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Inhibitory Potential of *Ferula assafoetida* Extract on L-type Calcium Channel Protein Revealed by Zebrafish Studies and Molecular Docking

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Ferula assafoetida is a part of many herbal formulations and hence it is pertinent to check the safety of its components specially to growing embryos. Zebrafish (*Danio rerio*) is considered to be one of the best models to study human embryonic development and metabolic pathways as its genome is fully sequenced and it possesses easily detectable developmental properties. In present study, the embryos of *Danio rerio* were treated with different concentrations of methanolic extract of *Ferula assafoetida* (MEFA) and its effects were checked at different post fertilization periods. Decreased heart beat rates, shrinkage of the chorion wall and other developmental abnormalities leading to the death of the embryos were observed. The methanolic extract of *Ferula assafoetida* was subjected to GC-MS to determine the different compounds present. Cardiotoxicity of these compounds were studied as it is one of the important factors for the retraction of a drug from the market. Molecular docking studies with L-type calcium channel (LTCC), a protein important for cardiac functioning, showed strong binding to the phytochemicals in the extract, with the maximum binding affinity observed with 26-hydroxycholesterol. The study proves that the methanolic extract of *Ferula assafoetida* contains phytochemicals which have the potential to cause cardiotoxicity in zebrafish embryos by interfering with the functions of LTCC possibly leading to arrhythmia. Altogether, our data suggest that the usage of these extracts in drug formulations should be done with caution. This is also indicative of the possible cytotoxic effect of the extract which could be tapped in the search for anticancer drugs.

Keywords: Embryotoxicity, *Ferula assafoetida,* Zebrafish, 26-Hydroxycholesterol, Cardiotoxic.

INTRODUCTION

The use of herbal extracts or its components directly or as part of other drug formulations has been on the rise in the past few decades. Though many medicinal plants are being increasingly used to extract components for treating various metabolic disorders and microbial infections, it is imperative that toxicological studies be done on these extracts to arrive at safe concentrations for drug formulations. In this context, the zebrafish model has emerged as a popular tool to perform toxicological studies owing to its extremely rapid embryonic development and the transparent nature of embryos [1]. A high resemblance of the developmental stages of embryos of zebrafish to that of higher vertebrates facilitates the process [2]. Preclinical embryotoxicity evaluation has emerged as an important methodology primarily to eliminate the side effects of drugs in clinical trials.

Ferula assafoetida is known for its medicinal properties from time immemorial. This small leguminous plant, a native of Afghanistan has been used both for culinary and medicinal purposes. It has been found to have antioxidant [3] and anti-convulsive [4] properties and also has good potential to treat Alzheimers disease [5]. About 180 different phytochemicals are responsible for the varied medicinal properties of members of this genus Ferula and one such is Ferutinin with good anti-proliferative effect [6]. Though the cytotoxic property of Ferula can be made use of in cancer therapy, one should arrive at the correct dose dependent *in vivo* effects of these compounds before inducting into treatment regimens for specific ailments. Studies have indicated that the plant also has cardioprotective effects at low concentrations and cardiotoxic effects at high concentrations [7]. Hence, before using for large scale herbal medicine manufacture, it is important to check the embryotoxic activity of the extracts. Studies done on zebrafish embryonic

development can be achieved much faster owing to their rapid development cycle. For example, the neural plate formation in zebrafish occurs at 10 h post fertilization, followed by organogenesis at 24 h post fertilization. The first heartbeat occurs at 30 h post fertilization in the zebrafish when compared to 10.2 days in rats [8]. Also, over 90% of the human open reading frames (ORFs) are homologous to genes in zebrafish [9] hinting the possibility of many conserved pathways in these organisms. The objective of the present study was to check the embryotoxic property of the methanolic extract of *F. assafoetida* (MEFA) by measuring its effect on hatchability, heartbeat rate and other growth parameters of the embryo of *Danio rerio* and also to find out role of potential phytochemicals involved in this by GC-MS and molecular docking studies.

EXPERIMENTAL

Soxhlet extraction: The seeds of *F. assafoetida* were procured from a local market in Bengaluru, India. These seeds were thoroughly washed with tap water, dried in shade, powdered using a grinder and stored in a sealed container at room temperature until use. The seed extract was prepared by performing 9 cycles of Soxhlet extraction by mixing 50 g of seed powder with 200 mL of methanol. The extracted material was stored in screw cap glass vials and refrigerated until use. 0.1 g of the plant extract was weighed and a few drops of dimethyl sulphoxide (DMSO). The volume was made up to 10 mL by adding distilled water so as to get a final concentration.

Maintenance of zebrafishes: Zebrafishes were bought from a local aquarium in Bengaluru, India and maintained at 28 ºC. A continuous circulating aerated and filtered water system was maintained for the zebrafish, which helped the elimination of the food remnants and excreta. The water used to maintain the zebra fishes was dechlorinated, which was achieved by ageing the water for 48 h. The pH was maintained between 6.8 to 7.5.

Breeding of zebrafishes and collection of embryos: Zebrafish mostly breeds at the onset of the light. The breeding tank was assembled and was placed inside the main tank. Two females and one male were transferred inside the breeding tank using a net and was kept overnight. Mating was allowed to occur undisturbed in the morning until the required numbers of embryos were laid. The eggs were collected using a strainer, which are found laid at the bottom of the fish tank. The eggs laid at the bottom of the fish tank were then washed thoroughly with distilled water. The embryos were then transferred to a Petri dish with embryo water which comprises of 10% Hank's solution with full strength calcium and magnesium with a pH of 7.2. Embryos were then observed under a microscope. Fertilized eggs and unfertilized eggs were separated using a pipette.

Treatment of zebrafish embryos with plant extract: Zebrafish embryo toxicity tests were carried out in accordance with Organization for Economic Cooperation and Development [10]. Four healthy eggs each were randomly taken into 3 polystyrene boxes containing 2 mL of embryo water and treated with methanolic extract of *Ferula assafoetida* (MEFA) at a concentration of 4 mg mL^{-1}. This was repeated with plant extract concentrations of 2, 1, 0.5, 0.4, 0.2, 0.1, 0.05, 0.04, 0.02, 0.01 and 0.005 mg mL^{-1}. Three boxes were kept as control which had 2 mL of embryo water only. These boxes with the zebrafish embryos were maintained for 72 h at 28.5 ºC, to check for the toxic effect of the plant extract on the embryos. Parameters like hatchability rate, heartbeat rate and other growth parameters were measured and recorded for the different treatment sets of embryos.

GC-MS analysis: GC-MS analysis was conducted on MEFA to know the details of the compounds present in this fraction. A sample (1 mL) was used for GC-MS analysis as previously described [11]. The column used was DB5MS and V5MS and the gas used was helium with a flow rate of 0.8 to 1.0 mL min^{-1}. GC-MS headspace was Perkin-Elmer Model (Clarus 680 Serial No: 680s10032401).

Molecular docking: The 3D structure of Cav1.2 protein (LTCC) of *Danio rerio* was not available and hence the homologous protein structure of *Oryctolagus cuniculus* (PDB ID:5gjv) with voltage dependent calcium channel subunits of *Danio rerio* was used for this study [12]. In this study, Autodock 4.2 [13], Autodock Vina [14] and PyMol were used to understand the protein-ligand binding sites, where Autodock and Autodock Vina are molecular docking tools and PyMol a molecular visualization platform. Protein structures were prepared using Autodock tools. Water molecules were removed from the protein structure as prior preparation. Polar hydrogen atoms were added to the protein. Protein structure was finally optimized by adding Kollman charges. The 3D structure of the ligands used in this study were retrieved from PubChem. All compounds were checked for the Lipinski's rule of five. The 3D structure of the ligands was converted into PDBQT format using Auto dock tools after the addition of gasteiger charges.

Autodock tools were used to develop the grid box with a spacing of 3.75 nm and grid points in X, Y and Z axis were 60 × 60 × 60. The grid center coordinates were placed as X = 164.168, Y = 170.721, Z = 175.341. The grid box was placed at the binding site of the protein which gives sufficient space for the ligand rotation. Autodock Vina was used for the docking process by bringing both the protein and ligand together in the binding site. Docking scores of the best poses docked into target protein were calculated. Docking poses of the target protein and ligand were visualized and imaged using PyMol.

RESULTS AND DISCUSSION

Effect of MEFA on embryonic development: Embryotoxicity studies were conducted on zebrafish using methanolic extract of *Ferula assafoetida* and observed at 24, 48 and 72 h for morphological changes. The embryos collected from the breeding tank of *Danio rerio*, were incubated and exposed to different concentrations of MEFA in their gastrula period at the stage called the Germ-ring stage where the embryos were approximately 5.7 h old. 100% mortality of zebrafish embryos were observed from concentrations starting from 0.2 mg mL^{-1} within 24 h of incubation. Survival rate after 72 h was only 25%, 50% and 75% at concentrations of 0.05, 0.01 and 0.005, respectively (Table-1). Embryos exposed to extracts of 0.1 mg mL^{-1} and below concentrations showed no mortality within

TABLE-1
DEVELOPMENTAL PATTERNS OF THE ZEBRAFISH EMBRYOS WHEN EXPOSED TO VARYING CONCENTRATIONS OF METHANOLIC EXTRACT OF *Ferula assafoetida* (MEFA)

MEFA (mg/mL)	24 h	48 h	72 h
4, 2 and 1	All died within 1h of incubation.	–	–
0.5, 0.4 and 0.2	All died overnight.	–	–
0.1	Stagnant growth- remained in the germ-ring at which we incubated.	Grown up to 21-26 somite stage (24 h)	All coagulated to death.
0.05	50% of the embryos had grown to 21-26 somite stage, 50% in germ-ring stage.	Slow growth rate. Had only grown up to prim-16 stage (31 h).	25% hatched. Rest were still in the prim-16 stage.
0.04	Stagnant growth- remained in the germ-ring stage at which incubated.	Slow growth rate. Had only grown up to prim-16 stage (31 h).	25% hatched,which grew up to the long-pec stage (48 h).
0.02	Stagnant growth, *i.e*., the germ-ring stage at which incubated.	Slow growth rate. Had only grown up to prim-16 stage (31 h).	50% hatched, which grew up to pec-fin stage (60 h).
0.01	100% of them had normal growth, *i.e*., till 21-26 somite stage.	Slow growth rate. Had only grown up to prim-22 stage (31 h).	50% hatched, which grew up to pec-fin stage (60 h).
0.005	100% of them had normal growth, *i.e*, till 21-26 somite stage.	Slow growth rate. Has only grown up to prim-22 stage (31 h).	75% hatched, which almost resembled protruding mouth stage (72 h).
Control	Grew up to the 21-26 somite cell stage	Hatched to grow up to long-pec stage.	Grew up to protruding-mouth stage.

24 h of incubation. The zebrafish embryo was considered as dead if it showed any one parameter of lethality, *i.e.* coagulation, non-detachment of the tail, somite disruption and lack of heartbeat. In zebrafish, early stages of development were found to be very sensitive to the environmental factors due to rapid changes in cellular differentiation, migration and tissue differentiation [15]. So, exposure to harmful substances may result in significant anomalies in the development of the embryo. Apparently, mortality was more evident with increasing concentration of the extract with all embryos treated with more than 0.1 mg mL^{-1} concentration of the extract dying within 24 h of exposure. The increase in the damage done to the embryos at increasing levels of the extract concentration is proof for the abortifacient activity of *F. assafoetida*.

Effect of MEFA on hatchability: Analysis of mortality rates of embryos showed that the rates were directly proportional to the concentration of the extract. Hatching was completed at 48 h post treatment exposure in control embryos. No hatching was observed in embryos exposed to different concentrations of the plant extract within 48 h of incubation. After 72 h of incubation, the hatchability rate was found to be reduced to 75% in embryos treated with 0.01 mg mL^{-1} extract and 25% in embryos treated with 0.05 mg mL^{-1} extract. Above this concentration, none of the embryos hatched even after longer hours of incubation (Table-1). A zebrafish embryo is considered as hatched when the entire body of larvae from tail to head is out of the chorion. The delayed hatching rates of the extract treated embryos strongly indicates growth retardation possibly due to interactions of the phytochemicals in various pathways of embryonic development. In a similar study, using aqueous extracts of *Derris elliptica*, progressive reduction in hatchability of zebrafish was observed with increase in the concentration of the extract [2]. Upon exposure of aqueous extract of *Momordica charantia* to zebrafish, the LC_{50} value was found to be about 150 µg/mL. Scoliosis of zebrafish larvae was also seen in higher concentrations [16]. *F. assafoetida* has been implicated as an antifertility agent in preventing pre-coitus pregnancy in Sprague-Dawley mice [17,18] and also as an abortifacient agent [19].

Effect of MEFA on the heartbeat rate of embryos: In this study, heartbeat rates of embryos were significantly affected by different concentrations of the extract. The embryos of the control group recorded the highest heartbeat rate of 186 beats per min (bpm) 72 h after fertilization. As the extract concentration increased the heartbeat rate decreased progressively - 180, 174, 162, 156 and 150 bpm for the embryos treated with extracts of 0.005, 0.01, 0.02, 0.04 and 0.05 mg mL^{-1}, respectively. No heartbeat rate was noted in embryos treated with extracts of concentrations above 0.1 mg mL^{-1}. This progressive reduction in heartbeat rates observed at the pharyngula stage of embryo, suggests the cardiotoxic nature of MEFA. Errors in cardiac functioning in the underdeveloped heart may induce abnormal heartbeats due to the failure of blood circulation. This can in turn lead to retardation of body growth caused by deficiency of nutrients [20]. In a study conducted on zebrafish embryos using safflower extract, depressed heart beat rate was observed. The study also observed the appearance of additional apoptotic cells mainly around the heart [21]. This may imply tissue damage. In another study, aqueous extract of *Ficus glomerata* had negative effects on embryonic development in zebrafishes. Abnormalities in hatchability, heartbeat rate, tail detachment, head-trunk angle, scoliosis/flexure, *etc*. and yolk sac edema were recorded [22]. Current findings also corroborate such deleterious effects of some components of *F. assafoetida* in the cardiac functions of zebrafish embryos. The structure and function of the 2-day postfertilization embryonic zebrafish heart is quite similar to that of the 3-week gestation human embryo heart. So, the findings can be significant when translated to higher vertebrates. Studies done using many other plant extracts have also reported delayed growth, abnormal movement, tail detachment, abnormal head-trunk angle, scoliosis/flexure and yolk sac oedema of the embryos as in the case of *Momordica charantia* extract [23]. These data point to the presence of embryotoxic chemicals in the methanolic extract of *F. assafoetida*. The possible reason for the embryotoxicity of various chemicals is their ability to cross the maternal bloodstream through the placenta to enter the bloodstream of the embryo. This can result in altered heartbeat, growth retard-

ation, altered hatchability rate and abnormality in body parts of the *Danio rerio* embryos.

Effect of plant extract on the growth and development parameters of embryos: One of the most distinct teratogenic effects of *F. assafoetida* extract was the delayed development. In the control, all the embryos incubated had normal growth and none of them showed any mortality. Within 24 h, all these embryos reached the 21-26 somite cell stage. The embryos hatched within 48 h of incubation to reach the long-pec stage. By the end of 72 h, all of them grew up to the protruding-mouth stage with a heartbeat rate of 186 per min. When treated with the Ferula assafoetida extract, 100% delayed development was noted at concentrations above 0.1 mg mL^{-1}. Negligible development, which was also delayed, was observed in embryos exposed to 0.05 mg mL^{-1} concentration as it grew up to the protruding-mouth stage in 72 h of incubation. The varying effects of the plant extracts at different concentrations are depicted in Fig. 1.

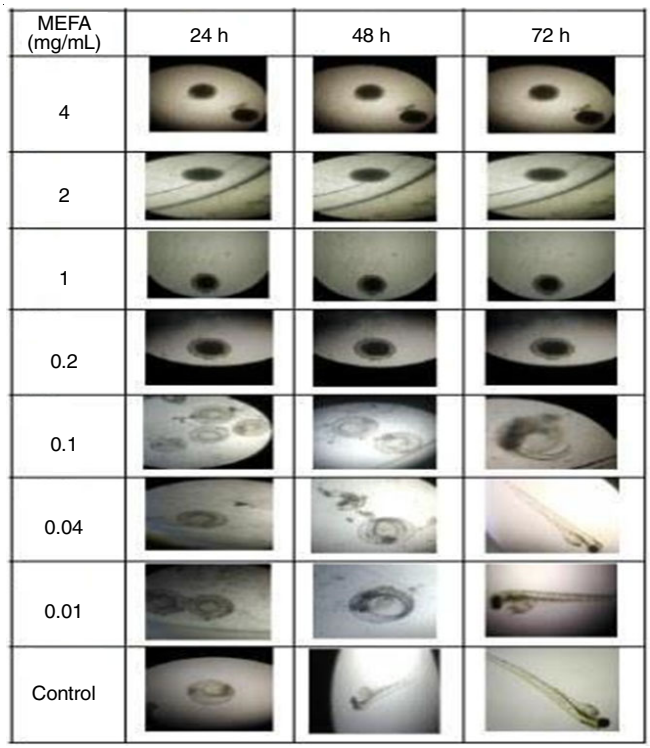

Fig. 1. Teratogenic effect of different concentrations of the methanolic extract of *Ferula asafoetida* (MEFA in mg mL^{-1}) on zebrafish embryos at different time intervals

The embryotoxic potential of some of the Ferula species were known in ancient days too. An extinct species of Ferula, which had abortifacient properties was speculated to be used in ancient times to prevent embryo implantation. This traditional medicine known as silphium in ancient herbal medicine collections and was thought to be highly effective in preventing pregnancy. This points to the embryo-toxic nature of some constituents of this plant. It is even believed that the plant was so much in demand that it has led to its extinction because of overuse. Once it had become extinct, asafoetida was used as a replacement which is believed to retain 50 % of embryotoxic properties of silphium [19]. It is also suggested that *F. tingitana*, which has been considered to have abortive properties [24] is a possible identity for this extinct silphium [25]. Recent studies have also showed cytotoxic effect of *F. tingitana* oil on hormone-responsive breast carcinoma cell line (MCF7), cervical carcinoma cell line (HeLa) and liver carcinoma cell line (HepG2) [26]. Another species of this genus, *Ferula communis* (giant fennel) has been found to contain some coumarin derivatives which can act as anticoagulants. When consumed by pregnant sheep, they gave birth to lambs with ataxia. These lambs died within a few hours after birth due to hemorrhage [27].

GC-MS and docking studies: Phytochemicals found in the GCMS chromatogram of the methanolic extract of *Ferula assafoetida* were further studied for their potential embryotoxicity. Fatty acids like decanoic acid, undecanoic acid, dodecanoic acid, tridecanoic acid, pentadecanoic acid, hexadecanoic acid, pentadecanoic acid and octadecanoic acid were found in MEFA. In general, free fatty acids (FFAs) can act as energy substrates. But if present in excess quantities, FFAs can accumulate in non-adipose cells and induce apoptosis leading to lipotoxicity. In an effort to study its effect on fetal development, mouse trophoblast stem cells when exposed to increasing doses of palmitic acid showed increased rate of apoptosis and decreased rate of proliferation [28].

in silico molecular docking was conducted to provide more evidence for potential interactions of the phytochemicals in the extract with proteins important in embryonic development. These compounds were subjected to molecular docking with the LTCC protein using Autodock Vina. The binding affinities of the docked compounds ranged from -4.7 kcal/mol to -8.8 kcal/mol as depicted in Table-2. The docking poses of the four compounds with maximum binding affinity are depicted in Fig. 2. 26-Hydroxycholesterol showed the highest docking score of -8.8 kcal/mol with one hydrogen bond. The docking poses of 26-hydroxycholesterol depicting the bond formation captured using Pymol is shown in Fig. 3. The second highest binding energy was -8.1 kcal/mol for lanost-7-en-3-

TABLE-2
BINDING AFFINITY VALUES FOR THE PHYTO-CHEMICALS PRESENT IN THE METHANOLIC EXTRACT OF *Ferula assafoetida* WITH THE L-TYPE CALCIUM CHANNEL (LTCC) PROTEIN

Phytochemicals as ligands	Binding affinity (kcal/mol)	No of H bonds formed
26-Hydroxycholesterol	-8.8	1
Lanost-7-en-3-one	-8.1	1
2-Bromo cholestane-3-one	-7.9	1
Cycloartenol	-7.8	0
2,2-Dibromocholestanone	-7.7	1
3-Chloro-5-cholestene	-7.5	0
N-(2-Hydroxyethyl) octanamide	-5.1	3
N,N-Bis(2-hydroxyethyl) decanamide	-5.7	3
Pentadecanoic acid	-5.7	2
Heptadecanoic acid	-5.1	1
Undecanoic acid	-5.1	1
Dodecanoic acid	-4.8	0
N-Decanoic acid	-4.8	0
Tridecanoic acid	-4.7	3

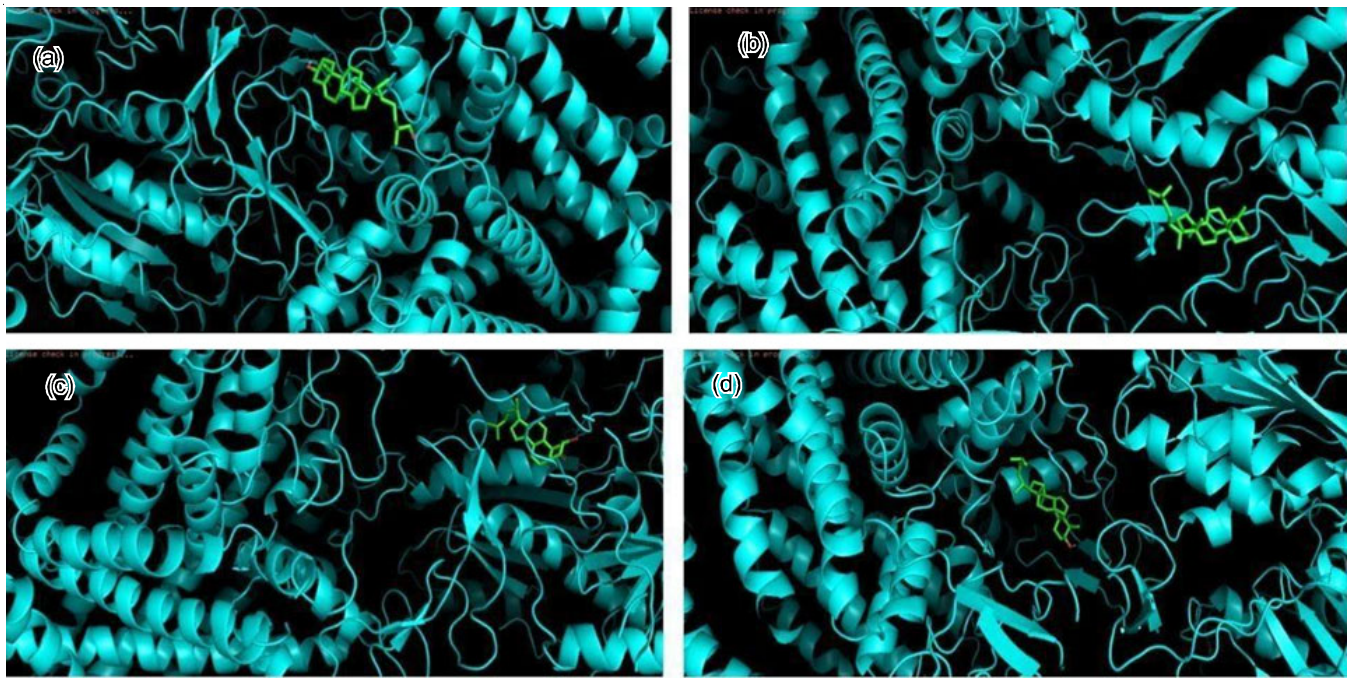

Fig. 2. Molecular docking of 4 prominent phytochemicals from *Ferula asafoetida* with the LTCC protein using Auto dock Vina (a) 26-hydroxycholesterol (b) lanost-7-en-3-one (c) 2-bromo cholestane-3-one and (d) cycloartenol

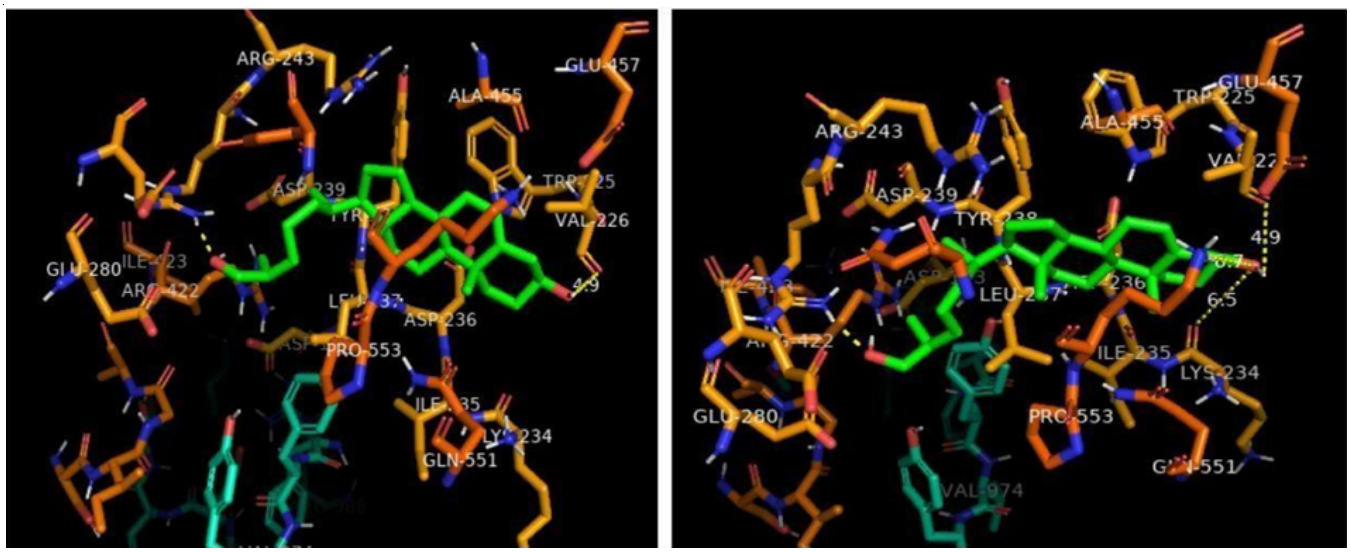

Fig. 3. Docking poses of 26-hydroxycholesterol with the LTCC protein depicting the hydrogen bond formation and weak interactions captured using Pymol software

one. Tridecanoic acid had the lowest binding energy of -4.7 kcal/mol.

One of the proteins involved in cardiac functions is LTCC (L-type calcium channel), which plays an important role in calcium influx in the sarcoplasmic reticulum and hence important in cardiac rhythm maintenance. The expression of LTCC was found to be higher in cardiac muscles and hence may be considered pharmaceutically important [29]. Of all the compounds docked with LTCC, 26-hydroxycholesterol (26-HC) gave maximum binding affinity (-8.8 kcal/mol). The presence of such phytochemicals, especially in high concentration could be one of the reasons for the cardiac malfunctioning of the zebrafish embryos leading to their death. The concept of free energy (ΔG) is used to determine the binding affinity of protein-ligand complex in docking studies. More negative the free energy, the stronger will be the protein-ligand interaction. Since 26-HC had the most negative binding affinity, it implies that it could bind strongly with the LTCC protein and potentially inhibit its function.

Calcium entry is an essential element of the heart beat by way of helping in functions like pacemaking, action potential, conduction and excitation-contraction (EC) coupling in the adult heart [30]. Calcium has also been implicated in the regulation of cellular growth and hypertrophy [31]. L-type calcium channels (LTCCs) constitute the prominent route for calcium entry into cardiac myocytes. Calcium-dependent path-

ways function as a unifying mechanism by which mutations in myofibrillar genes in humans lead to hypertrophy [32]. By way of gene mutation, LTCC activity was found to diminish normal cardiac growth during embryogenesis in zebrafish [33]. Since heartbeat rate was substantially affected in the zebrafishes treated with MEFA extract, we propose that chemicals like 26-HC together with the large number of free fatty acids may play a detrimental effect on embryonic cardiac activity.

26-Hydroxycholesterol (26-HC) belongs to the class of oxysterols and also known as 27-HC (when C-25 is asymmetric, stereochemistry is assumed to be 25R unless stated otherwise), which is the starting compound of the acidic pathway of bile acid biosynthesis [34]. 26-HC has been shown to have an antiproliferative effect as it inhibited rat and human myocyte proliferation [35]. Studies in human beings have shown that concentrations of total 26-HC have been found to be elevated in both CSF and the brain from Alzheimer's disease patients [31]. Recent studies in rodents have shown impairment of neuronal morphology and hippocampal spine density and levels of the postsynaptic protein PSD95 by 26-HC. This postsynaptic protein is very important for synaptic maturation and synaptic plasticity [34]. Present study shows that MEFA could also have deleterious effects on the nervous system of the zebrafish embryos due to components like 26-HC. Phytochemicals with teratogenic activities may have an anticancer potential as it suggests antiproliferative activity and antineoplastic activity [3]. Hence, the results of the present study can be carried forward in future to check the antitumor activity of these phytochemicals in *F. assafoetida* extract. The *in vivo* and *in silico* studies we had conducted in the present study points to the potential embryotoxicity of 26-hydroxycholesterol in *F. assafoetida* extract and corroborates the earlier findings.

Conclusion

The current study provides light on the inhibitory effect of one of components in *Ferula assafoetida viz.* 26- hydroxycholesterol on a protein, LTCC, vital to the cardiac functions in the embryo pointing to the cardiotoxic nature of the components of MEFA. Hence, care should also be taken before adding these extracts in formulations of drugs to be given to patients, especially during times of pregnancy. The embryotoxic effect of *Ferula assafoetida* can also be made use of in the future evaluation of its anticancer and apoptotic properties.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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