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COLLATERAL PROSPECTS OF POLYHERBAL FORMULATION AS ANTIDIABETIC AND APPRAISING ITS TOXICITY IN EARLY DEVELOPMENT OF ZEBRAFISH (DANIO RERIO) LARVAE

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ABSTRACT

Aim: To formulate a polyherbal formulation, evaluate their toxicity and antidiabetic potential level using zebrafish model.

Materials and Methods: The polyherbal formulation was prepared using leaves, flowers, fruits of these specific plants *Ficus religiosa, Allium sativum, Senna auriculata, Andrographis paniculata, Momordica charantia, Eugenia jambolana* (FASAME). The study is instigated by analyzing phytochemical constituents of the hydroalcoholic extracts. Further, its efficiency was determined by performing toxicity studies at varied quantities (100 µg, 70 µg, 50 µg, 10 µg, and 5 µg) and lethal effect was authenticated by calculating the viability of zebrafish .The antidiabetic potential of the formulation was established by β - galactosidase assay.

Results: The phytochemical assay exemplified the presence of flavonoids, steroids, terpenoid, saponins, resins, carbohydrates, proteins and essential oil . The efficacy of FASAME was evident among zebrafish larvae by a substantial increase in its viability at lower concentration. Conversely, the selective anti-diabetic activity of formulation authenticates its treatment towards diabetics.

Conclusion: The phytochemical analysis of FASAME drug has a mixture of phytochemical compounds and their IC50 value was found to be 74.39 µg/ml. The toxicity analysis on zebrafish larvae also showed higher viability at their lower concentration and proved to be a powerful antidiabetic drug for diabetics.

Keyword: Anti-diabetic activity, Polyherbal formulation, phytochemical, FASAME, zebrafish larvae.

INTRODUCTION

Diabetes mellitus is a chronic disease that last long with several disorder associated both metabolically and physically.[1] The control of diabetes mellitus is challenging and it is a universal problem. The successful natural approach of treating these diseases has paved the new way in drug discovery.[2] Although there are many drugs which are consumed orally or injected intravenously to control the blood sugar level, they may develop some side effects and are also of high cost. This condition leads to exploration of the traditional herbal plants which can have complete cure without any side effects. The Ayurvedic medicine has a primeval knowledge and considered to be the ancient remedial sciences that has persisted over many generations in many countries as traditional medicine. In India it was prevailed thousands of years ago, and it is acknowledged as the "Mother of All Healing".[3] In this system of medicine, nearly 600 different plants have capacity to overcome diabetes and it is scientifically reported in some of the Indian books like Charak, Samhita, Mahdhav Nidan and Astang Sanghra.[4] There are more than 12,000 plants that have shown good have ethnopharmacological effect against diabetes, but most of them lack in scientific authentication.[5]Recently certain therapies are using oral hypoglycaemic tablets which does not restores the normal glucose level and also the have side effects.[6] This is considered as an immense challenge which needs to resolved by exploring the efficiency of herbs which divulge conventional antidiabetic activity . The World Health Organization also supports and recommends several medicinal plants and investigating its potent in combination with modern medicines.[7] Herbs and phytochemicals production plays a main role in the detection of novel therapeutic compounds and also, they possess antioxidants, hypoglycemic, and antihyperlipidemic properties. *Sharangdhar Samhita*, an ayurvedic literature from 1300AD has noted the importance of polyherbalism.[8] Polyherbal formulations (PHFs) boost the therapeutic activity and condense the concentrations of single herbs and decreases the adverse effects. The PHF has enhanced and multi-targeted therapeutic properties than the single herb. The idea of polyherbal is different and it is evident that many herbs or medicinal plants have various bioactive compounds with different mode of mechanism. So, this can give a good synergic response and can act as effective drug to treat several disorders. The efficacy of new drugs is generally authenticated and promoted by clinical trials using animal models. The zebrafish model has become prevalent to carry out drug efficacy studies due to its desirable morphological characters like small, transparent body and large clutch size. Further it exhibits a greater genomic and physiological resemblances to humans .Drug monitoring and accessing its

effects can be performed extensively due to its transparent body features which can optimize optimal in vivo chemical screening and imaging. Hence these features enhance zebrafish as an optimal model to study developmental, physiological, and pathological processes [9]. Further various human diseases can be mimicked to understand their complications and mechanisms [10]. The present study is modulated to formulate a polyherbal formulation for antidiabetic activity and evaluate its toxicity using zebra fish as animal model.

MATERIALS AND METHODS

Collection of plant materials and Preparation of polyherbal formulation - FASAME

The plants like *Ficus religiosa* (leaves), *Allium sativum* (Bulb), *Senna auriculata* (Flower) *Andrographis paniculata* (leaves), *Momordica charantia* (Fruit), *Eugenia jambolana* (leaves) were collected in January to February from various places in Tiruchirappalli, Tamil Nadu (Fig. 1).

Figure 1. Collection of Herbal plants A. *Ficus religiosa (leaves), B. Allium sativum (Bulb), C. Momordica charantia (Fruit) D. Senna auriculata (Flower) E. Eugenia jambolana (leaves) F. Andrographis paniculata (leaves)*

These plants were identified based on their anatomical and morphological features at the Rapinat Herbarium and Centre for Molecular Systematic, St. Josephs College (Autonomous), Tiruchirappalli, Tamilnadu, India where, the voucher specimen number (AAJ 006) was assigned and deposited. The collected materials were washed thoroughly in running tap water to remove soil particles and other debris. The leaves, fruit, bulb and flowers of this specific plants were shade dried separately and ground to a fine powder using electric blender. The powdered materials were stored in an air tight container for further investigation.

Phytochemical extraction

Different ratio of powdered samples of *Ficus religiosa* – 15 g, *Allium sativum* – 5 g, *Senna auriculata* – 10 g, *Andrographis paniculata* – 10 g, *Momordica charantia* – 5 g and *Eugenia jambolana* – 5 g are used for extraction by soxhlet apparatus (Accumax ,India) at boiling temperature (78.2 ˚ C) and the solvent used for extraction was hydro alcohol which constituted of analytical grade ethanol, methanol and distilled water at 7 : 3. ratio for 1 hr (Fig.2).

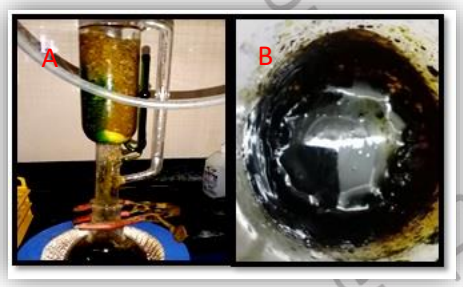

Figure 2. Extraction and formulation of FASAME - a polyherbal mélange

A-Soxhlet set up loaded with poly herbal hydroalcoholic suspension B- Condensed extract

The crude extract obtained were assessed for the qualitative phytochemical characterization for the identification of the various classes of active chemical constituents like resins, carboxylic acid, flavonoids, tannins, steroids, carbohydrates, glycosidase saponification, proteins, phenol, saponins, and gums using standard prescribed methods [11-13]. The positive tests were noted as weak (+), moderate (++), strong (+++) and absent (-).

Detection of Antidiabetic activity by β-galactosidase Assay

For the inhibition of β-galactosidase activity, a total of 0.5 mL of the different concentration of FASAME sample was pre-incubated with β-galactosidase in Na-acetate buffer at room temperature for 20 min. Then 0.5 mL of substrate mixture (8.3 mM ortho nitrophenyl β-D-galactopyranoside, 1 mM $MgCl_2$, and 0.1 M β-mercaptoethanol in 0.1 M Na-phosphate buffer, pH 7.0) was added to the sample mixture. After the incubation at 30°C for 20 min, the reaction was terminated with 0.5 mL of 0.5 M Na_2CO_3 buffer. Release of o-nitrophenol was recorded at 420 nm using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo scientific, USA). Each measurement was performed in triplicates using 96 well plate. Inhibition of enzyme activity was determined by using the following formula.

$$\text{Percentage of inhibition (\%)} = \frac{\text{Absorbance of control- Absorbance of test sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

Zebrafish Maintenance and Embryo Collection

Adult wild type zebrafish (Danio rerio) at 3-months of age of mixed sex were purchased from a local supplier (NSK Aquarium, Kolathur, Chennai,Tamilnadu,India).The fishes were maintained in closed ,9-L glass tanks (20 fish per tank) with charcoal-filtered tap water that endured the following physical parameters of 28 (± 0.5) ˚C temperature , pH 6.8–7.5 ,dissolved oxygen > 4 mg/L, conductivity 500–650 µS cm^{-1} ,total dissolved salt (TDS) 250 to 325 ppm) and salinity of 800–1200 µS/cm. An alternate 14/10 h light/dark cycle conditions were provided and Brine shrimp was feed twice daily at 9:00hrs and 16:00hrs. At night before fertilization, adult male and female zebrafish in 2:1 ratio were placed on opposite sides of a divider in a breeding tank. The next day morning, the zebrafish laid their eggs by natural mating soon after the exposure of first light. Embryos were collected within 30 min after spawning and rinsed with fresh water three times. The clean embryos were moved to tanks with the prepared embryo medium (14.61 g NaCl, 0.63 g KCl, 2.43 g mM $CaCl_2$ $2H_2O$, and 1.99 g $MgSO_4$, in 1 L of deionized water; stored at room temperature (~20–25° C) ;Diluted to 1X in deionized water prior to use) and cultured at 28°C for the subsequent experiments.

Zebrafish Embryo Toxicity

The test was carried out according to Organization for Economic Co-operation and Development (OECD) Test No. 236, 2013 (TIB Reg.No.219 /Res.25-09/09/2020). The median inhibitory concentration (IC_{50}) of the formulation was determined for 24hrs prior to the embryo toxicity studies .After elucidating an optimal threshold value, the healthy larvae were randomly divided into 24-well plates (5/well) and exposed to various concentrations of polyherbal formulation (0, 50, 100, 250 and 500 µg/mL) in 5 mL of fish water, in triplicate. The exposure lasted for 1 h in an environment at 28 ± 0.5°C under the same light/dark cycle (Fig. 3). After 1 h the solutions were replaced and dead embryos were discarded. The mortality rates were recorded, and examined using a stereomicroscope.

Morphology Score

Morphology scores were determined at 20 h post-treatment. Nine endpoints, including body shape, somite, notochord, tail, fins, heart, face, brain, and pharyngeal arches/jaws, were examined to evaluate the phenotypes of the zebrafish, and eight larval specimens per group were

used for scoring. Subsequently, the larvae were anesthetized with 0.25 mg/mL tricaine and were observed and photographed using an inverted microscope.

Behavioral Analysis

The locomotor response of the larvae to physiological stress was determined after exposing them to the formulation for 2hrs. Further the larvae (sets of 5) were transferred to 24-well plates with 0.5 mL of fish water and incubated for 30 min at 28°C. The behavioral study was observed for a duration of 10 mins. The swimming pattern was considered as the foremost parameter for the survival of larvae.

Statistical analyses

Data were analysed using SPSS software (Version 24) The results were presented as mean _ standard deviation, while the results with a p-value below 0.05 were considered significantly different.

RESULTS AND DISCUSSION

In this work we focused to develop a polyherbal formulation for the treatment of diabetes. And also to prevent the side effects that is been caused by use of allopathy medicines and to reduce the financial crisis of the diabetic person [14]. Therefore, we tested the antidiabetic effect of FASAME, a polyherbal formulation by β-galactosidase assay and their toxicity using zebra fish model. The FASAME is a mixture of phytochemicals that was extracted from several plants like *Ficus religiosa, Allium sativum, Senna auriculata, Andrographis paniculata, Momordica charantia, Eugenia jambolana*. The soxhlet apparatus are used to fraction this poly herbal mixtures.The phytochemical studies revealed the presence of flavonoids, steroids, terpenoid, saponins, resins, carbohydrates, proteins and essential oil in the hydroalcoholic extract of FASAME polyherbal formulation. (Supplementary Table 1). The inhibitory concentration of FASAME was calculated for toxcitity testing in zebra fish (Supplementary Table 2).

These plants have antidiabetic property and they exhibit various mechanisms. Some plants can synthesis insulin secretion from beta cells of islets of Langerhans, they supply essential elements like calcium, Zinc, Magnesium and copper for beta cells.[15] Furthermore certain plants has efficiency to prevent the conversion of starch molecules to glucose and also increase the capacity of bowl movement by proper digestion and excretion of urea.[16] Then these compounds

also has capacity to scavenge the reactive oxygen and free radicles so that they also act as potent anti-oxidising agent.In this present study the biologically active compounds present in the extracts interact with carbohydrate-hydrolyzing enzymes and promote antidiabetic properties. The observed variations in chemical composition of FESAME polyherbal formulation from different plants are not only due to the type of species and also to the selected part of plant and polarity of extraction solvents.[17]

Many plant tissue and its organs like seeds, stems, roots, cotyledons, vascular tissue and pollen has ability to produce β-galactosidases enzyme. This reduces β-D-galactosyl residues from β-D-galactosides.[18] This reduction process helps in removal of carbohydrates in intestine and decreases the sugar levels. Hence this mechanism plays major role and it is the important key point to use poly herbal formulation to control diabetes. Beta galactosidase assays results showed (Fig.3) the antidiabetic efficiency of FASAME polyherbal formulation.

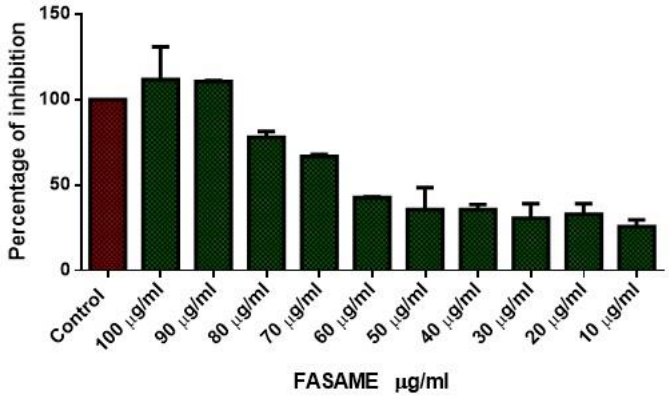

Figure 3. Percentage of inhibition of FASAME by β- galactosidase Assay

Finally, the toxicity analysis of polyherbal formulation using zebra fish larvae carried out, the results showed that the FASAME sample was not found to toxic up to the concentration of 100 µg/ml. But in 250 and 500 µg/ml there was mortality in fish larvae. Zebra fish is commonly known as Danio *rerio*, it is a small size fish with 2 to 4 cm length. It is a fresh water animal with short life cycle and so it is often used as experimental animals in research laboratories. It is largely used for toxicological studies and has 80% genetic similarity with humans.[19] Because of these reasons this fish is selected for toxicity studies. The IC_{50} was calculated and it was found to be 74.39µg/ml. The zebra fishes were exposed to different concentration. Finally, the toxicity analysis of polyherbal formulation using zebra fish larvae (Fig 4) carried out, the results showed that the FASAME sample was not found to toxic up to the concentration of 100 µg/ml. Finally, these zebra

fish is proved to be good model to study toxicity of polyherbal formulations. Thus, to avoid toxicity dosage of phytochemical plays important role and lower dosage is preferred to be safe for human consumption.

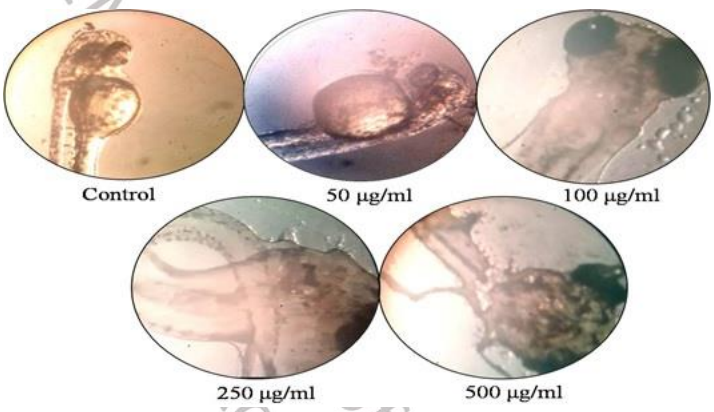

Figure 4. Effect of polyherbal formulation using zebra fish larva

Further studies should be applied to find the target compounds in these formulation using high throughput technologies.

CONCLUSION

In the current study the phytochemical analysis of FASAME drug showed a mixture of phytochemicals such as resins, steroids, carbohydrates, flavonoids, proteins and saponins. FASAME polyherbal formulation from various plant extracts have shown the antidiabetic activity of FASAME. The polyherbal formulation IC50 value was found to be 74.39 µg/ml. In addition, toxicity analysis with Zebra fish larvae showed a substantial increase in the percentage of viability at their lower concentration. Finally, these results showed that, FASAME polyherbal formulation could be a powerful antidiabetic drug for diabetics. The present examination has opened opportunities for further research, specifically with reference to the different dose studies and development of effective formulation for diabetes. Purification of the polyherbal extracts, formulation, and its evaluation through molecular studies will be a need for the future studies.

REFERENCES

1. Bailey CJ, Day C Traditional plant medicines as treatments for diabetes. Diabetes Care 1989; 12:553–64.
2. Singh D, Singh B, Goel RK, Traditional uses, phytochemistry and pharmacology of *Ficus religiosa*: a review. J. Ethnopharma 2011; 134(3): 565–583.
3. Malhotra V, Singh S, Tandon OP, Madhu SV, Prasad A, Sharma SB, Effect of yoga asanas on nerve conduction in type 2 diabetes. Indian. J. Physiol. Pharmacol 2002; 46:298–306.
4. Kumar CH, Kumar JNS, Ishaq BM, Rani GU, Prakash KV, Antidiabetic activity of a polyherbal preparation. Pharmacol 2010; (1):780-87.
5. Daisy P, Feril G, Kani J, Evaluation of antidiabetic activity of various extracts of *Cassia auriculata linn.* bark on streptozotocin-induced diabetic wistar rats. Int. J. Pharm. Sci 2012; 4(4):312-18.
6. Rohner A, Ried K., Sobenin, Igor AS,Heiner CB, Alain JN, A systematic review and meta-analysis on the effects of garlic preparations on blood pressure in individuals with hypertension. American J. Hypertension 2015; 28(3):414–423.
7. Simmonds M and Howes M. Plants used in the treatment of diabetes, in Traditional Medicines for Modern Time—Antidiabetic Plants. A. Soumyanath, Ed., vol. 6th, pp. 19–82. United States: CRC Press/Taylor and Francis Group; 2006.
8. Srivastava S, Lal VK, Pant KK, Polyherbal formulations based on Indian medicinal plants as antidiabetic phytotherapeutics. Phytopharmacol 2012; 2:1-15.
9. Santoriello C, Zon L I Hooked, Modeling human disease in zebrafish. J. Clin. Investig. 2012, 122; 2337–2343.
10. Lee J G, Cho H J, Jeong Y M, Lee J S, Genetic Approaches Using Zebrafish to Study the Microbiota–Gut–Brain Axis in Neurological Disorders. Cells. 2021, 10(3);566.

11. Debiyi O O , Sofowora F A, Phytochemical screening of medical plants, *Iloyidia*, 1978, 3; 234–246.
12. Roopashree T S , Dang R, Rani R H S, Narendra C, Antibacterial activity of antipsoriatic herbs: *Cassia tora*, *Momordica charantia* and *Calendula officinalis* Int. J. Appl. Res. Nat. Prod, 2008,. 1 (3); 20–28.
13. Jamous R, Zaitoun S, Husein A, Qasem I, and Ali-Shtayeh M, Screening for biological activities of medicinal plants used in traditional arabic palestinian herbal medicine, European Journal of Medicinal Plants, 2015,9(1): 1–13.
14. Bachok MF, Yusof BN, Ismail A, Hamid AA, Effectiveness of traditional Malaysian vegetables in modulating blood glucose levels. Asian Pacific J. Clini. Nutri. 2014; 23(3):369-76.
15. Boxi M, Rajesh Y, Raja Kumar V, Praveen B, Mangamma K, Extraction, phytochemical screening and in-vitro evaluation of anti-oxidant properties of *Commicarpus chinesis* (aqueous leaf extract). Int. J. Pharm. Biosci 2010; 1: 537–547
16. Zohary, Daniel, Hopf, Maria. Domestication of Plants in the Old World .3rd Ed. England:Oxford University Press; 2000.
17. Jamous R, Zaitoun S, Husein A, Qasem I, Ali M, Screening for biological activities of medicinal plants used in traditional *Arabic palestinian* herbal medicine. Europ. J. Medi. Plant 2015; 9(1): 1–13.
18. Wu A, Liu J, Isolation of the Promoter of a Cotton β- galactosidase Gene GhGal l and its Expression in Transgenic Tobacco Plants. Science in China Series C: Life Sci 2006; 54:234-245
19. Arome D, Chinedu E, The importance of toxicity testing. J. Pharma. BioSci 2014; 4:146–148.

FIGURE CAPTIONS

Figure 1. Collection of Herbal plants A. *Ficus religiosa* (leaves), B. *Allium sativum* (Bulb), C. *Momordica charantia* (Fruit) D. *Senna auriculata* (Flower) E. *Eugenia jambolana* (leaves) F. *Andrographis paniculata* (leaves)

Figure 2. Extraction and formulation of FASAME - a polyherbal mélange

Figure 3. Toxicity analysis of polyherbal formulation using larva of Zebrafish

Figure 4. Percentage of inhibition of FASAME by β- galactosidase Assay

Figure 5. Effect of polyherbal formulation using zebra fish larva