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Antimicrobial and hepatoprotective effect of ISM Drug in alcohol induced liver damage on Zebrafish larvae

Cynthia C*[1], Horne Iona Averal[2]

[1]Department of Biotechnology, Bishop Heber College, Tiruchirappalli, Tamilnadu, India
[2]Department of Zoology, Holy Cross College, Tiruchirappalli, Tamilnadu, India

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ABSTRACT

Liver cirrhosis occurs due to the extracellular matrix proteins accumulation which distorts the hepatic structure. The aim of our study is to investigate the antimicrobial and hepatoprotective effect of India Siddha Medicine(ISM) drug in alcohol-induced liver damage on zebrafish larvae. ISM drug, a hepatoprotective agent comprising of 15 herbs offers numerous formulations with advantages of hepatocellular rejuvenation has proven to be the most effective treatment to suppress liver damage. A series of analyses were carried out using alcohol-induced liver damage through herbal-based therapeutic drug and agar well diffusion method. For the hepatoprotective study, the zebrafish larvae were exposed to different concentrations of ethanol % (0.01, 0.05, 0.1, 0.5) and the treatment with 217.2 μg/ml of ISM drug has been evaluated. The outcome revealed the effect of ISM on alcohol-induced zebrafish larvae with a substantial increase in the percentage of viability. ISM drug showed antibacterial and antifungal activity in the diffusion method with a maximum zone of inhibition observed as 12 mm and 13.5 mm, respectively. Taken together, these results showed that the drug has hepatoprotective and antimicrobial activity which can be used for the treatment of liver cirrhosis.

*Corresponding Author

Name: Cynthia C
Phone: +91- 8220821265
Email: cynthiachainsam@gmail.com

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INTRODUCTION

The Liver is the curator of the consistency of the internal environment. As it does so, the liver detoxifies chemicals and metabolizes drugs. Liver injury is a disease that can be caused by drugs which can be life-threatening. Alcoholic Liver disease (ALD) was the foremost etiological factor of cirrhosis and liver-related morbidity among patients worldwide for decades. An early stage of ALD is Hepatic steatosis which may progress to fibrosis even cirrhosis, the loss of liver metabolic and synthetic function. During the drug development, Drug-induced liver injury (DILI) is a major problem (Lai *et al.*, 2018). The common drug causing DILI is Paracetamol, an analgesic that is safe when used at therapeutic doses but an overdose of it results in over 300 deaths in USA and half of it in UK. Amiodarone, valproic acid, vitamin A and methotrexate are the drugs that are linked to being causing cirrhosis (Vliegenthart *et al.*, 2014). It can be a result of severe acute injury, prolonged injury due to chronic hepatitis, or vanishing bile duct syndrome(VBDS).

This is the most striking form of cirrhosis that usually arises after acute cholestatic injury and has the possibility of progressing even after discontinuation of the medication.Problems in estimating the drug kinetic behavior are intensified by the

additional impairment of the kidney (Llanos *et al.*, 2009). The most adverse drug reactions observed in patients with liver disease is the electrolytic disturbances occurred due to furosemide.The recent studies showed that the plant, Phyllanthus drug is best known to cure Jaundice by inhibiting DNA-polymerase activity in virus and protecting the liver from further damage.Sarakkondraipulipatru known to reduce the enlargement of the liver. The Zebrafish organs specifically the liver has revealed multiple similarities with higher vertebrates. Hepatic stellate cells were recently found in zebrafish as myofibroblasts that became activated and secreted ECM on the hepatic injur (Howarth *et al.*, 2013). They metabolize drug-using similar pathways to humans as the expression and the function of orthologous chemokine receptorsin lower and higher vertebrates are highly similar. Zebrafish has emerged as a dominant model for biomedical research (Phillips and Westerfield, 2014). The main difference between mammals and zebrafish liver is the arrangement of hepatocytes which are in plate-like form and in tubular fashion respectively. In the present study was to investigate the antimicrobial and hepatoprotective effect of ISM drug for the treatment of alcohol-induced liver damage in zebrafish larvae.

MATERIALS AND METHODS

Preparation of ISM drug

ISM drug was prepared using various concentrations of 15 different herbal plant extracts in Table 1. The herbal plants such as Phyllanthus amarus, Fumaria vaillantii, Ionidium suffruticosum, Evolvulus alsinoides, Hibiscus rosa-sinensis, Wedelia calendulacea, Murrayak oengi, Hydrcotyle asiatica, Solanumnigrum, Androgra phispanic-ulata, Cuminum cyminum, Foeniculum vulgare, Vitisvinifera, Emblica officinalis, and Cyperus rotundus were collected from different places. The leaves were shade dried and ground to a fine powder using an electric blender. Then, the powders were stored in airtight polythene container.

Preparation of extracts by Soxhlet extraction method

The crude powders were defatted with one litre of petroleum ether using Soxhlet apparatus. After defatting, the extraction was carried out using 1000 ml of ethanol at 75°C for 4 h. After extraction, the samples were evaporated at the rotary evaporator to remain with important ingredients.

Maintenance and Embryo Collection

Zebrafish were kept under a 14/10 h light/dark cycle at constant temperature (28 ± 0.5°C) in a closed flow-through system with charcoal-filtered tap water. Brine shrimp were provided twice at 9:00 and 16:00 hours. In a ratio of 2:1, adult males and females were segregated on opposite sides of a divider in a breeding tank the night before fertilization. There was a mesh provided for the eggs to be laid by natural mating soon after the first light, otherwise, there was a probability of them consuming their own eggs in Figure 1. Embryos were collected within 30 min after spawning and rinsed with fresh water thrice. The clean embryos were moved to the tanks with embryo medium and cultured at 28°C for the subsequent experiments.

Liver damage analysis using zebrafish larvae

After three days post-fertilization the larvae were collected and separated into two groups in Figure 1. The first group of larvae was treated with various concentrations of ethanol (0.01, 0.05, 0.1 and 0.5) %. The second group of larvae was treated with similar concentrations of ethanol along with 217.2 μg/ml of ISM drug. The Petri plates were maintained at 25°C for 24 h and the viable larvae count was noted and were observed microscopically.

Antibacterial activity by agar well diffusion method

Petri plates containing 20ml nutrient agar medium were seeded with the 24hr culture of bacterial strains (Pseudomonas aeruginosa, E.Coli, Staphylococcus aureus, and Bacillus cereus). Wells were cut and different concentration of ISM drug (500, 250, 100, and 50) μg/ml was added. The plates were kept for 24 h incubation at 37°C. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells where the Gentamicin antibiotic (Ab) was used as a positive control.

Antifungal activity by agar well diffusion method

Petri plates containing 20ml potato dextrose agar medium were seeded with 5 days of fungal strains (Candida albicans, Aspergillus niger, and Aspergillus fumigatus) and treated with various concentrations of ISM drug and incubated at 28°C for 24 hours. The anti-fungal activity was assayed by measuring the zone of inhibition. Amphotericin B (AM) was used as a positive control (Wilkins and Pack, 2013).

Statistical analysis

The difference in estimated parameters between the groups was analyzed using one-way ANOVA with Bonferroni's test. Data expressed as mean ± SD. All parameters were analyzed at 95% confidence intervals and a P-value of <0.05 was considered to be sta-

Table 1: Ethnobotanical information of ISM drug

S. No.	Plant Name	Quantity per 5 ml
1.	Phyllanthus amarus	5 mg
2.	Fumariava illantii	5 mg
3.	Ionidium suffruticosum	5 mg
4.	Evolvulus alsinoides	5 mg
5.	Hibiscus rosa-sinensis	5 mg
6.	Wedeliacalendulacea	5 mg
7.	Murraya koengi	5 mg
8.	Hydrocotyle asiatica	5 mg
9.	Solanum nigrum	1 mg
10.	Andrographis paniculata	1 mg
11.	Cuminum cyminum	1 mg
12.	Foeniculum vulgare	1 mg
13.	Vitis vinifera	1 mg
14.	Emblica officinalis	1 mg

Table 2: Effect of ISM on alcohol-induced zebrafish larvae

S. No	Name of the tested sample	No. of Live larvae		No. of dead larvae		Mean value of Viability (%)	Mean value of Mortality (%)
1.	Control	10	10	0	0	100	0
2.	0.01 % of Ethanol	6	5	4	5	55	45
3.	0.05 % of Ethanol	2	3	8	7	25	75
4.	0.1 % of Ethanol	1	0	9	10	05	95
5.	0.5 % of Ethanol	0	0	10	10	0	100
6.	0.01 % of Ethanol + 217.2 μg /ml of ISM	8	7	2	3	75	25
7.	0.05 % of Ethanol +217.2 μg /ml of ISM	6	4	4	6	50	50
8.	0.1 % of Ethanol + 217.2 μg /ml of ISM	2	1	8	9	15	85
9.	0.5 % of Ethanol + 217.2 μg /ml of ISM	0	0	10	10	0	100

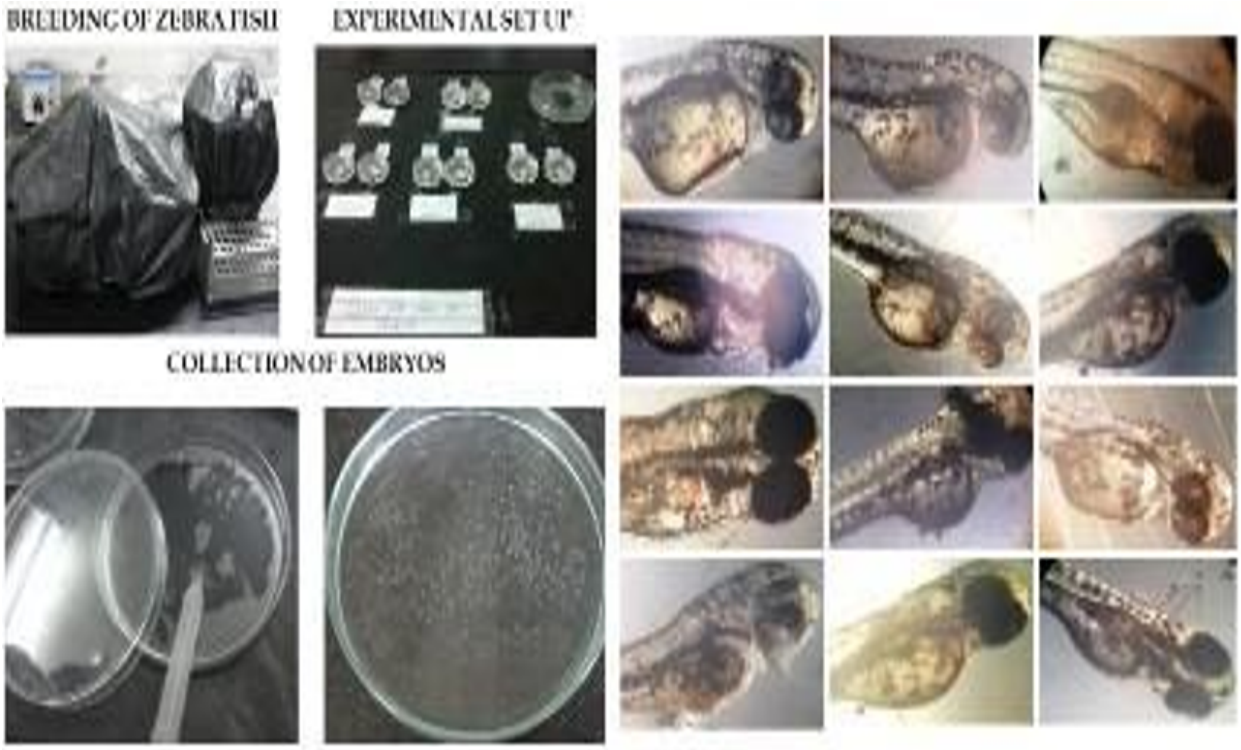

Figure 1: Collection of zebrafish embryos and ISM treatment

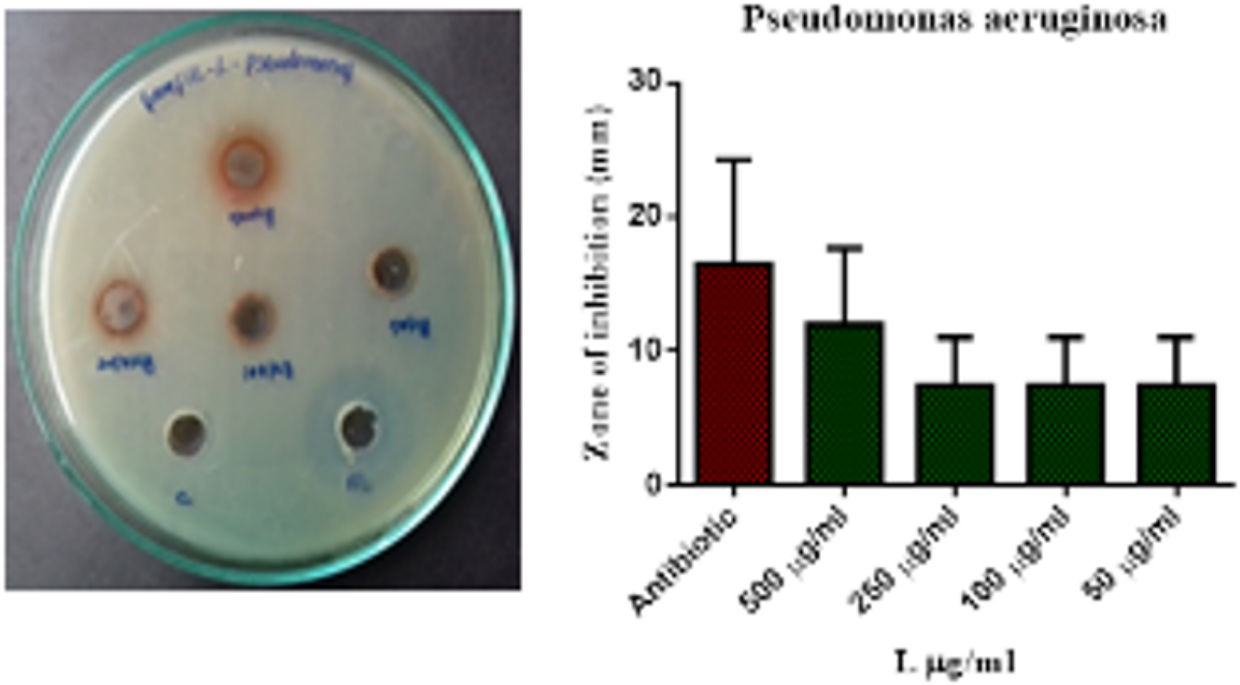

Figure 2: Effect of sample ISM against Pseudomonas aeruginosa

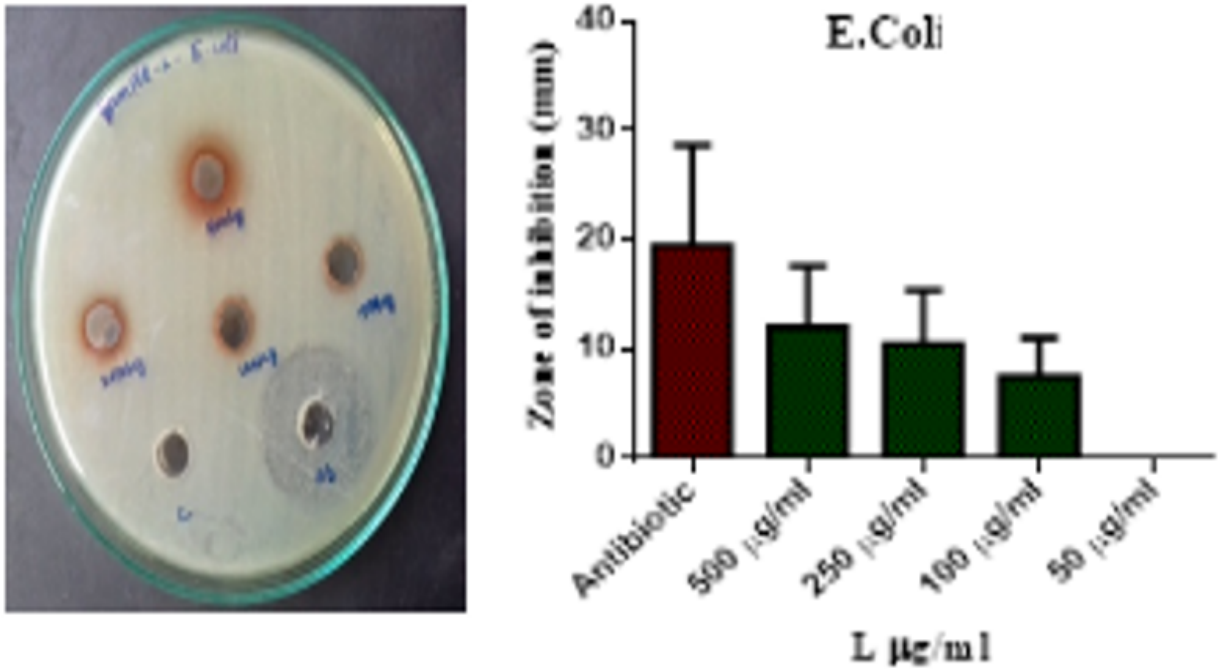

Figure 3: Effect of sample ISM against Escherichia Coli

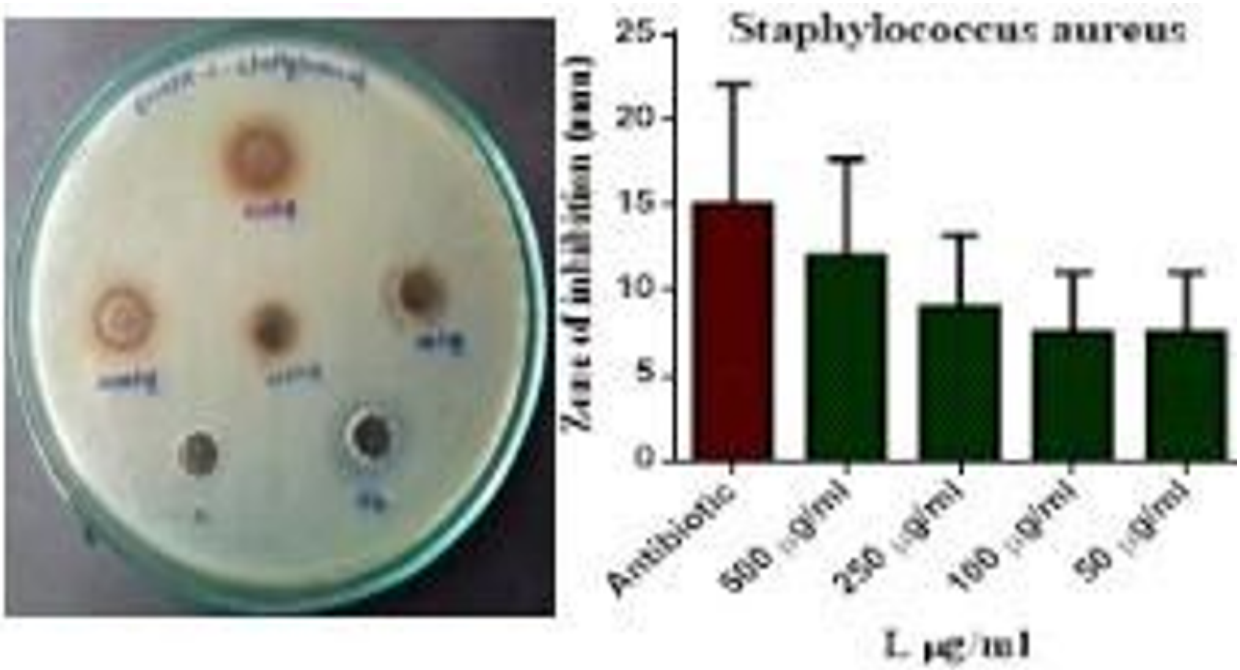

Figure 4: Effect of sample ISM against Staphylococcus aureus

tistically significant. Statistical analysis of the data was performed using Graph pad Prism version 6.00 for Windows, Graph Pad Software, San Diego California USA.

RESULTS AND DISCUSSION

Antibacterial activity of ISM drug

Using agar well diffusion method, four different bacterial strains (Staphylococcus aureus, E.coli, Pseudomonas aeruginosa, and Bacillus cereus) were plated with solutions of the ISM drug ranging (500 μg/ml, 250 μg/ml, 100 μg/ml and 50 μg/ml). After 24 h, the growth inhibition zones of the test strains determined showed that the drug exhibited selective antibacterial activity towards S. aureus, with a maximum inhibition zone of 12 mm and minimum inhibition zone of 7.5 mm Figures 2, 3, 4 and 5

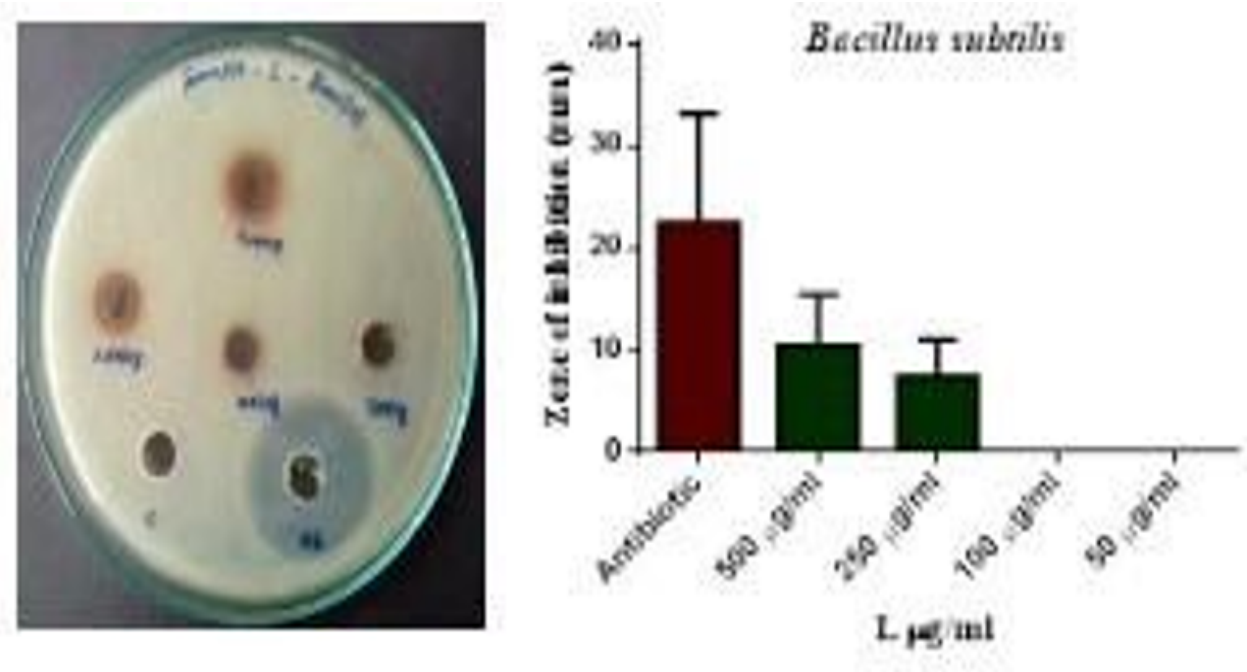

Figure 5: Effect of sample ISM against Bacillussubtilis

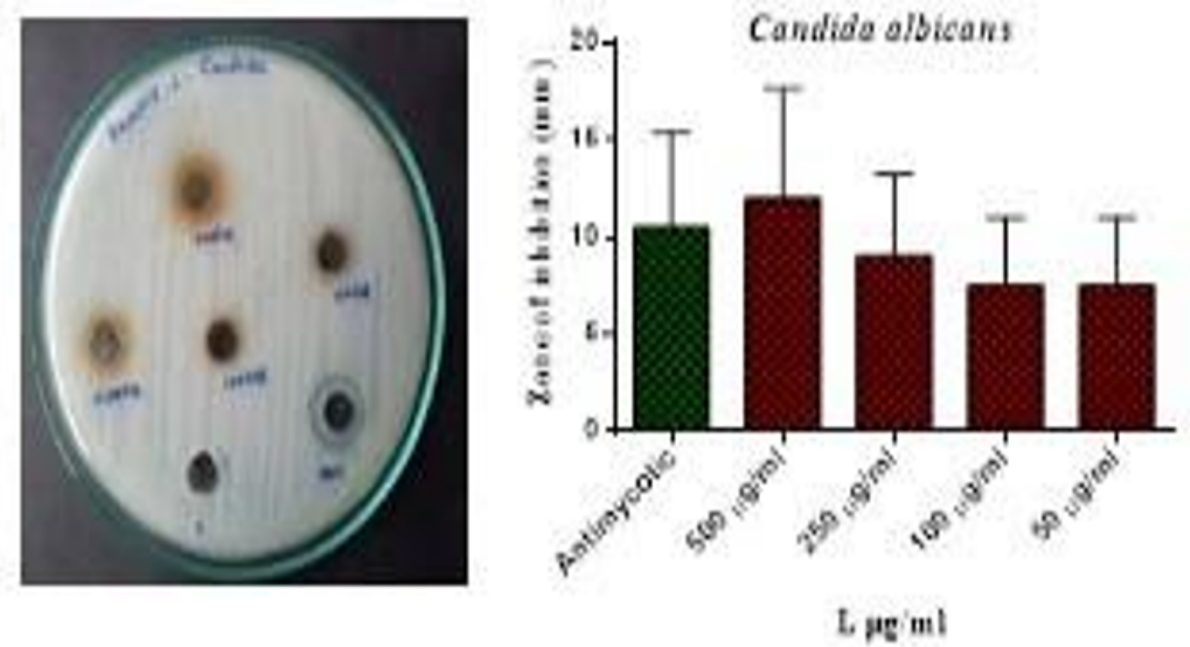

Figure 6: Effect of sample ISM against Candidaalbicans

whereas the other bacterial strains showed limited antibacterial activity.In our research study, the efficacy and safety of all the fifteen herbal products in the treatment of liver disease has been demonstrated along with antimicrobial effect by not leading to further microbial infections such as sepsis, an independent risk factor for multiple organ dysfunction. As elucidated above, the present study was done using Agar well diffusion method showed that Gram-positive bacteria were more sensitive to the tested ISM drug when compared to Gram-negative ones. Based on the interpretation, the drug indicated whether it is resistant (R), intermediate (I) and susceptible(S). The zone diameter for Staphylococcus species as well as for the other non-fermenting and enteric Gram-negative rods showed (R) of less than 12 mm, (I) of 14 mm and (S) of more than 15 mm when treated with 10 μg concentration of Gentamycin antibiotic.It has the ability to quickly develop resistance to antibiotics as they are highly adaptable pathogen responsible for severe infection in the United States (Moellering, 2012; Gould *et al.*,

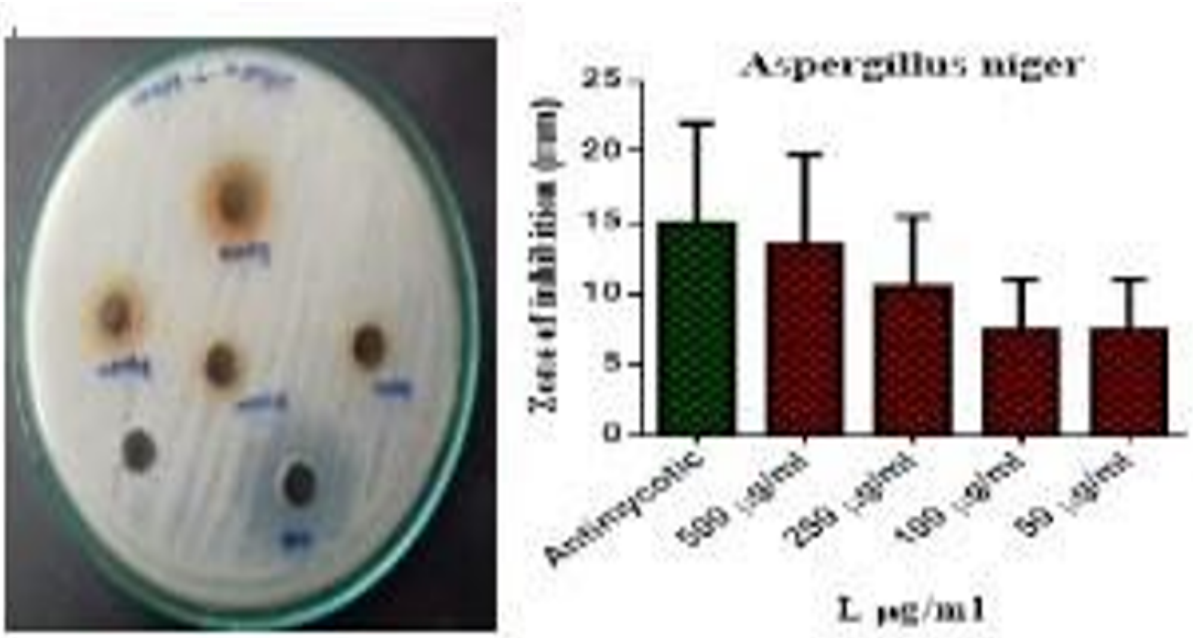

Figure 7: Effect of sample ISM against Aspergillusniger

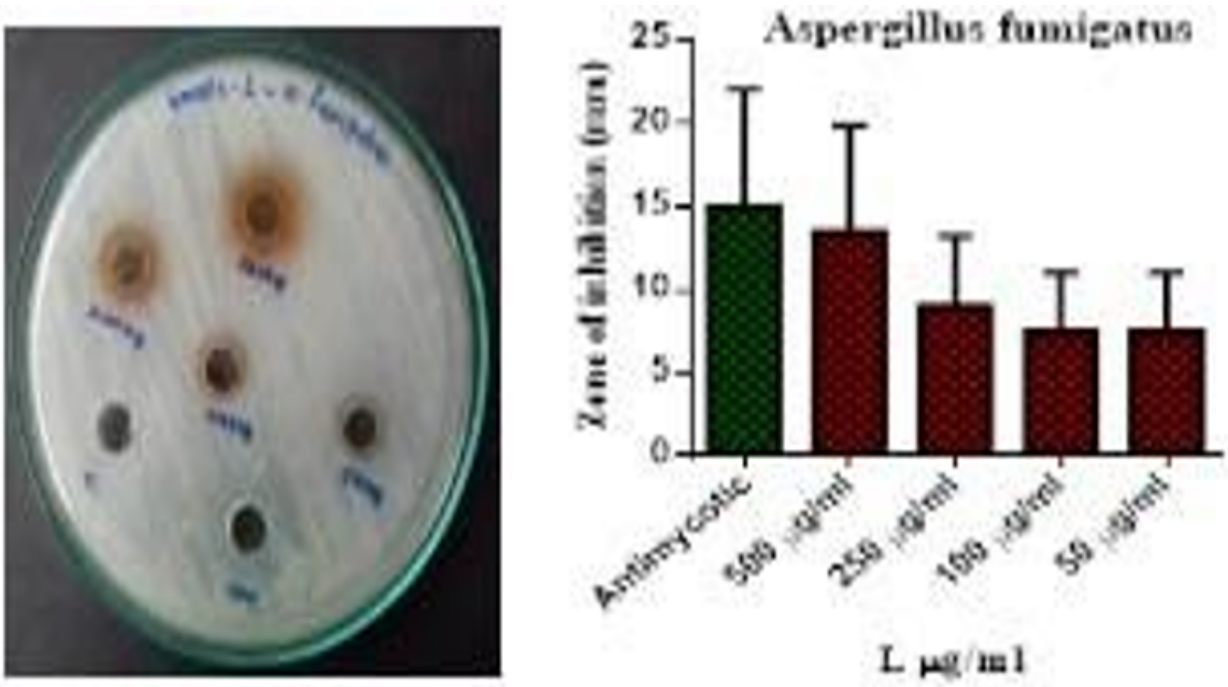

Figure 8: Effect of sample ISM against Aspergillusfumigatus

2012)

Antifungal activity of ISM drug

The ability of antifungal activity was evaluated to inhibit Candida albicans, Aspergillus niger and Aspergillus fumigatus. Following the similar method above, the drug concentrations were plated. Interestingly, after 24 h, the growth inhibition zones of the test spores were determined which revealed that the drugexhibited selective anti-fungal activity towards Aspergillus niger with amaximum inhibition zone of 13.5 mm and minimum inhibition zone of 7.5 mmFigures 6, 7 and 8. Also, there was a presence of fungal spore growth around the well diffusion. The zone of inhibition obtained by sample ISM against three fungal strains was obtained showing lesser activity . The bacteria constitutes of SepF protein involved in cell division which is essential for survival at a temperature greater

than 30°C.About 30% of cirrhotic patients.This classical medicine was already been introduced into the Japanese system 1500 years ago termed to be Kampko stands for Han method (Ohtake *et al.*, 2004). Salvia plebeia R.Br. (SPEE) is also another Chinese medicinal herb used to treat hepatitis, inflammatory disease (Basnet *et al.*, 2019). In in vitro experimental models, ethanol extracts of SPEE displayed antioxidant and anti-inflammatory effects.

The effect of ISM on alcohol-induced liver damage analysis

After studying different dosing regimens of ethanol concentrations in addition to treatment with ISM drug in larval zebrafish, the model was determined to be beneficial, showing antimicrobial and hepatoprotective effects with regard to liver injury (White *et al.*, 2013). Within the Pharmacovigilance, the time of onset was important in the causality assessment of drugs and suspected adverse reactions. The first scenario was treated with acute alcohol consumption that showed a decrease in the viability rate. The second scenario included a similar ethanol concentration with ISM drug which showed an increase in viability rate. Thus, the cohort resulted in the detection of alcohol-induced liver injury with high specificity and sensitivity. Alcohol ingestion with the ISM drug-induced a small, non-clinically relevant, increase in the viability rate Table 2.

Zebrafish larvae at 4dpf show distinct characteristics of swimming pattern with respective to light conditions (Persichino *et al.*, 2018). Here, zebrafish with the aim of developing a liver toxicity model to replace higher vertebrates was investigated. Firstly, larvae was treated with ethanol at various concentrations ranging from control, (0.01, 0.05, 0.1 and 0.5) %. Secondly, the larvae were treated with similar concentration in addition to ISM drug 217.2 μg/ml after 24 hours the effect of the ISM on alcohol-induced zebrafish was observed. The outcome showing a P-value of less than 0.05 at 95% confidence interval is closed a significant increase in the viability rate when been additionally treated with the ISM drug.

CONCLUSIONS

The Liver is vital for life. The study has been performed mainly focusing on liver injury through establishing a zebrafish model through long-term ethanol exposure which caused inflammatory liver damage. Zebrafish represents an alternative experimental model species to study liver injuryand helps to identify the disease mechanisms that are targeted therapeutically. Further pre-clinical studies are needed to verify the benefits of the Siddha system of Ayurvedic medicine over ALD. Hence, the major characteristic feature of Siddha materia medica is the utilization of mineral-based preparations.

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Conflict of interest

None

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