Mother Theresa Post Graduate and Research Institute of Health Sciences, Puducherry, India (Herbarium Number: DOPG/COP/2023/001). *Mentha spicata* is commonly called spearmint, native to Southern Asia and Europe. It belongs to the Plantae, Clade Tracheophytes, Angiosperms, Eudicots, Asterids, order Lamiales and family Lamiaceae[10].

2.2. Preparation of Mint distillate

The fresh leaves (Figure. 1) were detached from the whole plant and were washed with distilled water and shade dried (Figure.2). Drying process continued until removal of adhered water after washing. 800gms of dried fresh leaves are soaked overnight in 6000 ml distilled water (Figure.3). The soaked content is transferred into distillation assembly (Figure.4) and distilled at 85 ºC by hydro distillation method in clavenger apparatus.[11] The distillate was collected for 3 hours and stored in a well closed container (Figure.5) and subjected for physical characterization, volatile oil content and methanol content , further estimating its effect on HepG2 cell line by MTT assay. [12-14].

2.3. Characterization of Mint Distillates

The prepared Mint Distillate was subjected for characterizing Physicochemical Parameters as per the methods mentioned in IP (Indian Pharmacopoeia, 2010) viz., pH (using Table Top, portable digital pH meter), Density (using specific gravity bottle), Refractive Index (using Abbes refractrometer), Viscosity (using ostwald viscometer), Thermal analysis (TA Instruments: Q600SDT and Q20DSC) – Differential Scanning Calorimetry (DSC) & Thermo Gravimetric Analysis (TGA), Dielectric spectrometry (NOVOCONTROL Technologies GmbH & Co. Germany, Model: Concept 80) to determine the Dielectric permittivity and conductivity of mint distillate, Laser Confocal Raman Microscope with Spectrometer (Renishaw Metrological Systems UK, Model: Invia Reflex, Laser Excitation: 514/488nm and 785nm) and IR Spectroscopy (Thermo Scientific Nicolet IS5) as qualitative analysis for fingerprinting the phytoconstituents in mint distillate [15-19].

2.4. **Estimation of volatile oil content in Mint distillate**

Overnight soaked 30.66gms of mint leaves in 230ml of distilled water was completely transferred into Clevenger's apparatus and the distillate was collected. The Mint distillate collected in the receiver tube of Clevenger's apparatus, was allowed to settle for 30 mins. From the collected volatile oil (in terms of ml) on the top of water layer in the receiver tube, the amount of essential oil content in the mint distillate was calculated by following formula [13].

Amount of essential oil/volatile oil content in Mint distillate in % = Volatile oil in ml collected in receiver tube / Weight of Mint leaves taken in grams x 100

2.5. Estimation of methanolic content in Mint distillate by Gas chromatography

To detect the presence or absence of methanolic content in Mint distillate, 1µl of the prepared mint distillate along with methanol as standard reference was subjected for Gas chromatography as per the method mentioned in IP (Indian Pharmacopoeia, 2010). Estimation of methanolic content in Mint distillate was done by Netel Analyte GC2900A Gas Chromatograph instrument (India) with Flame Ionization detector equipped with autosampler. Initial temperature was 85ºC, Oven temperature was 180°C, Injection and detection temperature was 230°C. Length of the column (Column DB24) was 30m with internal diameter 0.25 µm and particle size 0.25µ. Carrier gas used was Nitrogen (2ml/min in volume/time) and the split was 1:25. Injected volume of sample and standard was 1µl. Standard and sample run time was 25mins [20].

2.6. Effect of Mint distillate on HepG2 cell line

The prepared mint distillate was subjected for Invitro hepatoprotective studies by MTT assay using HepG2 cell line along with standard drug silymarin as positive control. 70mM concentration of Isoniazid was used to induce toxicity to hepatic cell line. The concentration of the test sample Mint distillate and the standard drug silymarin used for the hepatoprotective study was 6.25µg/ml, 12.5µg/ml, 25µg/ml and 50µg/ml respectively (Table.3). MTT assay for hepatoprotective effect of Mint distillate compared with the standard drug was performed in triplicates.

100µl of diluted cell suspension (10,000 HepG2 cells/well) was added to each well of 96 well microtiter plates. After 24 hours, the supernatant was discarded. 70mM Isoniazid and 100µl of test drugs in different concentrations mentioned above were added to the wells. The plates were incubated in 5% CO_2 atmosphere at 37ºC for 24 hours. After incubation the test drug was discarded and 100µl of 0.05% MTT in Phosphate buffer saline was added to the wells. The plate is incubated at 37ºC in 5% CO_2 atmosphere. The supernatant was removed and 100µl of DMSO was added and the plate is shaken gently to solubilize the metabolite formazan. The absorbance is measured at 570nm. The test drug is replaced with standard drug Silymarin [21]. The percentage of viable cells was calculated below [22].

% of Viability = Absorbance of Sample/Absorbance of Control x 100

3. Results

3.1. Preparation of Mint Distillate

Mint distillate was prepared by overnight soaked *Mentha spicata* leaves followed by hydro distilling the soaked content using clavenger apparatus (Fig1-6).

3.2. Physical Characterization

Physical characterization of Mint distillate was determined using the parameters nature, colour, odour, taste, pH, viscosity, refractive index, specific gravity, thermal analysis, dielectric conductivity, thermogravimetry analysis, raman spectroscopy, FTIR spectroscopy and Gas chromatography to determine the *in-situ* production of methanolic content due to overnight soaking of mentha leaves, since during soaking, fermentation taken place results in the production of toxic *in-situ* methanol generation (Table 1&2, Graph.1, Spectrum 1, 2, 3 & 4, Chromatogram 1 & 2).

3.3. Invitro Hepatoprotective activity

In HepG2 cell line studies Mint distillate exhibited remarkable cytoprotective effect with EC_{50} value of 4.01µg/ml similar to the standard drug Silymarin (Table.3, Graph.2 & Figure 7 & 8).

4. Discussion

4.1. Physical Characterization of Mint Distillate

The amount of Mint distillate collected was 50ml. The concentration of phytomolecules in distillate is found to be 0.004mg/ml or 4µg/ml, calculated by evaporating 1ml of mint distillate followed by calculating the residue on dry weight basis. The distillate was subjected for Physical characterization as per IP (Indian Pharmacopoeia, 2010). The physical nature of the distillate is colourless liquid, aromatic odour, sour taste, pH 4.52 acidic in nature, viscosity 0.7732 poise, refractive index-1.333 and specific gravity 1.0211g/ml (Table.1).

4.2. Thermal Analysis

Thermal analysis of the mint distillate was carried out by differential scanning calorimetry and thermogravimetric analysis. DSC is used to analyse the temperature required for phase transition and to analyse whether the molecules in the sample are exothermic or endothermic in nature [23,24]. Thermogravimetric analysis was used to analyse the thermal stability or temperature at which the molecules in the sample are decomposed [25]. The Thermogravimetry graph (Graph.1) showed that at 103.45ºC, 99.73% of phytomolecules in Mint Distillate are decomposed and the decomposition temperature starts from 57.12ºC. The energy required for decomposition was 910J/g and 35J was required to decompose 26mg of sample. Moreover, at 90ºC, 50% of phytomolecules in mint distillate were decomposed. From these data it was understood that the phytomolecules in mint distillate are thermally stable only up to 57ºC and the distillation temperature should not exceed 57ºC to prepare Mint Distillate.

4.3. Dielectric Spectroscopy

Dielectric constant represents the index polarity of solvent that regulates the solubility of the drug. Conductivity is one of the properties of liquids to conduct the electrical flux [26,27]. The higher the dielectric constant value, is the character to dissolve polar substance in liquids having high dielectric constant values and non-polar molecules in liquids having low dielectric constant values. The dielectric spectrum (Spectrum.1) of Mint distillate showed the permittivity on the left side and conductivity on the right side. From the spectrum the permittivity values showed that at 1MHz the value is $0.1x10^3$, 100Hz - 2×10^3 and at 1Hz – 15×10^3 and the conductivity values showed at 1Hz is $550x10^{-9}$S/cm, 100Hz - $650 x10^{-9}$S/cm, 10KHz to1MHz – $700 x10^{-9}$S/cm. From these values it was interpreted that the phytomolecules in mint distillate have high permittivity values, that could soluble in solvents of high dielectric constant values and in terms of conductivity, the Mint distillate have insulative properties.

4.4. Raman & IR Spectroscopy

Raman and IR spectroscopy measures the difference in the vibrational energy levels in molecules [28]. In IR spectroscopy absorption takes place from polychromatic IR radiation in a molecule that causes changes in the dipole moment [29]. In Raman spectroscopy the monochromatic radiation causes inelastic scattering at the electron shell of the molecules resulting in changing of molecular polarizability [30]. The absorption of monochromatic radiation in Raman spectroscopy was 514/488nm and 785nm. The mint distillate consists of varied functional groups in phytomolecules and hence the Raman spectra (Spectrum.2) and IR Spectroscopy (Spectrum.3 & 4) will be used as fingerprint analysis that provides qualitative analytical data for identity and authenticity of the prepared herbal medicine. In addition, from the IR Spectroscopy it was interpreted that the The IR spectrum exhibited a broad absorption band between 3600–3450 cm^{-1}, indicative of O–H and N–H stretching vibrations, which confirms the presence of hydroxyl and amide functionalities. Additional bands observed in the 2600–2300 cm^{-1} region likely represent combination bands or overtones, while a distinct absorption at 2077 cm^{-1} suggests triple-bond stretching or overtone phenomena. Notably, the peaks at 1589 cm^{-1} and 1316 cm^{-1} were attributed to Amide II and Amide III vibrations, respectively, that strongly supports the presence of aliphatic primary amide groups in the distillate (Spectrum.4 & Table 2), identified by in-built library matching of the spectral datas.

4.5. Volatile oil content in Mint distillate

Clavenger apparatus is used for isolation of volatile oil by hydrodistillation method. The percentage yield of volatile oil in mint distillate estimated by calculating the amount of volatile oil (ml) collected in the receiver tube of clavenger apparatus with reference to the amount of mentha leaves (gms) taken for soaking.[13] The amount of volatile oil or essential oil content present in mint distillate was 1.63% (Figure 6).

4.6. Characterization of methanolic content by Gas Chromatography

Hence Mint distillate was prepared by overnight soaking Mentha leaves in distilled water, the absence or presence of trace amounts of methanolic content was needed to ensure as per regulatory guidelines to qualitatively check the in-situ methanolic production during overnight soaking of mint leaves in water due to fermentation, which was done by Gas chromatography studies as per the method mentioned in IP (Indian Pharmacopoeia, 2010). (Chromatogram.1) showed the presence of standard methanolic peak at retention time 5.47 mins with 100% peak area. Whereas (Chromatogram.2) showed the absence of methanolic peaks in the same retention time. From Gas chromatography studies it was observed that the mint distillate was free of methanolic content which is safe and devoid of toxicity due to *in-situ* methanol production during overnight soaking process in the preparation of mint distillate.

4.7. **Effect of herbal medicine Mint distillate on HepG2 cell line**

HepG2 and Hep3B are the cellular modals for Invitro liver toxicity experimental studies in new drug development, because of their characteristic drug metabolism potential.[33] When compared to Hep3B, HepG2 express the majority of drug metabolism enzymes, have varied chemosensitivity to therapeutic drugs and also due its diverse effects on cell cycle progression induced by different treatments. HepG2 human hepatoma cell line is widely used in drug metabolism, toxicity and liver function studies.[31] It is the alternative to human hepatocytes for *in-vitro* studies of normal hepatic cells because of aforesaid characteristic features and hence HepG2 cell line was selected for screening hepatoprotective efficacy of Mint distillate.

Exposure of Isoniazid to hepatocytes results in induction of hepatocyte apoptosis due to disruption of mitochondrial membrane potential and DNA strands break. It also causes macromolecular damage in hepatic cells. During metabolism by hepatic cells, Isoniazid is acetylated, converted into toxic acetyl isoniazid, oxidized into toxic hydroxylamine and hydrolysed into toxic hydrazine and these toxic metabolites induce toxicity to hepatic cells.[32] Hence, isoniazid (70mM concentration) was used as toxicity inducing agent against HepG2 cell line to screen the efficacy of mint distillate for its hepatoprotective action by MTT assay.

The exposed HepG2 cells to Isoniazid are treated with Mint distillate and standard drug in the concentration of 6.25µg/ml, 12.5µg/ml, 25µg/ml and 50µg/ml respectively. It was observed that mint distillate at 50µg/ml concentration showed maximum protective effect with 82.41% in presence of hepatotoxic agent Isoniazid whereas the standard drug Silymarin showed 86.98% of cytoprotective effect on HepG2 cell line at 50µg/ml concentration (Table.3, Graph.2, Figure.7&8). The median cytoprotective concentration observed for mint distillate is 4.01 µg/ml whereas for the standard drug silymarin the median cytoprotective effect found to be 2.02 µg/ml. The cytoprotective effect observed for the test drug (Mint distillate) and Silymarin was found to be in dose dependent manner for its hepatoprotective action screened by MTT assay.

5. Conclusion

Mint Distillate was prepared by laboratory process by distilling overnight soaked leaves of *Mentha spicata* in water. The prepared Mint distillate was subjected for physical property analysis, thermal analysis to determine the ideal temperature of distillation process and from thermal analysis it was understood that the distillation temperature should not exceed 57ºC to avoid thermal degradation of phytomolecules in mint distillate responsible for therapeutic efficacy. From the dielectric spectroscopic studies, it was observed that the mint distillate is having high dielectric permittivity values with no electric conductivity having insulative effect. Presence of Aliphatic Primary Amide group in Mint distillate was confirmed in IR spectroscopy studies and the IR and Raman fingerprints will serve as a reference spectral data's that provide the authenticity of the prepared herbal Mint distillate. The essential oil content in mint distillate was found to be 1.63%v/w and absence of toxic in-situ methanolic content was confirmed by Gas chromatography studies. Mint distillate on HepG2 cell line studies, the prepared herbal medicine showed 82.41% of cytoprotective effect at 50µg/ml in dose dependent manner in isoniazid induced toxicity in HepG2 cell line similar to the standard drug Silymarin. The median cytoprotective concentration was found to be 4.01µg/ml for the prepared Mint distillate whereas for standard drug silymarin the median cytoprotective effect was found to be 2.02 µg/ml. Mint distillate exhibited remarkable hepatoprotective action which has been scientifically validated by MTT assay.

ABBREVIATION

DSC: Differential scanning calorimetry, TGA: Thermal Gravimetric Analysis, FTIR: Fourier Transform Infra-Red Spectroscopy, GC: Gas Chromatography, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, HepG2: human hepatoblastoma cell line, DMSO: Dimethyl Sulfoxide.

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