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Lipid lowering effect of the nanoencapsulated essential oil of the leaves of *Myristica fragrans* Houtt.- In hyperlipidemia without cardio vascular complications

[1] Geethanjali M*, [2] Gazala Parveen, [3] Periyanayagam K and [4] Ramasamy.

[1] Assistant professor, Department of Pharmacognosy, Dr. M.G.R. Educational and Research Institute (Deemed to be University), Tamilnadu, India.

[2] Professor and Head, Department of Pharmacognosy, Sunrise University, Bagad Rajput Alwar, Rajasthan, India.

[3] Professor and Head (Retd), Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai, Tamilnadu, India.

[4] Assistant Professor, Department of Pharmacognosy, Arulmigu Kalasalingam College of Pharmacy, Srivilliputhur, Tamilnadu, India.

* **Corresponding Author:** E-Mail**:** mani.anjali93@gmail.com

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ABSTRACT

This study aims to evaluate the effect of the *in vivo* effect ofnanoencapsulated essential oil of *Myristica fragrans* leaves (Family: Myristicaceae) on the hyperlipidemic zebrfish larvae and its cardio vascular system.Initially the various constituents of the isolated essential oil of the leaves was determined by GC-MS. It was encapsulated with ethyl cellulose by solvent displacement technique. The**3R's** ethical principle (**R**eduction, **R**efinement, **R**eplacement) was implemented that help to minimize harms to vertebrate animals used in science. Larval toxicity study was carried out on5 dpf zebrafish (zf) larva cultured in E3 medium. Preliminary toxicological studies on embryo and larvae of zf (*Danio rerio*) is performed to evaluate toxicity and hazards to early life stages and embryo development. Assessment of its effect on the transparent hyperlipidaemic zf larvae by quantitative image analysis using Integrated Optical Density (IOD) after the determination of maximum non-lethal concentration (MNLC) and its effect cardiovascular system by cardiac function assessment(cardiac morphology,heart rate, blood circulation etc.).GC-MS study showed the percentage of sabinene(16.1),Eugenol (15.3),methyl eugenol (1.2) myristicin (8.3) Caryophylene (7.3) β myrcene (5.1)α,β pinene,D limonene,safrole (1.6) and traces of some monoterpenes. From the experiment it was observed that there was no mortality in 0.5 and 0.75µl/ml concentrations. But 5% and 10% mortality was observed at 1 and 2 µl/ml concentration respectively. No mortality was observed in both the control viz DMSO and embryonic medium.. Nano encapsulated essential oil of *Myristica fragrans*(NEEOMF) significantly decrease lipid levels dose dependently ($p<0.001$) when compared to lipid lowering drug ezitemibe. There was no CVS complications were observed. So it is concluded that formulation can be developed to inhibit lipid absorption with novel mechanism using the essential oil of the leaf.

Keywords: *Myristica fragrans; Zebrafish larvae; lipid lowering effect; Nanoencapsulation.*

1. INTRODUCTION

Bioactive compounds of plant origin especially essential oils have received great scientific attention in the recent decades as novel therapeutic agents that could aid the healing process owing to their antioxidant, anti-inflammatory and anti-microbial effects. Nutmeg, Myristica fragrans Houtt. Family Myristicaceae is an evergreen tree indigenous to the Maluku Islands of Indonesia, is extensively distributed to

Grenada, India, Mauritius, Sri Lanka, South Africa, and USA. In traditional Indian medicine, nutmeg has been used to treat indigestion, diarrhoea, parasites, plague, rheumatism paralysis, and other illness [1]. Several scientific reports say that nutmeg has potential antioxidant, antibacterial, antifungal, anti-inflammatory, antiulcerogenic, anticancer, aphrodisiac, and several other activities [2-5].Though there is traditional and experimental evidences to support various claims and benefits of this plant, still it needs proper evaluation and exploitation.

Hyperlipidaemia is an abnormally elevated level of lipids/or lipoproteins in the blood which is the most common form of dyslipidaemia and is the key risk factor for cardio vascular diseases and stroke. Now diseases associated with it is rapidly increasing. Experimental studies often use mice, rabbit and hamster fed high fat diets to induce hyperlipidaemia and lipid deposition in the blood vessels. Mammalian hyperlipidaemia models are consume more time, laborious and expensive [6]. *Invitro* cell culture models used for screening lack organ structures and extrapolation of its results to the whole organism is often challenging. Hence, *invivo* animal model with a detail analysis of lipid metabolism would be valuable for lipid metabolism and for lipid lowering drug screening. The three **Rs**" (**R**eduction, **R**efinement, **R**eplacement) of ethical principle is implemented that help to minimize, harms to animals used in science. In this study we used zebrafish larvae which is an emerging novel preclinical *invivo* model that support rapid decision making in the early phases of drug discovery process and amenable to high throughput screening (HTS) with numerous advantages. *Danio rerio* (Zebrafish) is a model organism for investigating *invivo* assessment of drug efficacy toxicity, safety [7]. The merit of this model is morphological and molecular basis of tissue and organs is identical or similar to other vertebrates including humans. Many important genes' sequence and presumed function of vertebrates are conserved in the zebrafish. [8]

Since most of the research works are carried out using other parts of *M.fragrans* and the leaves were not much concentrated on research work. So we have studied leaves especially its volatile. The essential oil of the leaves and mace of *M. fragrans* reported to contain myristicin,sabinene, eugenol,limonene [1]. Though there are traditional and experimental evidences to support various claims and benefits of this plant still it needs proper evaluation and exploitation. Hence we planned to identify the various components present in the isolated essential oil of the leaves of *M.fragrans.* Further we aim to study its effect on hyperlipidaemic zebrafish larvae in nanoencapsulated formulation.

2. MATERIALS AND METHODS

2.1. Collection and Authentication of the Plant

Leaves of the plant *M.fragrans* selected for our study was collected from elappara Idukki District, Kerala, India during the month of July 2018 and was authenticated by Dr.Stephen, Department of Botany, American college, Madurai 625 002.

2.2. Isolation ofthe leaf essential oil

The shadow dried leaf powder was sieved in a no.60 sieve and the volatile is extracted by using Clevenger apparatus by hydrodistillation method.The volatile oils stored in well-closed completely filled containers and away from light in refrigerator.

2.3. GC-MS analysis

JEOL GC MATE 11 model GC-MS was used, front inlet temp : 220°C, Column : HP 5 Ms Helium gas,flow rate 1ml/min, Mass analyser , detector photon multiplier tube, Quadrupole with double focusing mass analyser.

2.4. Preparation of Nanoencapsulated Essential Oil

100mg of ethyl cellulose was dissolved in 8ml of DMSO using a magnetic stirrer. 5µl of *M.fragrans* leaf VO was dissolved in 1ml of DMSO. Then both the Ethyl cellulose solution and VO solution were mixed together. Then the solution were added drop wise to 30ml of distilled water using a syringe with constant stirring with the help of magnetic stirrer. The formation of nanoparticles was observed using a stereomicroscope. [9-11]

2.5. Measurement of Nanoparticles

Scanning Electron Microscope SEM is a method for high-resolution imaging of surface. The sample was placed on a glass slide (1×1cm), after rinsing the slide with ethanol. A drop of nanoparticles was evenly distributed over the glass slide and allowed to dry in air. The NPs were subjected to SEM analysis under ambient conditions [9,12].

2.6. Preliminary Toxicological Studies Of NEEOMF leaves On The Embryo And Larvae Of Zebrafish

Toxicology through intensive studies has traditionally focused on the effects of chemicals on living organisms which was done by one chemical at a time. Such approaches show the mode of action of many chemicals and provide a detailed mechanistic understanding of the molecular target of toxicity for some as the cost of this approach is high. Toxicology studies rely on the utility of vertebrate animals which is an expensive

undertaking in both time and cost with debatable predictive power in case of safety aspects for human. To streamline the drug development timeline prioritize drug candidates for animal testing and reduce an unnecessary cost for mammalians studies, drug screening assay using zebrafish is becoming increasingly popular [13].

2.6.1. Zebrafish Larvae Handling

An average of 250-300 embryos from 2-3 pairs of zebrafish after nature matting. Embryos were maintained at 28°C in E3 medium. Embryos hatch from the chorion at around 48hour post fertilisation (hpf) and the larvae are incubated at 28°C. By 5-6dpf the yolk has been depleted and larvae must now eat to acquire nutrients(Fig -1)

2.6.2. Whole Embryo Culture Toxicity Study

Materials

Fertile eggs, E3 embryo medium (standard medium), Dimethyl Sulphoxide (DMSO), glass petridishes, research microscope (laboscope microscope with microphotography), incubator, micropipette, NEVOMFand standard podophyllotoxin.

Collection of eggs

Eggs were collected from natural spawning and reared in embryo medium at pH 7.2, and kept in an incubator at 28±0.5°C for our assay. The developmental stage of the embryos was determined using microscope. (Hisaoka, KK and Battle, HI 1958).

At around 2– 4 h postfertilization, only the fertilized eggs (blastula stage) were selected. The fertilized eggs were collected and rinsed several times with tap water.

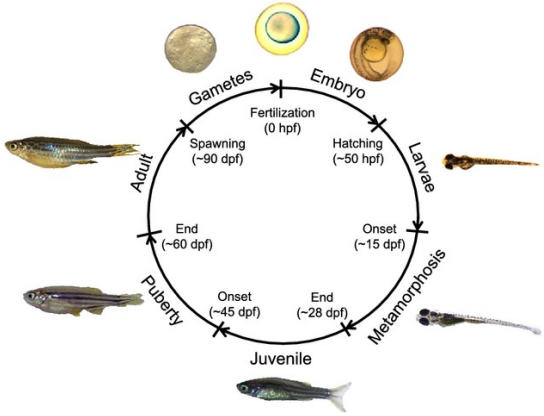


Figure -1

2.6.3. Experimental design

The eggs were transferred to each of the glass petri-dishes (3 per dish) containing different concentrations of NEVOMF (0.5, 0.75, 1 and 2 µg/ml) dissolved in 1% DMSO at 28°C as well as DMSO control . Embryo medium served as the over-all control .Standard podophyllotoxin of concentration 10 µg/ml was taken as positive control. Occasional stirring was done to ensure even distribution of the chemical. The maximal acceptable toxicant concentration (MATC) was calculated according to (Dave, G *et al.,* 1987) by scoring the malformations. The development of blastula eggs was monitored at specified time points (12, 36, 60 & 80 hrs) under microscope. Endpoints used for assessing the effect of drug during the major organ is visible included edema, eye malformation, bent tail, undulated notochord, twisted notochord and death. Malformations were also noted and described among the juveniles from the control 1% DMSO treated and standard podophyllotoxin.

2.6.4. Larval Toxicity Study

Materials

Zf larvae of 5dpf, E3 embryo media (standard medium), 1% DMSO, glass petridishes, research microscope (laboscope microscope with microphotography), incubator, micropipette, NEEOMFand standard podophyllotoxin.

Experimental design

Healthy 5dpf zf larvae were selected and used for larval toxicity study. About 5 larvae were released in the embryonic medium (10 ml) taken in a petridish, in triplicate. Various concentration of NEEOMF (0.5 and 0.75, 1 and 2 mg/ml) dissolved in 1% DMSO in the embryonic medium were tested. Two controls were used DMSO and embryonic medium respectively. Podophyllotoxin (10 µg/ml) was used as standard toxin (graph1).

Observation of Feeding Habit

5dpf zf larvae was fed with yolkmass in a petridish. After allowing time for its absorption (2-3 hrs), larvae from the petridish was collected and examined under research microscope to see the presence of intestinal yolkmass. Yolkmass within the intestinal lumen was detected and the expulsion of unabsorbed food material was also seen, which shows that the nutrient has been absorbed in the intestine and reaches the blood circulation.

2.7. Assessment of Effect Of NEEOMF On Zebrafish Blood Lipids

Even though lipids play a vital role in the cellular function, it is not surprising that defects in lipid metabolism underlie many human diseases. More than a third of adults and 17% of children are currently classified as obese. This study was focussed on the inhibition of absorption of dietary lipid in the intestine of zf that provides an *invivo* environment as well as the potential for high throughput drug screening.

Zebrafish larvae hyperlipidemia model was validated for pharmaceutical testing using 5marked human lipid lowering drugs silverstatin, lovastatin (HMG-CoA reductase inhibitors), ezetimibe (the Niemann-Pick C1 like 1 [NPC1L1] protein cholesterol transporter blocker), bezafibrate (peroxisome proliferator-activated receptor[PPAR-a]agonists) and hydrodesoxycholic acid (inhibiting bile acid formation and dissolving fat) were tested in this model and reported that all the drugs significantly reduce lipid level in a dose dependent mannar after 24h and 48h treatment indicating that zebrafish hyperlipidemia model is suitable for *invivo* screening and assessment of hypolipidemic drug with different mechanisms. When drugs and food are provided simultaneously, a major confounding variable is the impact of a drug on feeding behaviour such that additional assays are required to determine the amount eaten in each treated group. This method has the advantage that it does not require such assays since feeding is performed prior to drug treatment. zebrafish larvae were fed with egg yolk for 48hr, followed by drug treatment for 24hr and 48hr at 3 concentrations. At the end of treatment, ORO was used to stain the lipids of Zf larvae. Fifteen to twenty zebrafish larvae from each group were randomly chose for ORO image acquisition. Zebrafish larvae were immobilized in 3% methyl cellulose and images were acquired in the identical lighting intensity under light microscope installed with a high speed video camera linked with computer system. When viewed dorso-laterally, the blood vessel of an 8 or 9 dpf Zebrafish larvae was situated posterior to cloacal pore and predominantly anterior to the tail fin. Quantitative image analysis of ORO was performed and Integrated Option Density (IOD) data were expressed as mean ±SEM. The effect of test drug EELA was calculated based on the following formula.

Drug effect on lipids lowering (%) = (1-IOD compound/IOD vehicle) X 100.

A positive percentage means that the tested drug could reduce lipids level and a negative percentage had no lipid lowering effect in this model.

2.8. Effect of NEEOMF on the CVS (Heart Rate, Cardiac Morphology And Blood Circulation) of Zebrafish Larvae

Cardiac function assessment

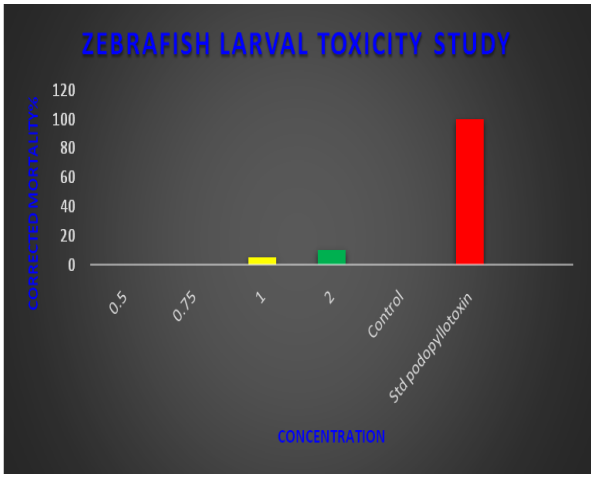
Hyperlipidemia zebrafish larvae of 5dpf were placed in 96 well plates, at 1 larva per well which different concentration of NEEOMF (0.5, 0.75, 1 and 2mg/ml) (test). The larvae fed with normal diet (paramecia) in embryonic medium serve as the control. The cardiac function assessment was performed for normal diet and yolk mass fed larvae. All zf larvae were exposed for 3 hrs. Then they were immobilized in methylcellulose (3% w/v) and the heart rate per minute for each larva was recorded by eye under a research microscope. Cardiac morphology and blood circulation were observed. The test was performed in duplicate.

2.9. STATISTICS

One-way ANOVA was used for quantitative analysis, all data were presented as mean± SEM, and results were statistically compared between drug-treated and vehicle-treated zebrafish groups.

3. RESULTS

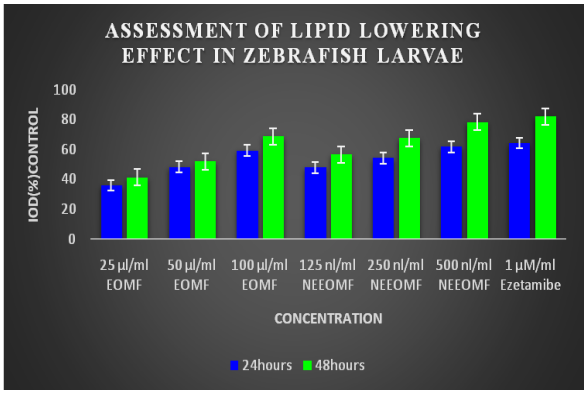
Pale yellow colour essential oil was obtained. (1.1%) GC-MS showed the presence ofsabinene(16.1),eugenol (15.3),methyl eugenol (1.2) myristicin (8.3) Caryophylene (7.3) β myrcene (5.1)α,β pinene,(1.01) D limonene (1.7), safrole (1.6) and traces of some monoterpenes. Traces of Cubenol, 1-methyl-4-(1-ethylethylidene,4,6-Octadienoic acid are present. SEM examination of prepared NEEOMF was found to be 142nm(Diameter),54.1(width). From the toxicity study it was observed that there was no mortality in 0.5 and 0.75µl/ml concentrations. But 5% and 10% mortality was observed at 1 and 2 µl/ml concentration respectively. No mortality was observed in both the control viz DMSO and embryonic medium. 100% mortality was observed in the standard podophyllitoxin at 0.5µg/ml concentration (Graph 1).



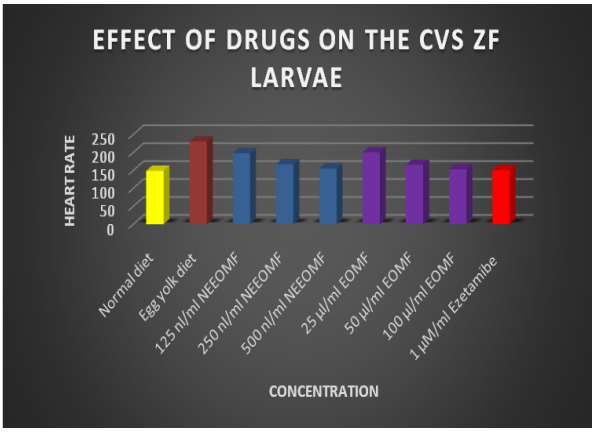
Graph – 1: Zebrafish larval toxicity study

When developing zebrafish hyperlipidemia model for lipid lowering drug screening and efficacy zf appearance and clearance time of stain up lipid were determined initially 5dpf zf larvae were fed with 0.1% egg yolk and ORO was stained for imaging under a dissecting stereomicroscopy at 24h and 48h of feeding followed by quantitative analysis it was found that the lipids in zebrafish gut and vasculature increased with time that was

consistent with the previous report, that indicated that a time dependent increase in whole larval triacylglycerol content was correlated with the increased level of ORO staining. At 48h of feeding engorged uniformly. From these results feeding for 48h was chosen for experiments. After removing egg yolk for 24h and 48h the lipids of blood and gut vessel were abundant" and the lipids of blood and gut vessels were scarcely any until 96h after removing egg yolk. Hence drug treatment for 24 and 48h were selected for quantify drug efficacy. We treated with hyperlipidemia zf larvae with25,50, 100µl/ml of EOMF, 125, 250, 500 nl/ml of NEEOMF and 1µM/ml ezetamibe which has been shown to reduce lipids humans and in mammal models. After 24h treatment the tested and standard drug significantly reduce hyperlipidemia zf larvae lipid levels in the blood by 35.9.1±0.16, 48.2±0.93, 59.2±0.72, 47.8±0.88, 54.1±0.9, 61.7±0.73, 64.2±0.12respectively after treatment for 48h effect on lipid lowering were 41.2±1.3, 51.8±1.02, 68.6±1.07, 56.6±1.02, 67.5±0.18, 78.3±0.25, 81.9±1.2 respectively the results were statistically significant ($p<0.001$) (Graph 2).



Graph – 2: Assessment of lipid lowering effect in zebrafish larvae.



Graph - 3: Effect of drugs on the CVS zebrafish larvae.

5dpf larvae were fed with lipid rich diet containing 25, 50,100 µl/ml of EOMF, 125, 250, 500 nl/ml of NEEOMF, larvae fed with normal diet (paramecia) was also observed. From the result it was observed that there was increase in heart rate after a lipid rich diet in the zf larvae (230.8±2.1) compared with the normal diet fed larvae (149.1±1.9) moderately elevated heart rate (198.3±1.6, 200.2±2.4) was observed in the zf treated with 125nl/ml NEEOMF, 25µl/ml EOMF in 1% yolk mass. But dose dependent decrease in elevated heart rate were observed in the 50, 100µl/ml EOMF, 250, 500 nl/ml of NEEOMF and 1 µM/ml ezetamibein yolk mass (Graph 3).

4. DISCUSSION

It was reported that Myristicacae family members have phytoconstituents useful in the treatment of various diseases and was also claimed that these plants merit detailed study which can prove useful in the discovery of lead compounds leading to novel and more efficacious drugs. Leaves of M.fragrans of this family, is traditionally known to be useful for the treatment of wide panel of diseases. As reported earlier several reports have been published on the effects of the plant extract and chemical constituents on different biological activities *in vitro* and *in vivo*.

We performed larval toxicity assay after hatching using 5dpf, larvae was odserved that 0.5, 0.75µl/ml concentration showed no mortality and 5%, 10% mortality at 10µl/ml concentration. This study showed no significant mortality or malformation in zf larvae at 24h exposure at normal concentration but showed moderate toxicity in higher concentration so this evaluation no embryo toxicity or larval toxicity and toxicity was dose dependent.

Our investigation was initiated with the objective of developing an ideal model for hyperlipidemia that would closely reflect the natural metabolic characteristic of human hyperlipidemia and respond to the pharmacological treatment. It was reported that in zf lipids are mostly stored as TAGs (triacylglycerols) in visceral, intramuscular and subcutaneous adipocyte depots but less in blood vessels as main storage sites. The zf larvae are small, translucent enabling non-invasive visualization of internal organs in vivo in high resolution under microscope. It was observed that the lipids of gut and blood vessel increased with feeding time and blood lipids engorge uniformly at 48 h of feeding. In our model NEEOMF leaves significantly decreased zf larval lipid levels dose dependently ($p<0.001$) both after treatment for 24h and 48 h. Our results support the hypolipidemic effects of the NEEOMF. In addition, the cardiac function assessment was performed for normal diet and yolk mass fed larvae. All zf larvae were exposed for 3 hrs and the heart rate

per minute for each larva was recorded by eye under a research microscope. Cardiac morphology and blood circulation were observed. It was found that all cardiac functions were normal, thus showing that NEEOMF of the leaves has not affect the cardiac function.

5. CONCLUSION

It will be imperative to develop a new model of modern pharmacology based on traditional pharmacognosy. Nevertheless we have developed a new model of modern pharmacology based on traditional Pharmacognosy. The NEEOMF can be considered as a cocktail of phytoconstituents that act on multiple pathways that initiate and maintain lipid lowering activity. NEEOMF significantly decrease lipid levels dose dependently ($p<0.001$) when compared to human lipid lowering drug ezitemibe. So it is concluded that the lead compounds can be developed to inhibit lipid absorption with novel mechanism. There was no CVS complications were observed. So it can be concluded that NEEOMF of the leaves lowers lipid levels without affecting the cardiac function of the zebrafish larvae in the tested concentration without toxicity. Further investigation is suggested using animal models and clinical trials.

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