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A Recent Trends on Pharmacological Screening Methods of Hepatoprotective Potential of Herbs by Using Zebrafish Model

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ABSTRACT

Effective hepatoprotective agents are desperately needed, as liver disorders are a major worldwide health problem. A useful model organism for researching many facets of liver development, function, and illness is the zebrafish. In terms of anatomy, function, and ability for regeneration, the livers of zebrafish and humans are quite similar. Through the use of chemical poisons, genetic engineering, and other techniques, researchers have effectively caused liver damage in zebrafish, enabling the investigation of the disease's etiology and course. The zebrafish model serves as a link between in vitro tests and in vivo research on mammals. This model's range of applications and research tractability make it a potent tool. This review emphasizes how studies on zebrafish have yielded important information on the different herbs used to treat liver disease in humans. In order to find possible therapeutic agents for human liver illnesses, hepatoprotective research in zebrafish models offer important insights into the liver-protective properties of different chemicals, herbal extracts, and medications. Furthermore, zebrafish models provide a practical and affordable substitute for conventional mammalian models in the investigation of liver toxicity and function.

Keywords: Hepatoprotective, Zebrafish, Liver injury, Herbal extracts, Anti - inflammatory, Antioxidant.

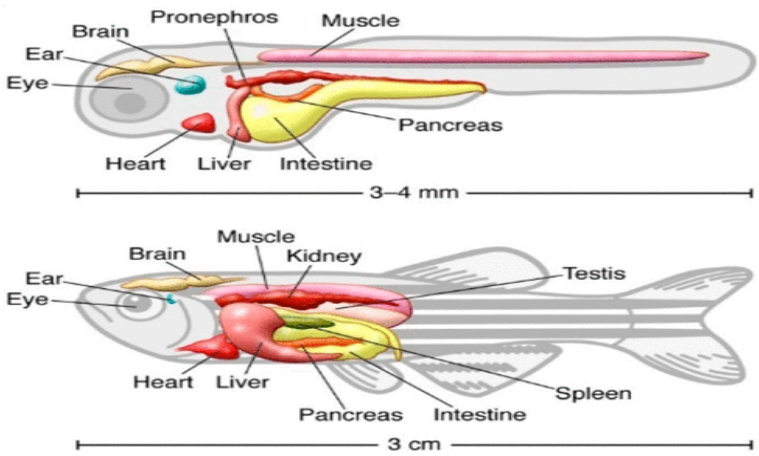
INTRODUCTION:

The human liver is a vital organ that carries out several physiological tasks. Albumin, prealbumin, plasma fibrinogen, transferrin, transport protein, phospholipids, and essential fatty acids are among the serum proteins that are primarily synthesized there. The liver is the primary organ for detoxification and is involved in the metabolism of nutrients. In humans, disorders related to development, infection, immunological response, metabolism, and cancer all target the liver and are linked to morbidity. When compared to other organs, the liver's capacity for regeneration is astounding [(1)]. Many medications have mild to severe hepatotoxicity, and one of the main reasons that authorized medications are withdrawn is drug-induced liver damage (DILD). However, due to a lack of better substitutes, certain life-saving medications with hepatotoxic side effects are still in use. There aren't any well-known, clinically approved substances with shown hepatoprotective action at the moment [(2)]. When the liver is unable to detoxify free radicals, such as reactive oxygen species (ROS), or other harmful metabolites from pharmacological compounds, drug-induced liver damage occurs. This kind of liver damage is becoming a bigger issue in science, medicine, and public health [(3)]. One common and extensively used analgesic and antipyretic medication is acetaminophen (paracetamol). At therapeutic dosages, paracetamol is harmless; but, at greater dosages, it damages the livers of both humans and experimental animals. According to reports, the most common cause of drug-induced acute liver failure is paracetamol overdose. Acetaminophen-induced liver damage is still linked to significant morbidity and mortality and has been linked to numerous emergency hospital admissions for conditions like cirrhosis, hepatitis, and hepatic transplant [(4,5)]. Although N-acetylcysteine (NAC) is utilized as a specialized antidote for poisoning caused by paracetamol, it has significant negative side effects, such as the potentially fatal anaphylactic reaction [(6)]. A helpful model that enables researchers to perform in-depth genetic and embryological analysis is the zebrafish (Danio rerio). Zebrafish are particularly effective systems for investigating the development and morbidity of the liver since they are thought to be a modal representative of the human liver in hematology investigations. Zebrafish may be bred and maintained for relatively little money, and hundreds to thousands of embryos can be collected quickly for analysis [(1)]. Although rats have long been employed in liver toxicology research, small fish, like zebrafish, have recently been adopted as animal models because to their benefits, which include quick generation times, high fertility, and low operating costs for housing space and daily upkeep. As a result, in some

vertebrate toxicological investigations, zebrafish are a more useful animal model than rats [3]. Compared to inbred rodent strains, the most widely used laboratory zebrafish strains are outbred, which helps to prevent strain-specific effects [1].

In order to better understand liver function, find possible treatments, and lessen the requirement for mammalian models, zebrafish are used in hepatoprotective activity research. In accordance with the Committee for the Purpose of Control and Supervision of Animal Experiments' (CPCSEA) standards. This study's primary goal was to ascertain how plant methanolic extracts affected the liver damage caused by acetaminophen in young zebrfish (Danio rerio) [3].

ZEBRAFISH



ANATOMY OF ZEBRAFISH

HISTORY:

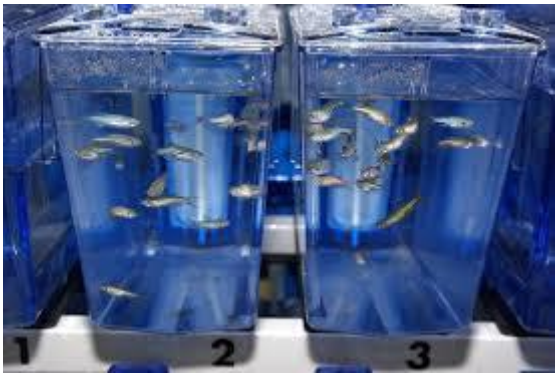
IN YEARS	DESCRIPTION
1822	Zebrafish where first described by british zoologist Francis Hamilton as (“cyprinus rerio”) [7]
1860	Introduced to the aquarium trade and became popular among hobbyists [8]
1960	Scientist began using zebrafish as a model organism for research, particularly in the fields of development biology and genetics [9]
1970	Zebrafish used to study embryonic development and tissue regeneration [10]
1980	First zebrafish mutants where, generated allowing researches to study specific genetic trials [11]
1990	Become a popular model for studying human diseases, such as cancer, cardiovascular disease and neurological disorders [12]
2000	Zebrafish genome was sequenced making it easier to study gene functions and behaviours [13]
Present day	Widely used in research, with applications in fields, like toxicology, pharmacology, biotechnology [14]

BIOLOGY:

- ✓ **Scientific name** : Danio rerio
- ✓ **Family** : Cyprinidae
- ✓ **Order** : Cypriniformes
- ✓ **Class** : Actinopterygii
- ✓ **Genus** : Danio
- ✓ **Species** : Danio rerio

ZEBRAFISH MAINTENANCE:

As stated, zebrafish (Danio rerio) were bred, grown, and housed at 28.5°C in 10-liter tanks under conventional conditions. Every zebrafish experiment complied with the guidelines set forth by the CSIR-Institute of Genomics and Integrative Biology's Animal Ethics Committee (IAEC) and the suggestions made by the government of India's Committee for the Purpose of Control and Supervision of Animal Experiments (CPCSEA) [(2)].



BREEDING AND EMBRYO COLLCTION:

Overnight, the male and female fish were housed in separate breeding tanks with separators. The day-night cycle-14hours of light and 10hours of darkness-was rigorously upheld. Divisions were eliminated the following day, right before the day cycle began. Embryos were obtained from fish that were permitted to mate. After sorting, about 100 embryos per 90mm petriplate were maintained in the E3 buffer at 28°C. To prevent the production of melanin pigment, 0.003% PTU was administered to each experiment one day after fertilization [(2)].

HERBAL PLANT:

S. No	Plant Name & Family	Parts Used	Extract Used	Secondary Metabolites	Chemical Constituents	Uses
01	*Acanthus ilicifolius linn* **Family:** Acabthacea	Leaves	Alcohol, Aqueous, Methanol	Alkaloids, Flavonoids, Tannins, Phenol	Acancifolioside, Acteoside Isoacteoside, Acanthaminoside	Hepatitis, Joint inflammationAsthma (2-6)
02	*Acanthacea-Andrographis Paniculata* **Family:** Acanthaceae	Leaves	Alcohol	Flavonoids, Alkaloids	Andrograpanin, Androgrphiside, Andrigrapholide	Common cold, Diarrhoea, Fever, Jaundice, Antioxidant (7-9)
03	*Rhinacanthus nasuta* **Family:** Asteraceae	Root	Methanol	Alkaloids, Terpenoids, Flavonoids, Coumarin	Naphthoquinone ester, Rhinacanthin-C-D and –N	Scabies, Eczema and Various skin condition (7-9)
04	*Eclipta alba* **Family:** Asteraceae	Fresh leaves	Alcohol	Alkaloids, Terpenoids, Flavonoids, Steroids	Wedololactone, Demethyi wedelolactone Hentri acontanol	Hepatic disorder, Jaundice, Ulcers (7-9)
05	*Calendula officinalis* **Family:** Asteraceae	Flowers	Hydro alcoholic	Flavonoids, Triterpenes, Quinones	Alpha-cadinol, Trans-βocimene, Carvenone	Dermatitis, Anti-inflammatoryAntibacterial (7-9)
06	*Piper nigrum* **Family;** Asteraceae	Root	Aqueous, Ethanol, Chloroform	Flavonoids, Alkaloids, Terpenoids	B Caryophyllene, Limonene, Sabinene	Stomachache, Worms, Increased appetite (2-6)
07	*Beta vulgaris* **Family:** Amaranthaceae	Root	Ethanol	Flavonoids, Saponins, Polyphenols	Predominantly, Ellagic acid, Gallic, Cafferic acids	Anticancer, Antioxidant, Anti-inflammatory (2-6)
08	*Achillea millefolium* **Family:** Asteraceae	Aerial parts	70% Aqueous methanol	Flavonoids, Terpenoids, Cumarins	Artemisia ketone, Camph or linalyl acetate	Anti-hemorrhagic, Healing, Analgesic properties (2-6)
09	*Terminalia chebula* **Family:** Combretaceae	Seed	Methanol, Ethanol, Chloroform	Flavonoids, Alkaloids, Terpenoids	Chebulagic acid, Chebulinic acid, Gallic acid, Ellagic acid, Tannic acid	Increased appetite, Digestive aid, Liver stimulant (7-9)
10	*Apium graveolens l.* **Family:** Apiaceae	seed	Methanol	Alkaloids, Terpenoids, Flavonoids, Coumarin	Caffeic acid, Chlorogenic acid, Apiin, Apigenin, Rutaretin	Anti-inflammatory Arthritis, Rheumatism and Gout (7-9)
11	*Apocynum venetum l.* **Family:** Apocynaceae	leaves	Aqueous, Methanol, Ethanol	Flavonoids, Terpenoids, Cumarins	Vanillic acid, Adhyperforin, Hyperforin, Phytol	Anti-inflammatory Neurological diseases, High cholesterol (7-9)
12	*Trianthema portulacastrum l.* **Family:** Aizoaceae	leaves	Aqueous-methanol, Ethanol Chlorofrom	Flavonoids, Saponins, Polyphenols	Trianthrnol, 3-acetyleuritolic acid, 5,2-dihydroxy-7-methoxy-6,8-dimethylflavone	Analgesic, Stomachic, Laxative, anti-inflammatory (7-9)
13	*Sarcostemma brevistigma*	Stem bark	Ethyl acetate	Alkaloids, Flavonoids, Tannins	Aldehyde, Alkanes, Alkyl	Anti-diabetic, Throat and mouth infection,

	Family: Asclepiadaceae			Phenol	halides, Aromatics, nitro compounds	Jaundice [7-9]
14	*Cleome viscose l.* **Family:** Capparidaceae	leaves	ethanol	Flavonoids, Alkaloids, Saponins	Palmitic acid, Stearic acid, Oleic acid, Linoleic acid	HypertensionMalaria, Neurasthenia, Wound healing [7-9]
15	*Salacia reticulate* **Family:** Celasteraceae	root	Methanol, Ethanol	Alkaloids, Flavonoids, Terpenoids	1,3-diketones, Dulcitol, Phlobatanin, Triterpenes, Glycosidal tannins	Asthma, Rheumatism, Hemorrhoids, Itching and Swelling [7-9]
16	*Feijoa sellowiana* **Family:** Myrtaceae	Fruits peel	Methanol	PolyphenolsCarbohydrates, Vitamin A	Phenolic acids, Dietary fiber, Vitamin C, Potassium	Cholera, Dysentery [7-9]
17	*Ficus religiosa* **Family:** Moraceae	Leaves	Methanol	Flavonoids, Alkaloids, Saponins	Phenols, Steroids, β-sitosteryl-D-glycoside	Asthma, Diabetes, Diarrhea, Epilepsy [7-9]
18	*Fagonia schweinfurthii* **Family:** Zygophyllaceae	Whole plant	Ethanol	Total phenol, Flavonoids	Triterpenoids, Saponins, Sterols, Terpenoids, Coumarins, Glycoside	Jaundice, Diabetes, Joint pain, Dropsy, Cough [7-9]
19	*Acacia nilotica l.* **Family:** Fabaceae	Aerial parts	Methanol	Carbohydrate, Cardiac glycoside, Saponins, Tannins	Gallic acid, Dicatechin, Quercetin, Robidandiol	Oral and dental hygiene, Burn injuries, Skin infection [7-9]
20	*Astragalus kahiricus* **Family:** Fabaceae	Roots	Ethanol	Triterpenes, Flavonoids, Phenolic compounds	Amino acid, Trace elements, Anthraquinones	Immune system, Upper respiratory infection [7-9]
21	*Daucus carota* **Family:** Apiaceae	Seeds	Methanol	Monoterpenoids, Flavonoids, Quercetin	Phosphorus, Potassium, magnesium	Urinary calculus, Cystitis, Gout, Cancer [7-9]

Distribution and habitat:

It grows wild in a wide range of ecologies at elevations of up to 2000 meters. Sri Lanka, Bangladesh, Bhutan, Thailand, China, Indonesia, Pakistan, Malaysia, Nepal, Cambodia, and Vietnam are among its original countries. Madhya Pradesh, Uttar Pradesh, Punjab, and Maharashtra are the states in India where it is most frequently found.

Ecology: It is primarily found in teak-associated monsoon, mixed deciduous, or dry deciduous dipterocarp forests.

Biology: October through November is when it flowers, and November through December is when it bears fruit. In November, the tree sheds its leaves, and new ones emerge alongside flowers. limitations of biophysics.

Altitude: 0-2000m

Mean annual rainfall: 900-3000 mm

Mean annual temperature: 22-28°C

Soil type: It grows well loamy fertile soil with good drainage [15].

USES:

Abdominal disorders, bacterial infections, colds, sore throats, conjunctivitis, diarrhoea, dysentery, fever, gastric ulcers, headaches, heart diseases, hookworm, hypertension, jaundice, leprosy, nosebleed, edema, pneumonia, and skin diseases are just a few of the many illnesses that are commonly treated with herbs in traditional medicine across many continents [16].

MATERIALS AND METHODS:

There are several processes involved in the Soxhlet apparatus plant extraction process. First, the necessary quantity (about 10-20 grams) is weighed after the plant material has been dried and reduced to a fine powder. After that, the condenser is attached to the top of the device and the round-bottom flask is attached to the heating mantle to complete the Soxhlet apparatus. The plant material is put in a filter paper thimble and put into the Soxhlet apparatus after the solvent (such as ethanol, methanol, or hexane) has been poured to the round-bottom flask. The required compounds are then extracted over a period of hours (often 6-12 hours) by heating the solvent until it boils and rises into the condenser, where it cools and condenses before dropping back into the round-bottom flask. The final plant extract is obtained by filtering the extract through filter paper and evaporating the solvent either by air drying or using a rotary evaporator [17].

ANIMALS:

Zebrafish were kept in accordance with the committee on protocols and Institutional Animal care guidelines and utilized as animal models. The zebrafish were fed a typical fish diet. Adult zebrafish embryos were extracted using standard protocol [18].

PREPARATION OF METANOLIC, ETHANOIC, CHOLROFROM, ETHER EXTRACT OF HERBAL SEED:

In order to extract methanol using a Soxhlet apparatus, the chopped and shade-dried seeds were ground into a powder and run through a 40-mesh filter. In a rotary vacuum evaporator operating at reduced pressure, the solvent from the methanolic extract was totally eliminated and condensed to dryness at 40◦ educed pressure. In comparison to the dried starting material, the herbal seed produced a brown, semisolid residue of methanolic extract that weighed 9.0% w/w [19].

PREPARATION OF SUSPENSIONS:

Dimethyl sulfoxide (DMSO) was used to dissolve the methanolic extract of herbs (MEH). To get a stock solution of 1 mg/ml, the volume was increased to 10 ml and kept at -20°C before use. In order to get varying concentrations (15,250,500 µg/kg), further dilutions were prepared [19].

ACUTE TOXICITY STUDIES:

The acute oral toxicity study was conducted in accordance with Test No. 423 of the OECD's (Organization for Economic Cooperation & Development) criteria for chemical testing. The herbs' methanol extract was dissolved in water. The study employed five increasing doses of herb methanol extract (10, 50, 100, 500, and 1000 mg/kg). Seven fish were housed in each 4-liter glass tank following seven days of acclimation. For this test, 42 zebrafish in total-including a control-were used. Fish were not fed during the test. Semistatic testing was used, and the solutions were changed every 24 hours. Every 24 hours, the mortality and condition of the fish were assessed. The experiment lasted for ninety-six hours. Every 24 hours, measurements were made of the water's temperature (23±0.5◦C), PH (8.3 to 8.61), and oxygen saturation (above 60%). Oral administration of the extract increased in dosage upto 200 mg/kg [19].

METHODOLOGY:

In –vitro hepatoprotective activity by using a zebrafish:

i) DPPH radical scavenging activity:

1. By monitoring the drops in the absorbance of the metabolic solution of DPPH, the methanol extract of herbs (MEH) seed's capacity to scavenge free radicals was determined spectrophotometrically. One milliliter of METC extract at different concentrations (25, 50, 100, 200, 400, and 800 µg/ml) was combined with one milliliter of DPPH methanolic solution (100µM). Likewise, 1 ml of DPPH solution was combined with 1 ml of a methanolic ascorbic acid solution (100µg/ml).

2. As a control, 1 milliliter of DPPH (100µM) methanol solution was used. Following mixing, each solution was left in the dark for 20 minutes, and the absorbance at 517 nm was measured (Blois, 1958). The following formula was used to compute the scavenging activity, which was then represented as a percentage of inhibition. The experiments were conducted in triplicate [19].

$$\text{Scavenging \%} = \frac{\text{Absorbance of control-absorbance of test} \times 100}{\text{Absorbance of control}}$$

ii) Nitric oxide radical scavenging activity:

1. The Griess'reagent was used to measure the nitric oxide radical scavenging activity. 5 ml of sodium nitroprusside solution (5Mm) in standard phosphate buffer solution (pH 7.4) was incubated with 5 ml of each extract solution at varying concentrations (25, 50, 100, 200, 400, and 800 µg/ml) for 5 hours at 25◦C.

2. The same procedure was used to incubate 5 milliliters of ascorbic acid solution (100 µg/ml) in standard phosphate buffer solution (PH 7.4) with 5 milliliters of sodium nitroprusside mm) in the same solution.

3. Control tests were also carried out without the extract and using an equivalent volume of buffer. The absorbance was measured at 546 nm when 0.5 ml of the incubation mixture was combined with 0.5 ml of the griss'reagent (sulfanilamide 1%, O-phosphoric acid 2%, and naphthyl ethylene diamine dihydrochloride 0.1%). Scavenging activity was represented as a percentage of inhibition, and the experiments were carried out in triplicate [19].

REDUCING POWER:

Different extract concentrations (25, 50, 100, 400, and 800 µg/ml) in standard phosphate buffer solution (PH 6.6) were added to 2.5 ml of solutions, and the mixture was incubated for 20 minutes at 50◦c with 2.5ml of potassium ferricyanide solution (1%w/v). Likewise, a solution of ascorbic acid (100µg/ml) was incubated. Following incubation, each tube received 2.5ml of a 10% trichloroacetic acid solution, which was then centrifuged for 10 minutes at 650 rpm. The absorbance was measured at 700 nm after 5ml of the upper layer solution was combined with 5ml of deionized water and 1ml of ferric chloride solution (1%w/v) [19].

STATISTICAL ANALYSIS:

One-way analysis of variance (ANOVA) was used for the statistical analysis. The mean± SEM is used to represent the values. Turkey's multiple comparison test was used to assess the mean values of the groups treated with varying dose levels of extracts and positive controls. Significant was defined as $P<0.05$ [19].

ROUTE OF ADMINISTATION:

Immersion (Waterborne Exposure): Zebrafish are exposed to the test compound dissolved in water [20].

Intraperitoneal (IP) Injection: Test compounds are injected into the abdominal cavity of zebrafish [21].

Oral Gavage: Test compounds are administered directly into the stomach of zebrafish using a glass micropipette [22].

Microinjection: Test compounds are injected into specific tissue or cells of zebrafish embryos using a microinjector [23].

Other Routes:

- ✓ Intramuscular injection.
- ✓ Topical application.
- ✓ Food based delivery

DOSING VOLUMES:

Immression: 1-5ml of water per fish [20].

Intraperitonial Injection: 1-5ml per fish [21].

Oral Gavage: 1-5 ml per fish [22].

Microinjection: 1-100 ml per embryo [23].

DOSING FREQUENCY:

Acute Exposure: Single dose [20].

Chronic Exposure: Multiple doses over several days or weeks [24].

SAMPLING TIMES:

Acute Exposure: 1-24 hours after exposure [20].

Chronic Exposure: 24-72 hours after final exposure [24].

U**SEFUL TOOLS FOR ANALYSE OF ZEBRAFISH LIVER:**

Forward Genetics: In genetic research, forward genetics is a method that identifies the genes causing a specific phenotype or characteristic without first understanding how the genes function. Based on a phenotype-driven methodology, forward genetics uses mutagenic agents like N-ethyl-N-nitrosourea or retroviral insertions to cause random mutation in zebrafish [25].

Reverse Genetics: Disrupting target genes in zebrafish has always been more challenging than doing the same in mice. Established embryonic stem cell lines that enable homologous recombination with targeting vectors - a helpful method for mice are absent from Zebrafish. This limits the use of genetic recombination techniques. Targeting induced local lesions in genomes is known as tilling.

The method of the ZFN system (zinc Finger Nucleases).

The transcription activator-like effector nuclease technique, or TALEN System.
Coloured regularly interspaced short palindrome repeats, or CRISPR/Cas9, is a technique [24].

TRANSGENESIS:

Plasmid DNA encoding exogenous genes was microinjected into fertilized eggs to create the first transgenic zebrafish in 1988. A zebrafish strain that consistently expressed GFP was then reproduced in 1995. Transgenic zebrafish generation is a crucial method for viewing cells and examining gene activity. But these approaches are time-consuming and can provide low success rates [25].

CONCLUSION:

In conclusion, plants contain a variety of natural compounds that may be beneficial to human health. An effective method for assessing the hepatoprotective properties of herbs is the zebrafish model. Developing an efficient treatment plan and improving patient outcomes require a thorough understanding of liver disease. A major obstacle in drug development is drug-induced liver toxicity, which necessitates careful preclinical assessment. The hepatocytes of zebrafish and humans differ physiologically from one another. Variations in hepatocyte metabolism, drug metabolism, and detoxification pathways can impact how zebrafish react to different hepatotoxic substances and have an impact on how experimental results are interpreted. Herbal extracts like curcumin, silymarin, and andrographolide have been shown in recent research to be effective in preventing liver disease. For screening herbal substances, the zebrafish model provides a thorough and economical method. According to the results of these studies, using herbs to prevent and treat liver disease may be a beneficial strategy. To completely understand the hepatoprotective mechanism of herbs utilizing the zebrafish model, more research is required [26].

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