

MEDICAMENT FOR THE TREATMENT OF LIVER DISEASE, LIVER INJURY AND/OR HEPATOTOXICITY, BASED ON PLANT MATERIAL FROM THE *RAUWOLFIA SPP*, *ECLIPTA SPP* AND *PHYLLANTHUS SPP*

5 BACKGROUND

The application relates to the use of plant material selected from the group comprising *Rauwolfia spp*, *Eclipta spp* and *Phyllanthus spp* for the manufacture of a medicament for the treatment of liver disease, liver injury and/or hepatotoxicity. Polyherbal formulations utilise the concept of synergies as a result of positive herb-herb interaction. Without being bound by any theory, the
10 phytochemical constituents from one plant get activated in the presence of constituents from another plant.

EP0 890 360 B describes a polyherbal formulation for the treatment of conditions associated with hepatitis E and B virus infections. The formulation comprises 25-250 mg of extracts of each of the
15 following plants: *Rheum emodi* Wall., *Phyllanthus amarus* Linn., *Eclipta alba* Hassk., *Andrographis paniculate* Nees and *Picrorhiza Kurroa* Royle ex Benth.

US 6,136,316 provides a further polyherbal composition useful for treating Hepatitis E virus infection including acute liver failure. The formulation comprises extracts of *Rheum emodi* Wall.,
20 *Phyllanthus amarus* Linn., *Eclipta alba* Hassk., *Andrographis paniculate* Nees, and *Picrorhiza kurroa* Royle ex Benth.

The existing formulations and compositions however are complex mixtures, and with increasing numbers of ingredients comes an increase in the cost of manufacture, an increased risk of adverse
25 side effects, and importantly, an increased risk of herb-herb interactions that are not synergistic and may in fact be detrimental to the overall activity of the formulations by negating the individual effects of the ingredients.

Furthermore, existing formulations fail to take a holistic approach to treatment, often focusing on
30 singular aspects of the infection or disease. Liver diseases constitute a spectrum of conditions, encompassing viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD),

cirrhosis, and liver cancer. The effectiveness of current treatments varies based on the specific disease and its stage, presenting challenges and gaps in addressing these complex health issues.

5 One significant challenge lies in the limited treatment options for advanced liver diseases, particularly in cases of cirrhosis and liver cancer. Often, liver transplantation emerges as the sole viable option at this stage, underscoring the pressing need for alternative interventions.

10 Another hurdle in liver disease treatment relates to the side effects and tolerability of existing medications. Some drugs may induce significant side effects or prove poorly tolerated by certain patients, leading to non-compliance with prescribed treatment regimens and hindering overall efficacy. Furthermore, concerns about drug resistance continue to pose a threat to sustained therapeutic outcomes.

15 The emergence of non-alcoholic fatty liver disease (NAFLD) as a global health concern further highlights the gaps in current treatment approaches. While lifestyle modifications such as diet and exercise stand as primary recommendations, there exists a notable lack of targeted pharmacological interventions.

20 There is a need to provide new polyherbal formulations that seek to mitigate or overcome one or more of the above issues, and to provide new ingredients in the formulations with previously unknown activities for the treatment of liver disease, liver injury and/or hepatotoxicity.

SUMMARY

25 In one aspect of the invention there is provided a polyherbal formulation (PHF) comprising, or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- 30 c) plant or plant parts of the *Rauwolfia* family, especially *R serpentina*, in an amount of at least 25 %, preferably at least 27 %.

This PHF is preferably liver specific (i.e. a hepatic PHF) for the treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof.

- 5 In one embodiment, in relation to plant and plant parts, the PHF consists only of plant or plant parts of the *Eclipta* family, the *Phyllanthus* family, and the *Rauwolfia* family.

Preferably the PHF comprises not more than 70%, and preferably not more than 60%, plant or plant parts of the *Eclipta* family; not more than 30 % plant or plant parts of the *Phyllanthus* family;
10 and not more than 36 % plant or plant parts of the *Rauwolfia* family.

In a more preferred embodiment of this aspect of the invention there is a provided PHF comprising 43% to 46% plant or plant parts of the *Eclipta* family, 23% to 26% plant or plant parts of the *Phyllanthus* family and 29% to 32% plant or plant parts of the *Rauwolfia* family.

15 In a most preferred embodiment of this aspect of the invention the plants or plant parts are of *Eclipta alba*, *Phyllanthus amarus* and *Rauwolfia serpentine*.

The plant may be the whole plant; the plant parts may be one or more of fruit, seeds, rhizome,
20 buds, leaves, husk, bark, petals and petal extracts, fruit cover, seed cover and stems.

In each of the above mentioned embodiments the PHF may further comprise one or more secondary metabolites selected from flavonoids, saponins, coumestan, phenols, alkaloids, tannins and lignans.

25 In another aspect of the invention there is provided a use of a polyherbal formulation in the preparation of a medicament for the treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof, wherein the medicament comprises, or consists of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least
30 30%, preferably at least 35 or 40%; and

- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %.

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In another aspect of the invention there is provided a method of treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof comprising the administration of a polyherbal formulation comprising or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %.

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In another aspect of the invention there is a provided a polyherbal formulation comprising or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %.

for use in treating liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof.

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In each of the aspects relating to treatment of liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof, the PHF preferably comprises 43% to 46% plant or plant parts of the *Eclipta* family, 23% to 26% plant or plant parts of the *Phyllanthus* family and 29% to 32% plant or plant parts of the *Rauwolfia* family.

Further aspects of the present invention and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

5 BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the cytotoxicity of PHF, specifically the percentage inhibition of PHF. Specifically, it supports the IC_{50} determination of PHF towards epithelial cells. The IC_{50} of PHF towards epithelial cell lines was determined at 46.21 μ g/mL.

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FIG. 2 illustrates the cellulose fiber content in PHF.

DEFINITIONS

By “polyherbal formulation” (PHF) it is meant a preparation that uses more than one herb as a component for increased therapeutic effectiveness and decreased toxicity of individual herbs. The term “formulation” is used interchangeably with “composition”, and the abbreviation PHF is used interchangeably with polyherbal formulation. It is intended to be specific for the treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof, and may be referred to herein as a liver specific PHF, or hepatic PHF.

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By “plant and plant parts” it is meant either the whole plant, pieces of the whole plant (eg a stem of a plant), or parts of a plant including but not limited to fruit, seeds, rhizome, buds, leaves, husk, bark, petals and petal extracts, fruit cover, seed cover and stems. Once the plant and plant parts are obtained, cut in to smaller pieces if need be, and then preferably dried to a powder, they may be referred to herein as “processed plant or plant parts”.

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By “derived from” in the context of plant parts, it is meant that the parts are either produced by the plant and isolated or collected (eg seeds, fruits) or the parts are extracts of the plant (eg petal extracts) or the parts are removed from the plant.

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For purposes of interpreting this specification, terms used in the singular will also include the plural and vice versa.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude further additives, components, integers or steps.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 5\%$, in some instances $\pm 1\%$, and in some instances $\pm 0.1\%$ from the specified value, as such variations are understood by the skilled person to be appropriate to perform the disclosed methods.

Throughout this disclosure, various aspects of the disclosure can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosure. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

The term "pharmaceutically acceptable" as used herein pertains to compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgement, suitable for use in contact with the tissues of a subject (e.g. human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

As used herein, "pharmaceutically acceptable excipient" means a pharmaceutically acceptable material which is included in the composition for a purpose other than pharmaceutical efficacy

(this is not intended to exclude materials which may have some biological effect). Suitable carriers, excipients, etc. can be found in standard pharmaceutical texts, for example, Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990.

5 As used herein, "preventing" or "prevention" is intended to refer to at least the reduction of likelihood of the risk of (or susceptibility to) acquiring a disease or condition (i.e., causing at least one of the clinical symptoms of the disease not to develop in an individual that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease). Biological and physiological parameters for identifying such patients are provided
10 herein and are also well known by physicians. The skilled artisan will appreciate that "prevention" is not an absolute term. In particularly preferred embodiments, the methods of the present invention can be to prevent or reduce the severity, or inhibit or minimize progression, of a symptom of a disease or condition as described herein. As such, the methods of the present invention have utility as treatments as well as prophylaxes.

15 The terms "treatment" or "treating" of a subject includes delaying, slowing, stabilising, curing, healing, alleviating, relieving, altering, remedying, less worsening, ameliorating, improving, or affecting the disease or condition, the symptom of the disease or condition, or the risk of (or susceptibility to) the disease or condition. The term "treating" refers to any indication of success
20 in the treatment or amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; lessening of the rate of worsening; lessening severity of the disease; stabilization, diminishing of symptoms or making the injury, pathology or condition more tolerable to the individual; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating. Treatment may not necessarily result in
25 the complete clearance of a disease or disorder but may reduce or minimise complications and side effects of infection and the progression of a disease or disorder. The success or otherwise of treatment may be monitored by, amongst other things, physical examination of the individual, CT scan, MRI, or blood biomarkers.

30 The term "therapeutically-effective amount," as used herein, pertains to that amount of an active compound, or a material, composition or dosage form comprising an active compound, or a

treatment regime and the components thereof, which is effective for producing some desired therapeutic effect, commensurate with a reasonable benefit/risk ratio, when administered in accordance with a desired treatment regimen.

- 5 A subject may be a eukaryote, an animal, a vertebrate animal, a mammal, a rodent (e.g. a guinea pig, a hamster, a rat, a mouse), murine (e.g. a mouse), canine (e.g. a dog), feline (e.g. a cat), equine (e.g. a horse), a primate, simian (e.g. a monkey or ape), a monkey (e.g. marmoset, baboon), an ape (e.g. gorilla, chimpanzee, orang-utan, gibbon), or a human.

10 DETAILED DESCRIPTION

This application aims to provide a further polyherbal formulation (PHF) to be used in the holistic treatment of liver diseases liver injury and/or hepatotoxicity using combinations of ingredients not all known to previously have any effect or role in those conditions. For example, the PHF of the
15 invention includes plants from the *Rauwolfia* family, and in particular *Rauwolfia serpentina*, which has historically been employed in the treatment of sleeping disorders and insomnia. The PHF of the invention also seeks to be being highly effective and being easy to prepare and having reduced side effects compared to existing PHFs.

20 PHF according to the present application comprises or consists of three medicinal plants from the *Rauwolfia* family, *Eclipta* family and *Phyllanthus* family, and in particular *Rauwolfia serpentina*, *Eclipta alba* and *Phyllanthus amarus*. The plant *Rauwolfia serpentina* belongs to the family of *Apocynaceae*, *Eclipta alba* belongs to the family of *Asteraceae* and *P. amarus* belongs to the family of *Phyllanthaceae*. In a preferred embodiment, while the PHF may contain other
25 components and active ingredients, it only contains plant or plant parts from the *Rauwolfia* family, the *Eclipta* family and the *Phyllanthus* family.

For the preparation of the drug or pharmaceutical according to the application, the plant or plant parts are cut into smaller pieces if need be, and then preferably dried to be processed plant or plant
30 parts. In a further preferred embodiment, the plant parts are also sterilized. Sterilization can be

readily performed by conventional known means, but pasteurization by means of microwave and/or by means of UV irradiation is preferred.

Whole plants or any parts of the plants may be utilised in the PHF of the invention, including combinations of one or more of fruit, seeds, rhizome, buds, leaves, husk, bark, petals and petal extracts, fruit cover, seed cover and stems. Collected plant parts are preferably washed, cut into small pieces, dried, and crushed or ground in to powder form by conventional means. The powdered plant and plant parts can then be used in the PHF formulation.

10 Accordingly, there is provided a polyherbal formulation comprising, or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- 15 c) plant or plant parts of the *Rauwolfia* family, especially *R serpentina*, in an amount of at least 25 %, preferably at least 27 %.

Preferably, the PHF comprises:

- not more than 70%, and preferably not more than 60%, plant or plant parts of the *Eclipta* family;
- 20 not more than 30 % plant or plant parts of the *Phyllanthus* family; and
- not more than 36 % plant or plant parts of the *Rauwolfia* family.

Even more preferred, the PHF comprises 43% to 46% plant or plant parts of the *Eclipta* family, 23% to 26% plant or plant parts of the *Phyllanthus* family and 29% to 32% plant or plant parts of the *Rauwolfia* family. For a 100mg sample of the PHF of this embodiment, it preferably comprises 44.5 mg to 44.85 mg of plant or plant parts of the *Eclipta* family, 24.5 mg to 24.6 mg of plant or plant parts of the *Phyllanthus* family and 30.6 mg to 30.7 mg of plant or plant parts of the *Rauwolfia* family.

30 The plant or plant parts of the PHF are most preferably plant and plant parts from *Eclipta alba*, *Phyllanthus amarus* and *Rauwolfia serpentine*. In turn, it may be the whole plant utilised in the

PHF of the invention, or a part of the whole plant such as a branch or stem of the plant, or a part of the plant such as fruit, seeds, rhizome, buds, leaves, husk, bark, petals and petal extracts, fruit cover, and seed cover.

- 5 The plant and plant parts can be obtained by any known manner. They are then preferably dried by known drying means and powdered by known means to provide what shall be referred to herein as processed plants/plant parts.

10 The PHF suitably has a pH of from 4.5 to 6.5 (preferably from 4.5 to 5). Further, PHF according to the application is dissolved in a solvent. Preferred solvents include water, chloroform, ethanol and the ethyl ethers. For human formulations, the PHF is prepared with pharmaceutically acceptable excipients.

15 In addition to the plant and plant parts in the PHF, the PHF may also include other non-plant derived active ingredients one or more secondary metabolites selected from flavonoids, saponins, coumestan, phenols, alkaloids, tannins and lignans.

20 The PHF may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the processed plant or plant parts from the *Rauwolfia* family, the *Eclipta* family and the *Phyllanthus* family with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the processed plants/plant parts with liquid carriers or finely divided solid carriers or both.

25 Formulations may be in the form of liquids, solutions, suspensions, emulsions, elixirs, syrups, tablets, lozenges, granules, powders, capsules, cachets, pills, ampoules, suppositories, pessaries, ointments, gels, pastes, creams, sprays, mists, foams, lotions, oils, boluses, electuaries, or aerosols.

30 Formulations suitable for oral administration (e.g. by ingestion) may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the tartrate salt; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an

oil-in-water liquid emulsion or a water-in-oil liquid emulsion; as a bolus; as an electuary; or as a paste.

Uses of the PHF

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It is envisaged that the PHF of the invention will be useful for the treatment of a liver disease, hepatic injury, or hepatotoxicity. Liver diseases constitute a diverse spectrum of conditions, encompassing viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), cirrhosis, and liver cancer.

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Viral hepatitis may be any virus known to cause inflammation of the liver hepatocytes, but is commonly Hepatitis A virus, Hepatitis B virus or Hepatitis C virus.

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The emergence of non-alcoholic fatty liver disease (NAFLD) as a global health concern further highlights the gaps in current treatment approaches. While lifestyle modifications such as diet and exercise stand as primary recommendations, there exists a notable lack of targeted pharmacological interventions.

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In particular, in one aspect, PHF of the invention is expected to offer an alternative option for treating nonalcoholic fatty liver disease NAFLD, which is a continuous spectrum of diseases characterised by excessive lipid accumulation in hepatocytes. It progresses from simple liver steatosis to non-alcoholic steatohepatitis (NASH) and in more severe cases, to liver fibrosis and cirrhosis.

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In another aspect, the PHF of the invention is also expected to offer an alternative option for treating fatty liver disease that is the most common chronic liver disease in Western society. It is not caused by alcohol or hepatitis virus, but by the accumulation of fatty acid in liver parenchymal cells in the form of triglycerides. In particular, histological fat accumulation in the liver is a feature of non-alcoholic fatty liver disease and manifests as various symptoms including simple steatosis, steatohepatitis and cirrhosis. Also, fatty liver disease increases insulin resistance, which promotes

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decomposition of adipose tissue. The resulting increased fatty acid supply to the liver promotes fat oxidation that induces fat accumulation in the liver cells.

In another aspect the PHF of the invention is expected to offer an alternative option for providing protection against these processes. Evaluation of hepatic content of MDA by lipid peroxidation can directly damage hepatocytes as well as increase hepatic inflammation and can mediate fibrogenesis.

In another aspect the PHF of the invention is expected to offer an alternative option for treating hepatic steatosis that is the hallmark feature of NAFLD, whereby fat droplets accumulate in the form of triglycerides in hepatocytes. NAFLD is histologically diagnosed when accumulation occurs in >5% of hepatocytes.

Further to the above, in another aspect the PHF of the invention is expected to be an alternative therapeutic in the following conditions described below. Triglycerides in the livers with NAFLD derive from esterification of glycerol and free fatty acids (FFAs). Triglyceride accumulation occurs when the rate of import or synthesis of FFAs by hepatocytes exceeds the rate of export or catabolism. Obesity and particularly insulin resistance (IR) are tightly associated with the genesis of NAFLD. Over expression of tumour necrosis factor (TNF)- α activates I κ B kinase β , which plays an important role in IR development by inhibiting the phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2. IR leads to an increase in the liver triglyceride level and ultimately liver steatosis through various mechanisms. Insulin fails to suppress adipose tissue lipolysis via hormone-sensitive lipase, resulting in increased efflux of FFAs into the circulation and consequent uptake by the liver.

In another aspect the PHF of the invention is expected to be beneficial on the metabolic alterations in the lipid profile, glucose homeostasis and the functional and structural changes of hepatocytes in NAFLD induced by a high fat diet.

In another aspect the PHF of the invention is expected be an alternative therapeutic with Hepatitis virus inhibitory activity, preferably Hepatitis A, B or C virus inhibitory activity, and most

preferably Hepatitis B virus (HBV) inhibitory activity, and to treat liver fibrosis, cirrhosis and NAFLD.

Accordingly there is provided a use of a polyherbal formulation in the preparation of a medicament for the treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof, wherein the medicament comprises, or consists of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %.

In another aspect of the invention there is provided a method of treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof comprising the administration of a polyherbal formulation comprising or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %.

In another aspect of the invention there is provided a polyherbal formulation comprising or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %

for use in treating liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof.

In each of the aspects relating to treatment of liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof, the PHF preferably comprises 43% to 46% plant or plant parts of the
5 *Eclipta* family, 23% to 26% plant or plant parts of the *Phyllanthus* family and 29% to 32% plant or plant parts of the *Rauwolfia* family.

In an embodiment of each of the above aspects of uses of the invention, the subject is infected with a virus that causes hepatitis, preferably selected from Hepatitis A virus, Hepatitis B virus and
10 Hepatitis C virus; most preferably Hepatitis B virus (HBV). The HBV life cycle comprises key stages, notably attachment and replication of viral DNA, with the end stage associated with liver cell damage. HBV induces liver inflammation, leading to potential long-term consequences, including the progression from inflammation to fibrosis and, in severe cases, cirrhosis, negatively impacting liver function.

15 A comprehensive approach to combat HBV therefore involves adopting a strategy that addresses viral replication and entry, with a focus on eliminating the virus while mitigating liver damage. Central to this approach is the reduction of inflammation and the facilitation of liver cell recovery. In pursuit of this holistic strategy, the 3 ingredients of the PHF of the invention have been selected
20 because of their unique properties to control HBV infection and promote liver cell repair.

Accordingly, there is provided a use of a polyherbal formulation in the preparation of a medicament for the treatment of Hepatitis Virus infection, in a subject in need thereof, wherein the polyherbal formulation of the medicament comprises, or consists of:

- 25 a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount
30 of at least 25 %, preferably at least 27 %.

In another aspect of the invention there is a provided a polyherbal formulation comprising or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- 5 b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %

for use in treatment of Hepatitis Virus infection in a subject in need thereof.

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The Hepatitis Virus is preferably selected from Hepatitis A virus, Hepatitis B virus and Hepatitis C virus; most preferably Hepatitis B Virus (HBV).

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The present disclosure should in no way be limited to the exemplary implementations, examples, and techniques illustrated in the drawings and described below.

EXAMPLES

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The invention will now be described by reference to the following non-limiting examples. A number of different plants and combinations were tested, but the PHF of *Rauwolfia* spp, *Eclipta* spp, and *Phyllanthus* spp exhibited the most promising activity for a spectrum of liver conditions.

In vitro studies evaluated individual plants but found their efficacy to be limited. Moreover, if only two ingredients were used, such combinations had lower efficacy.

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PHYSICAL CHARACTERISTICS OF PHF

Moisture content

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The moisture content of PHF was measured by placing different masses of PHF powder in an accurately weighed moisture disc. For estimation of loss on drying, it was dried at 105 °C for 3 hours in a hot air oven, cooled in a desiccator for 30 minutes and weighed without delay. The loss of weight was calculated as the content of air-dried material (as a percentage).

pH determination

The pH of PHF was determined by preparing stock solutions containing different percentages of PHF. The stock solutions were prepared by dissolving the desired masses of PHF in 100 mL of distilled water. The pH of these stock solutions was determined using a standard simple glass electrode pH meter.

PHYTOCHEMICAL ANALYSIS

10 Total alkaloid content

PHF powder (25, 50 and 100 mg) was added with 5 mL of PBS (pH 4.7), 5 mL of 0.1 % Bromocresol green solution and 4 mL of chloroform into a separatory funnel. Once the BCG-chloroform complexes are formed, (1, 2, 3, and 4) mL of chloroform was added consecutively to extract the complexes along with vigorous shaking. The extract was collected in a 10 mL volumetric flask and the volume was marked up by chloroform. The absorbance of complexes in chloroform was determined against blank at 470 nm by a visible spectrophotometer. Atropine standard solutions (0.5, 1.0, 1.5, 2.0 and 2.5) mg/mL were prepared by adhering to the procedures mentioned above. The atropine calibration curve was plotted against absorbance at 470 nm spectrophotometrically. Total alkaloid content (TAC) was determined by the equation shown below and expressed as mg of atropine equivalent (AE) per gram of crude extract (John et al., 2015).

$$\text{TAC} = \frac{C \times V}{m}$$

where C is the concentration result obtained from the calibration curve, V is the volume of the crude extract stock solution and m is the weight of PHF powder used.

Total flavonoid content

The rutin stock (1 mg/mL) was made by dissolving 100 mg of rutin in 100 mL of methanol and then diluted to (0.02, 0.04, 0.06, 0.08 and 0.10 mg/mL) rutin standard solutions. Each standard

solution was added with 0.1 mL of 20 % methanolic aluminium chloride and a drop of acetic acid. The final volume (5 mL) was made up by CH₃OH. Upon completion of 40 minutes incubation, the absorbance of rutin standard was determined at 415 nm by a visible spectrophotometer. Thereafter, PHF powder (25, 50 and 100 mg) was added with the same analytical reagents as described. After 40 minutes, the absorbance of each concentration was determined at 415 nm. Total flavonoid content (TFC) was then computed by the equation below and expressed as mg of rutin equivalent (RE) per gram of crude extract (Rao et al., 2016).

$$TFC = \frac{C \times V}{m}$$

where C is the concentration result obtained from the calibration curve, V is the volume of the crude extract stock solution and m is the weight of PHF powder used.

Total phenol content

PHF powder (25, 50 and 100 mg) was added with 1 mL of Folin-Ciocalteu reagent and 2 mL of 7.5 % sodium carbonate. Total volume was made to 7 mL with addition of dH₂O and kept for 2 hours incubation in the dark at room temperature. The absorbance was measured at 760 nm by a visible spectrophotometer. Gallic acid standard solutions (0.02, 0.04, 0.06, 0.08, 0.10 mg/mL) were prepared by the same analytical reagents mentioned and gallic acid standard curve was plotted against blank and the corresponding absorbance was measured at 760 nm. Total phenol content (TPhC) was computed by the following formula and expressed in mg of gallic acid equivalents (GAE) per gram of crude extract (Shamsa et al., 2008).

$$TPhC = \frac{C \times V}{m}$$

where C is the concentration result obtained from the calibration curve, V is the volume of the stock solution and m is the weight of PHF powder used.

Total tannin content

PHF powder (25, 50 and 100 mg) was added with 500 µL of FCR, followed by the addition of 1 mL 7.5 % Na₂CO₃ solution and 8 mL dH₂O. The reaction mixture was allowed to stand for 30 minutes at room temperature. The supernatant was obtained by centrifugation and absorbance was

determined at 725 nm. Tannic acid standard solution concentrations (0.02, 0.04, 0.06, 0.08, 0.10, 0.2, 0.4, 0.6, 0.8, and 1.0) mg/mL were prepared by the same analytical reagents mentioned and a tannic acid standard curve was plotted against blank and the corresponding absorbance was measured at 725 nm. Total tannin content (TTC) was computed by the following formula and expressed in mg of tannic acid equivalents (TE) per gram of crude extract (Frederick and V. Mani, 2016).

$$TTC = \frac{C \times V}{m}$$

where C is the concentration result obtained from the calibration curve, V is the volume of the stock solution and m is the weight of PHF powder used.

Total protein content

PHF powder (25, 50 and 100 mg) was added with 2 mL of alkaline copper sulphate reagent, mixed well by a magnetic stirrer at 300 rpm and incubated at room temperature for 10 minutes. After that, 0.2 mL of FCR was added, followed by incubation at room temperature for another 30 minutes. The absorbance of stem crude extract against blank was determined at 660 nm by a visible spectrophotometer. Bovine serum albumin standard solutions (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) was prepared with the same protocol and the BSA standard curve was plotted against 660 nm. Total protein content (TPC) was computed by the equation below and expressed as mg of BSA equivalent (BE) per gram of crude extract (Prabhavathi et al., 2016).

$$TPC = \frac{C \times V}{m}$$

where C is the concentration result obtained from the calibration curve, V is the volume of the stock solution and m is the weight of PHF powder used.

Total saponin content

PHF powder (25, 50 and 100 mg) was dissolved in 10 and 20 mL of 20 % ethanol, respectively. Then, the mixture was heated in a hot water bath at 55 °C for 4 hours of continuous stirring. The

residue obtained after vacuum filtration was re-extracted with 10 and 20 mL of 20 % C₂H₅OH and decreased to 4 and 6 mL over a hot water bath at 80 °C. The concentrated solution was mixed vigorously with 2 and 6 mL of diethyl ether each in a separating funnel. About 4 and 8 mL of butanol were added to the collected aqueous layer. It was then washed with 2 and 6 mL of 5 % w/v aqueous sodium chloride. The whole mixture was heated over a hot water bath and later oven-dried at 40 °C to a constant weight.

Cytotoxicity

- 10 A PHF liver composition for 100 mg was prepared with a combination of 44.75 mg of *Eclipta alba*, 24.58 mg of *Phyllanthus amarus* and 30.66 mg of *Rauwolfia serpentina*. (all in the dried state).

The cytotoxicity of PHF powder was assessed by a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity of PHF powder at different concentrations was performed on A2780 epithelial cell line.

A2780 epithelial cell line was revived and thawed; transferred into falcon tube; 3 mL of fetal calf serum was added; 4 mL of complete growth RPMI-1640 with 10 % FCS was added and the cells were centrifuged at 1500 g for 6 minutes. The supernatant was decant; pellet was suspended with 6 mL of 1 X Dulbecco's Phosphate Buffer Saline (DPBS) and centrifuged at 1500 g for 6 minutes. Following centrifugation, supernatant was decanted and the cells were suspended with 4 mL of complete growth RPMI-1640 with 10 % FCS. The cells were seeded to T-25 flask. About 6 mL of complete growth RPMI-1640 with 10 % FCS was added and incubated in 5 % CO₂ incubator at 37 °C. The cells were monitored regularly for formation of 100 % confluent monolayer.

Extract Preparation

The PHF stock solution was prepared by dissolving the PHF powder in dimethyl sulfoxide. Various concentrations of PHF suspension were prepared by two-fold dilution.

Cytotoxicity Determination

About 200 µL of freshly harvested A2780 epithelial cell line was seeded into each well of 96 well microtiter plate. Then, the wells with cell lines were added with 100 µL of complete growth media.

- 5 The respective plate was incubated at 37 °C for 24 hours in a humidified 5 % CO₂ incubator to attain 100 % confluent cells.

- After 24 hours, the test plate was returned to aseptic condition and the media from epithelial cell lines was decanted. Then, 100 µL of each PHF concentration was added to the test wells and 100
10 µL of complete growth was added to the negative and cell control wells. The plate was incubated at 37 °C in a humidified 5 % CO₂ incubator for 2 hours. After 2 hours, PHF samples from the epithelial cell line were decanted and the cells were washed thrice with 1 X DPBS.

- About 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT Dye
15 Solution was added to each well in a dark condition; the plate was wrapped with aluminium foil and incubated at 37°C for 4 hours in a humidified 5% CO₂ incubator. After incubation, the formation of formazan crystals was photographed using an inverted phase-contrast microscope. Later, 100 µL of the solubilization solution was added to each well. The plate was incubated for 15 minutes and the content of the wells was mixed to get a uniformly colored solution. The
20 absorbance value was taken at wavelength 570 nm.

Major structural components of PHF

- Fourier Transform Infrared Spectroscopy analysis was performed for PHF powder using the FTIR
25 facility. About 0.5 mg of PHF powder and 1 g of anhydrous potassium bromide (KBr) was made into pellet by hydraulic press. The sample pellet was transferred to the sample holder and FTIR analysis was performed for PHF powder.

IN VITRO ANTIOXIDANT ACTIVITY

30

ABTS antioxidant assay

The working solution was prepared by mixing 1 mL of ABTS solution with 88 mL of 50 % ethanol. Various concentrations of PHF powder were prepared by two-fold dilution. L-Ascorbic acid was used as a positive control.

5

The positive control was prepared by adding 250 µL of each L-ascorbic acid concentration and 1250 µL of ABTS working solution. The negative control was prepared by adding 250 µL of methanol and 1250 µL of ABTS working solution. The test sample was prepared by adding 250 µL of different concentrations of PHF suspension with 1250 µL of ABTS working solution, and allowed to stand for 4 minutes at room temperature. The absorbance was read at 734 nm in a visible spectrophotometer. The results have been expressed as ascorbic acid equivalent which was used as a standard. The radical scavenging activity in percentage of PHF powder and ascorbic acid were calculated by the following equation:

10

$$\text{Percentage of radical scavenging activity (\%)} = \frac{Ac - As}{Ac} \times 100$$

15

where Ac is the absorbance of control and As is the absorbance of test samples. The percentage of ABTS radical scavenging activity was plotted and the IC₅₀ were determined.

DPPH antioxidant assay

20

The different concentrations of PHF suspension were added with 2 mL of 0.3 mM DPPH. The reaction mixture was incubated in the dark at room temperature. Exactly 30 minutes after incubation, the absorbance was measured at 517 nm by a visible spectrophotometer.

25

Ascorbic acid was used as positive control. Each ascorbic acid dilution was added with 2 mL of 0.3 mM DPPH and the absorbance was also measured at 517 nm after 30 minutes of incubation at dark. The DPPH (0.3 mM) was employed as control whereas CH₃OH was used as blank. The radical scavenging activity in percentage of PHF powder and ascorbic acid were calculated. The percentage of DPPH radical scavenging activity was plotted and the IC₅₀ were determined.

30

Immunomodulatory effect of PHF

Peripheral blood mononuclear cells were freshly isolated and seeded (2×10^6 cells/well) into 12 well culture plate. Different concentrations of PHF liver extract were used and the PHF treated cells were served as positive control. The untreated cells were served as negative control. The supernatant was collected at 24, 48 and 72 hours post stimulation. The supernatants were screened for IL2, IL4, and IFN γ cytokines.

ANTI-HBV ACTIVITY

10 *In silico* anti-HBV activity

Protein preparation

Initially required protein sequences were downloaded from Genbank (NCBI) and were subjected to protein blast. The sequences with high similarity index were then taken into consideration and used to perform protein modelling. Different conforms of the same protein were identified using uniprot database analysis and used for homology protein modelling. Protein modelling of different conforms was carried out using Swiss model and the models were analyzed using structural assessment tool in the Swiss bioinformatics database. Conforms showing high Ramachandran plot value and low clash score were selected for further process. Qmean value of the model was also estimated to analyze the integrity of the developed model (Homology protein structure).

Table 1 illustrates the protein modelling of different conforms analysed using structural assessment tool in the Swiss bioinformatics database.

25 **Table 1**

Protein	Model	Ramachandran flavoured (%)	Clash score	Q Mean
Human Hepatitis B Virus polymerase	1	89.10	8.83	0.43
	2	85.58	8.65	0.39
	3	84.30	10.20	0.37

	4	90.91	10.66	0.30
Sodium taurocholate co- transporting polypeptide (ntcp)	1	94.35	16.53	0.59
	2	90.62	4.53	0.41
	3	89.41	5.09	0.31

Selected conformers: Human Hepatitis B Virus polymerase - 1

Preparation of ligand and target protein

- 5 Generated protein models were utilized for the process of docking. Three dimensional protein structures were analyzed for errors using pymol software. Similarly, the ions, molecules and standard inhibitors if present were also removed using pymol visualization tool.

Anti-HBsAg of PHF

- 10 Equal volume of HBsAg positive plasma and PHF were mixed and incubated at 37°C for 5 days. The mixture was assayed daily using commercial HBsAg ELISA Kit for 5 days. Tubes containing plasma with solvent alone were used as a control. The binding effects of the extracts were analyzed everyday.

In vivo toxicity study

In vivo evaluation of the acute toxicity of the test substance in mice

- 20 Animals were observed for the sign of toxicity including changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory and behavior patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep. Internal organs (brain, heart, lung, spleen, stomach, kidney, liver) were dissected, cleaned of any fat, weighed and observed macroscopically.

In vivo evaluation of the sub-acute toxicity of the test substance in mice

Subacute oral toxicity study of PHF was performed using female Swiss albino mice according to the OECD guideline 407. A total of 18 female mice were used for the study. The mice were randomly divided into 3 groups of 6, and the amount of PHF to be administered to the mice per unit weight for each of the 3 groups was 100mg/kg, 200mg/kg and 400mg/kg.

Experimental design of sub-acute toxicity study

Table 2 illustrates the experimental design of the three groups.

Table 2

Group-I	100mg/kg group receives PHF orally for 60 days (n=6)
Group-II	200mg/kg group receives PHF orally for 60 days (n=6)
Group-III	400mg/kg group receives PHF orally for 60 days (n=6)

Experimental protocol

PHF was made a suspension with Tween 80, a Polysorbate that is a nonionic surfactant and emulsifier, a synthetic compound in the form of a viscous, water-soluble yellow liquid. Volume was made up with distilled water and administered to 3 different groups of mice daily for up to 60 days consecutive days, with each group being administered different dosages, namely 100 mg/kg, 200 mg/kg and 400 mg/kg. The mice in all of the groups were maintained under the same conditions with normal food and water. They were observed for mortality, signs of toxicity and behavioral changes. Body weight changes and food intake were also recorded from the day of the study. On Day 0 and 60, they were weighed, urine sample and blood samples were collected for various biochemical analyses and sacrificed by an overdose inhalation of the anesthetic ether and it was followed by histopathological studies.

Biochemical parameters and haematology

5

On Day 0 and Day 60, urine samples and blood samples of mice in all groups were collected. The blood is used for hematological study like red blood count (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, white blood count (WBC), lymphocyte, monocyte, eosinophils and neutrophil and blood Serum and urine sample used for various biochemical parameters like alkaline phosphates (ALP), serum urea, serum glucose, serum cholesterol, serum total protein, serum total bilirubin, serum aspartate aminotransferase (AST), serum creatinine analyse.

10

15 *Histopathological Studies*

Necropsy and gross examination of internal organs (liver, heart, kidney, uterus, brain, spleen, lungs, stomach, ovary, pancreas, trachea, ileum, oesophagus, large intestine, aorta, bone and skin) were carried out. Then, the aforesaid internal organs were dissected, cleaned of any fat, weighed and observed macroscopically. Finally, they were fixed in a 10% buffered formalin solution for histopathological examination.

20

In vivo evaluation of the effect of the test substance for liver fibrosis in rats

25 Table 3 illustrates the experimental design of the six groups.

Table 3

Group-I	Normal rats receive vehicle orally and normal diet pellets. (n=6)
Group-II	Control receives vehicle orally and paracetamol (1gm/kg). (n=6)
Group-III	Standard Silymarin (200mg/kg) and paracetamol (1gm/kg). (n=6)
Group-IV	Paracetamol (1gm/kg) and PHF (100mg/kg). (n=6)
Group-V	Paracetamol (1gm/kg) and PHF (200mg/kg). (n=6)

Group-VI	Paracetamol (1gm/kg) and PHF (400mg/kg). (n=6)
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Treatment protocol

- 5 Animals are divided into six groups each group comprising of six animals. All the animals except the normal group were given paracetamol (1gm/kg, p.o.) daily for 7 days.

Group I received only vehicle for 7 days and served as Normal control. Group II served as Negative control and received paracetamol (1gm/kg, p.o.) for first 7 days. Group III served as the positive control and received paracetamol for first 7 days and Silymarin (200mg/kg, p.o.). Group IV received paracetamol (1gm/kg, p.o.) for first 7 days as well as lower dosage of PHF (100mg/kg) from Day 0 to Day 12. Group V received paracetamol (1gm/kg, p.o.) for first 7 days as well as moderate dosage of PHF (200mg/kg) from Day 0 to Day 12. Group VI received paracetamol (1gm/kg, p.o.) for first 7 days as well as higher dosage of PHF (400mg/kg) from Day 0 to Day 12.

15

Blood biochemical analysis

On Day 13, blood samples of rats in all groups were collected. The serum was separated and used for the various biochemical parameters like alkaline phosphates (ALP), serum total protein, serum total bilirubin, serum AST, serum alanine aminotransferase (ALT), Globulin, Albumin, Gamma glutamyl transferase (GGT) analyses.

20

Histopathological studies

- 25 Livers were collected in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6 μ m in thickness were cut and stained with hematoxylin and eosin dye, and were observed microscopically for histopathological changes.

30

Gene expression assay of the liver RT-PCR

Livers were collected and used for Gene expression assay like Collagen 1alpha 1, Tissue inhibitors of metalloproteinases-1, Transforming growth factor beta.

5

In vivo evaluation of the effect of the test substance for liver cirrhosis in rats

Table 4 illustrates the experimental design of the six groups.

10 **Table 4**

Group-I	Normal rats receive vehicle orally and normal diet pellets. (n=6)
Group-II	Control receives vehicle orally and 8 % carbon tetrachloride (CCl ₄) i.p (1ml/kg). (n=6)
Group-III	Standard Silymarin (200 mg/kg) and 8 % CCl ₄ i.p (1ml/kg). (n=6)
Group-IV	8 % CCl ₄ i.p (1 ml/kg) and PHF (100 mg/kg). (n=6)
Group-V	8 % CCl ₄ i.p (1 ml/kg) and PHF (200 mg/kg). (n=6)
Group-VI	8 % CCl ₄ i.p (1 ml/kg) and PHF (400 mg/kg). (n=6)

Treatment protocol

15 Animals are divided into six groups with each group comprising 6 rats. All the animals except normal control will be intoxicated with CCl₄ (1 ml/kg, p.o. 8 %) 2 times a week (Monday & Thursday) for 8 weeks. Group I received only vehicle 4 times a week (Tuesday, Wednesday, Friday & Saturday) and served as normal control. Group II served as negative control and received CCl₄ (1 ml/kg, p.o. 8 %) 2 times a week (Monday & Thursday) for 8 weeks. Group III served as the positive control and received Silymarine (0.2 gm/kg) 4 times/week (Tuesday, Wednesday, Friday & Saturday) for 8 weeks. Group IV received lower dose treatment, Group V received moderate dose treatment and Group VI received higher dose treatment 4 times/week (Tuesday, Wednesday, Friday and Saturday) for 8 weeks. Groups III to VI are treated with the same amount of CCl₄ as Group II.

20

Blood biochemical analysis

On Days 0, 28 and 56, blood samples of rats in all groups were collected. The serum was separated and used for the various biochemical parameters like alkaline phosphates (ALP), serum total protein, serum total bilirubin, serum AST, serum ALT, Globulin, Albumin, and Gamma glutamyl transferase (GGT).

Histopathological studies

Livers were collected in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6 μm in thickness was cut and stained with hematoxylin and eosin dye, were observed microscopically for histopathological changes.

Gene expression assay of the liver RT-PCR

Livers were collected and used for gene expression assay like P53, TGF β 1, TIMP1, MMP2 & Coll α .

RESULTS AND DISCUSSION

The MTT assay was performed on A2780 epithelial cells and THP-1 phagocytic cells. A2780 epithelial cell line is a human ovarian cancer cell line established from tumour tissue of an untreated ovarian endometroid adenocarcinoma patient and it is used in toxicity testing.

Physical Characteristics

Moisture content

Table 5 illustrates the moisture content of PHF across various masses.

Table 5

Mass	Moisture content (%)			Mean	SD
1g	7.1	6.6	7.5	7.07	± 0.368
100mg	13	12	12.4	12.47	± 0.411
50mg	15.2	15.8	15.4	15.47	± 0.249
25mg	20	21.2	20.4	20.53	± 0.499

pH

Table 6 illustrates the pH of PHF across various percentages.

5 **Table 6**

Percentage (w/v)	pH
1	4.94 – 4.97
10	4.5 – 4.54
0.1	5.27- 5.35
0.05	5.81 -5.88
0.025	6.22- 6.29

Heavy metal analysis

Table 7 illustrates the heavy metal screening of PHF across various test parameters.

10

Table 7

Test parameter	Result (mg/kg) PHF	Reference range in plants (mg/kg)
Cd	N.D. (<0.1)	0.3
Pb	3.41	10
Mn	87.28	15-100

Cu	12.49	20
Na	1691.80	3000
K	22988.25	17000
Zn	37.26	15-150
Ni	3.60	0.1-5
Mg	4330.02	2000
Fe	3175.42	50-250

Solubility of cellulose fiber

Table 8 illustrates the solubility of PHF in various solvents.

5 **Table 8**

Solvent	Solubility
Water	Completely soluble
Chloroform	
Ethanol	
Diethyl ether	

Phytochemical analysis

Table 9 illustrates the phytochemical content of PHF across various samples.

10

Table 9

Sample	Alkaloids (mg/g)	Flavonoids (mg/g)	Total phenol (mg/g)	Tannin (mg/g)	Total protein (mg/g)	Saponin (mg/g)
PHF	44 to 46	123 to 125	51-53	27-29	14-16	72-74

PHF (100 mg)	4 to 6 mg/100mg	9 to 11 mg/100mg	3 to 5 mg/100mg	2 to 4 mg/100mg	1 to 3 mg/100mg	7 to 9 mg/100mg
PHF (50 mg)	1 to 3 mg/50mg	4 to 6 mg/50mg	1 to 3 mg/50mg	1 to 3 mg/50mg	0.5 to 1 mg/50mg	3 to 6 mg/50mg
PHF (25 mg)	0.5 to 2 mg/25mg	2 to 3 mg/25mg	0.5 to 2 mg/25mg	0.5 to 2 mg/25mg	0 to 0.5 mg/25mg	1 to 3 mg/25mg

Note. Flavonoids- Rutin equivalent; Total phenol- Gallic acid equivalent; Tannin- Tannic acid equivalent and Total protein- BSA equivalent. Data present the mean of $\pm$ standard error.
* $p < 0.05$.

5

In vitro antioxidant activity

Table 10 illustrates the amount of antioxidant presence in PHF across various samples.

Table 10

Sample	Ascorbic acid equivalent (mg)/g of plant	
	ABTS	DPPH
PHF	109.13 $\pm$ 0.46	126.12 $\pm$ 0.4
PHF (100 mg)	11.04 $\pm$ 0.05	12.85 $\pm$ 0.04
PHF (50 mg)	4.98 $\pm$ 0.06	6.52 $\pm$ 0.07
PHF (25mg)	2.54 $\pm$ 0.05	3.31 $\pm$ 0.08

10

The antioxidant activity of PHF powder was evaluated using DPPH and ABTS free radical scavenging assays. The ABTS assay is based on the generation of a blue/green ABTS $\cdot^+$ that can be reduced by antioxidants; whereas the DPPH assay is based on the reduction of the purple DPPH $\cdot$ to 1,1-diphenyl-2-picryl hydrazine. Both assays revealed that PHF is a potent antioxidative agent and the antioxidant activity of PHF increased significantly when the concentration of PHF sample was increased. PHF may exhibit antioxidant property due to the presence of secondary metabolites and other major components in it.

15

Immunomodulatory effect of PHF

Table 11 illustrates the cytokine induction by various plant extracts.

Table 11

Plants	IFN γ (ng/ml)			IL 2 (ng/ml)			IL 4 (ng/ml)		
	24hr	48hr	78hr	24hr	48hr	78hr	24hr	48hr	78hr
<i>P. amarus</i>	17.65	111.10	49.57	50.09	249.60	768.90	89.78	226.45	873.24
<i>E. alba</i>	18.12	114.65	48.88	89.27	281.60	987.54	156.14	213.09	890.09
<i>R. serpentina</i>	-	-	-	91.55	342.10	884.32	86.45	345.32	764.91
PHF	76.90	155.54	-	100.05	231.00	4802.36	309.68	856.34	1823.77

5

Anti-HBV activity

In-silico anti-HBV activity

- 10 The docking results of Hepatitis B Virus Polymerase against the selected ligands are summarized in Table 12. The binding energy (Kcal/mol), RMSD value and hydrogen bonds formed were used to evaluate binding affinity of the inhibitors.

Table 12 illustrates the docking results of selected ligands against Hepatitis B Virus Polymerase.

15

Table 12

Compound Name	Binding Energy (kcal/mol)	RMSD value	Hydrogen bond interaction		
			Number of hydrogen bonds	Bond Length Å	Interacting residues
Niranthin	-5.9	0.000	3	3.319, 3.166	ASN 400
				3.500	TYR 647

Nirtetralin	-5.3	2.220	2	3.511	LEU 364
				4.034	GLY 439
Hinokinin	-6.1	1.640	1	3.425	LYS 668
Geraniin	-7.6	0.000	2	3.138	TYR 538
				3.230	LYS 576
Lignan	-5.3	0.000	2	3.324, 3.298	LYS 668
Wedelolactone	-6.5	0.000	1	3.941	LYS 668
Luteolin	-6.7	1.263	1	2.994	ARG 502
Apigenin	-6.7	0.000	2	2.222	ASP 418
				2.164	LYS 603
Coumestans	-7.2	0.0789	2	2.228	ASP 418
				2.164	LYS 603
Demethylwedelo lactone	-7.9	0.000	1	2.187	SER 709
Reserpine	-6.9	0.000	1	3.415	ASN 400
Rescinnamine	-5.7	0.000	1	3.885	LYS 668
Serpentine	-6.9	0.000	1	4.255	PHE 658

The docking results of Sodium taurocholate co-transporting polypeptide (ntcp) against the selected ligands are summarized below in Table 13. The binding energy (Kcal/mol), RMSD value and hydrogen bonds formed were used to evaluate binding affinity of the inhibitors.

5

Table 13 illustrates the docking results of selected ligands against Sodium taurocholate co-transporting polypeptide (ntcp).

Table 13

Compound Name	Binding Energy (kcal/mol)	RMSD value	Hydrogen bond interaction		
			Number of Hydrogen bonds	Bond Length Å	Interacting residues
Niranthin	- 4.8	0.000	1	3.375	SER 255
Nirtetralin	- 6.1	0.000	1	3.975	TRP 305
Hinokinin	-	1.513	1	3.808	PHE 304

	7.4				
Geraniin	- 7.7	0.000	1	3.506	ASN 271
Lignan	- 5.8	0.683	1	3.904	GLY 144
Wedelolactone	- 6.6	0.000	1	2.235	GLY 144
Luteolin	- 7.1	0.000	1	3.154	ASN 247
Apigenin	- 6.9	0.000	1	3.054	THR 268
Coumestans	- 8.6	0.777	1	4.206	SER 255
Demethylwedelolactone	- 7.6	0.000	1	2.730	ASN 103
Reserpine	- 7.1	0.405	1	3.100	ARG 251
Rescinnamine	- 6.6	0.455	1	2.148	GLY 144
Serpentine	- 7.0	2.593	1	3.288	PHE 231

Virtual screening of the fourteen selected phytochemical compounds was carried out using molecular docking analysis. Initially, two proteins, namely Human Hepatitis B Virus polymerase and sodium taurocholate co-transporting polypeptide (ntcp), were selected as the target for the process. The gene sequence of the protein targets was taken from Uniport web server and all the conformers present for a single sequence were also taken into consideration (different variants of same sequence). Then the sequences of different conformers of the same protein were subjected to protein homology modelling.

10

Finally, additional charge and torsion were added to both the three dimensional structure file of the ligands and the target proteins were added to evaluate binding affinity, followed by evaluating the binding efficiency between the molecules of interest. Docking boundaries were created so as

to target the required active site of the protein identified. The results were interpreted by comparing the binding energy of the different ligands with minimum inhibition constant and maximum number of bonds formed. Upon analyzing the binding energy of the docking result of the ligands against Hepatitis B virus polymerase, Demethylwedelolactone had the lowest binding energy of - 7.9 Kcal/mol, followed by Geranin with -7.6 Kcal/mol, Coumestane with -7.2 Kcal/mol, Reserpine and Serpentine with -6.9 Kcal/mol. Similarly upon considering the number of hydrogen bond formed, Niranthin with three hydrogen bonds followed by Nirtetralin, Geraniin, Lignan, Apigenin and Coumestans, all had formed two hydrogen bonds with Hepatitis B Virus Polymerase.

Finally, upon close observation and considering all the parameters including RMSD value, both Demethylwedelolactone and Geranin were found be effective towards Hepatitis B Virus Polymerase. Similarly, interactions between the ligands and the target protein sodium taurocholate co-transporting polypeptide (ntcp) were also evaluated and Coumestans showed a highest binding energy of -8.6 Kcal/mol, followed by Geraniin with -7.7 Kcal/mol, Demethylwedelolactone with -7.6 Kcal/mol, Hirokinin with -7.4 Kcal/mol and Apigenin with -6.9 Kcal/mol. When considering the number of hydrogen bonds formed, all 13 ligands had one hydrogen bond with the target protein ntcp. Finally, it was revealed that both Coumestans and Demethylwedelolactone were found to be effective against the target ntcp. Overall, the study concluded with a positive result that almost all of 13 ligands showed lower binding energy and better binding affinity towards both of the target proteins. In particular, phytochemicals such as Coumestans, Geranin and Demethylwedelolactone were found to be highly effective against both the target proteins, namely Human Hepatitis B Virus polymerase and ntcp.

Anti-HbsAg of PHF (Invitro)

Table 14 illustrates the anti-HbsAg activity of PHF across various concentrations.

Table 14

CONCENTRATIONS OF STANDARD IN ng/ml	PERCENTAGE OF INHIBITION
100	81
50	73
25	54
12.5	39
6.25	31
3.13	23
1.56	19
0	-

PHF has the anti-HbsAg activity towards the tested virus. As reported in Table 14, it can be seen that PHF at a concentration of 100 ng/mL inhibits the virus by 81 %.

5 *In vivo* toxicity study

In vivo evaluation of the acute toxicity of the test substance in mice

In the acute toxicity study, oral administration of 2000 mg of PHF per kg of body weight to mice did not result in any mortality. Mice did not show any visible signs of toxicity such as changes in skin, eyes, fur and mucous membranes. Further, these rats did not show any behavioural changes including salivation, sleep, coma, lethargy, diarrhoea. The animals were found to be normal throughout the study period. There are no such changes in histopathological observation of internal organs. Thus, the LD₅₀ was found to be more than 2000 mg/kg.

Spleen

Examination of H&E stained sections of experimental mice spleen showed the characteristics of normal spleen tissue architecture, such as no parenchymal and capsular fibrosis, no lipidosis, no splenic necrosis, and no vacuolization of splenic hitiocytes.

Stomach

Examination of H&E stained sections of experimental mice stomachs showed the characteristics of normal stomach tissue architecture with no pathological changes seen, such as no ulceration, no hyperemia, bleeding and no epithelial cell degeneration and necrosis were observed in the gastric mucosal layer.

10 *Kidney*

Examination of H&E stained sections of experimental mice kidneys showed the characteristics of normal kidney tissue architecture, such as prominent critical tubules and bowman's capsules, normal glomerular structure and no renal tubular damage, no haemorrhages, no infiltration of neutrophils increase, no tubular dilatation no glomerular atrophy, and no interruption in the basement membrane around the necrotic tubules.

In-vivo evaluation of the sub-acute toxicity of the test substance in mice

20 *Effect of different doses of PHF on body weight and food intake*

Table 15 illustrates the effect of different doses of PHF on body weight and food intake.

Table 15 supports that there are no changes in body weight and food intake as compared to Day 0.

Table 15

Group	Animal Body Weight (% change from Day 0 to Day 60)	Food Intake (% change from Day 0 to Day 60)
PHF 100mg/kg	-2.83	20.8
PHF 200mg/kg	-8.22	12.0

PHF 400mg/kg	0.00	0.0
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Effect of different doses of PHF on Serum Biochemical Parameters

Tables 16 and 17 illustrate the effect of different doses of PHF on Serum Biochemical Parameters.

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Tables 16 and 17 support a significant increase ($p < 0.05$ to $p < 0.001$) in serum levels of glucose, Cholesterol, Total protein, Total bilirubin, AST, Creatinine and urea as compared to Day 0. There is no significant increase in the level of ALP as compared to Day 0.

10 **Table 16**

Group	Glucose (% change from Day 0 to Day 60)	Cholesterol (% change from Day 0 to Day 60)	Total protein (% change from Day 0 to Day 60)	Total bilirubin (% change from Day 0 to Day 60)	AST (% change from Day 0 to Day 60)	Creatinine (% change from Day 0 to Day 60)
PHF 100mg/kg	5.86	22.19	506.98	-0.13	137.27	-45.84
PHF 200mg/kg	2.96	28.62	533.23	-5.49	3.54	-27.65
PHF 400mg/kg	11.33	10.50	557.63	-5.32	-4.07	-42.51

Table 17

Group	ALP (% change from Day 0 to Day 60)	Urea (% change from Day 0 to Day 60)
PHF 100mg/kg	-43.08	19.88
PHF 200mg/kg	-5.26	18.15
PHF 400mg/kg	-11.11	17.21

15 *Effect of different doses of PHF on Urine Biochemical Parameters*

Table 18 illustrates the effect of different doses of PHF on Urine Biochemical Parameters.

Table 18 supports a significant ($p < 0.05$ to $p < 0.001$) increase in glucose, total protein as compared to Day 0 whereas there is no change in total bilirubin & urine pH and there is absence of blood and leucocytes in urine as compared to Day 0.

Table 18

Group	Glucose (% change from Day 0 to Day 60)	Total protein (% change from Day 0 to Day 60)	Total bilirubin (% change from Day 0 to Day 60)	Urine pH (% change from Day 0 to Day 60)	Blood (% change from Day 0 to Day 60)	Leucocytes (% change from Day 0 to Day 60)
PHF 100mg/kg	40.69	-78.50	1.32	1.97	A*	A
PHF 200mg/kg	42.92	-86.22	9.77	0.56	A	A
PHF 400mg/kg	35.51	-76.96	0.78	1.68	A	A

*A stands for “Absent”, which means there were no changes in the % from Day 0 to Day 60.

Tables 19 and 20 illustrate the effect of different doses of PHF on Hemogram (CBC). They support a significant ($p < 0.05$ to $p < 0.001$) increase in hemoglobin, total count, neutrophils, eosinophils, RBC count & Mean corpuscular volume, Mean Corpuscular Haemoglobin, Mean Corpuscular Hb Concentration, Platelet Count, Packed Cell Volume as compared to Day 0 whereas lymphocytes did not show any significant increase as compared to Day 0. The results in Tables 19 and 20 show % change from Day 0 to Day 60.

Table 19

Group	Haemoglobin	Total Count	Neutrophils	Lymphocytes	Eosinophils	RBC Count
PHF 100mg/kg	-12.16	23.02	94.44	-0.74	1067.00	-7.25

PHF 200mg/kg	-2.83	8.65	-23.75	-3.32	85.99	3.91
PHF 400mg/kg	0.63	48.14	63.28	0.93	1328.45	-7.57

Table 20

Group	Mean Corpuscular Volume	Mean Corpuscular Haemoglobin	Mean Corpuscular Hb Concentration	Platelet Count	Packed Cell Volume
PHF 100mg/kg	2.35	-7.32	-7.09	31.43	-2.49
PHF 200mg/kg	2.02	-6.51	-6.92	18.25	3.73
PHF 400mg/kg	4.93	-6.38	-10.68	17.17	2.52

5

Table 20 supports a significant increase in Mean corpuscular volume, Mean Corpuscular Haemoglobin, Mean Corpuscular Hb Concentration, Platelet Count, Packed Cell Volume as compared to Day 0.

10 Hematological parameters examination can be used to identify any harmful effect of foreign compounds including in polyherbal formulation on blood. Mice have small RBC counts compared with that of most other mammalian species, with an average MCV of 45 to 55 fL in health. Based on Table 20, the animal treated with PHF 400mg/kg, the MCV count and Mean Corpuscular Haemoglobin(pg) increased but such increase is within the normal range.

15

The result of sub-acute toxicity study of PHF on mice at 100mg/kg, 200mg/kg and 400mg/kg did not produce any sign of toxicity and death in the animals. The treatment with PHF does not result in any significant change in body weight and food intake in animals. The haematological parameters are used to study the extent of toxicity of PHF. In this study, administration of PHF in female mice for a period of 60 days produced a significant change in all blood parameters except the lymphocytes.

20

The functionality of the liver was assessed by the blood serum. A reduction in serum levels of total proteins, bilirubin and albumin depicts reduced function, which is evident in liver damage or

diseases. Serum parameter and histology of the liver in the female mice did not produce any changes before and after treatment. Thus it can be concluded that the PHF is safe to use.

Effect of different doses of PHF on Histopathological changes

- 5 Examination of H & E stained sections of experimental mice internal organs (liver, heart, kidney, uterus, brain, spleen, lungs, stomach, ovary, pancreas, trachea, ileum, oesophagus, large intestine, aorta, bone and skin) showed the characteristics tissue architecture without any changes in their normal histopathological architecture.

10 ***In vivo evaluation of the effect of the test substance for liver fibrosis in rats***

Effect of PHF on Serum Biochemical parameters

Table 21 and 22 present data in terms of % change from Day 0 to Day 12.

- 15 Table 21 supports a significant ($p < 0.05$ to $p < 0.001$) decrease in serum ALP & SGOT of the treatment group, as compared to the control group, whereas there is significant ($p < 0.05$) increase in serum albumin of treatment group as compared to control group.
- 20 Table 22 supports a significant ($p < 0.05$ to $p < 0.001$) decrease in serum SGPT & total bilirubin in the treatment group as compared to the control group. Whereas there is significant ($p < 0.05$) increase in serum total protein & globulin of treatment group as compared to control group.

Table 21

Treatment Group	ALP	Albumin	SGOT
Normal	146.98	-10.7	470.4
Control	10749.10	-81.7	802.9
Standard	322.97	-16.6	-10.1
T1 (100mg/kg)	1211.71	-68.7	-22.5
T2 (200mg/kg)	-25.01	-55.3	160.2

T3 (400mg/kg)	-4.54	-35.2	-72.6
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Table 22

Treatment Group	SGPT	Total Protein	Total Bilirubin	Globuline
Normal	172.24	15.21	6.69	27.43
Control	5803.29	-72.73	1251.29	-67.59
Standard	357.23	32.51	186.68	58.69
PHF (100mg/kg)	10.43	-13.80	151.81	18.38
PHF (200mg/kg)	735.07	4.49	23.52	36.69
PHF (400mg/kg)	500.70	-3.57	42.66	12.91

5 *Effect of PHF on Histopathological studies*

- Effect of different doses of PHF on paracetamol induced liver damage in white albino rats 40 X magnified photographs of liver from different treatment groups stained with haemotoxoline and eosin. Normal group shows a portal vein constriction and control group shows a portal vein dilation and also there were completely alerted hepatic cells architecture, centrilobular necrosis, hepatic steatosis and macro vascular fatty changes noticed. But in treatment using 100mg/kg, 200mg/kg and 400mg/kg, this showed almost normal architecture and absence of centrilobular necrosis and hepatic steatosis.
- ALP, a hydrolysable enzyme excreted through the bile, is present in biliary cells. Hepatotoxicity causes the biliary congestion leading to the inability of excretion of the ALP from the body that leads to elevation of the ALP level in control group. PHF treated groups reduced the ALP level significantly and in a dose dependent manner comparable to the control group.
- The PHF treated groups recovered from the rise in liver marker in blood which leaks from hepatocytes, and from the decrease in protein level.

In vivo evaluation of the effect of the test substance for liver cirrhosis in rats

Effect of PHF on Serum Biochemical parameters

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Table 22 supports significant ($p < 0.05$ to $p < 0.001$) decreases in serum ALP & SGOT of treatment group as compared to control group, whereas there is significant ($p < 0.05$ to $p < 0.001$) increase in serum albumin of treatment group as compared to control group.

10 Table 23 illustrates the effect of PHF on Serum Biochemical parameters.

Table 23

Treatment Group	ALP (% change)		Albumin (% change)		SGOT(% change)	
	Day 28	Day 56	Day 28	Day 56	Day 28	Day 56
Normal	-4.10	-31.28	-93.76	-95.06	6.84	-14.74
Control	-43.42	305.31	-94.54	-98.89	309.46	197.72
Standard	-79.13	-90.31	-87.36	-87.72	297.38	-8.99
T1 (100mg/kg)	-54.55	-91.57	-94.59	-96.00	35.49	-49.17
T2 (200mg/kg)	-74.13	-93.54	-94.38	-97.09	25.60	-78.74
T3 (400mg/kg)	-78.32	-95.79	-93.15	-94.82	27.45	-48.59

15 Table 23 supports significant ($p < 0.05$ to $p < 0.001$) decreases in serum SGPT & total bilirubin in treatment group as compared to control group. Whereas there is significant ($p < 0.05$ to $p < 0.001$) increase in serum total protein of treatment group as compared to control group.

Table 24 illustrates the effect of PHF on Serum Biochemical parameters.

20

Table 24

Treatment Group	SGPT (% changes)		Total Protein (% changes)		Total Bilirubin (% changes)	
	Day 28	Day 56	Day 28	Day 56	Day 28	Day 56
Normal	2.44	7.97	-4.36	26.32	-7.74	37.42
Control	25.57	49.67	-37.33	-49.76	256.08	1254.05
Standard	-26.81	-9.04	4.74	68.88	170.14	415.97
T1 (100mg/kg)	-53.81	-63.41	-24.95	2.15	245.19	685.19
T2 (200mg/kg)	-16.80	-72.37	-19.92	17.70	229.37	637.76
T3 (400mg/kg)	13.49	-34.70	-14.39	91.88	221.09	727.34

Table 24 supports significant ($p < 0.05$ to $p < 0.001$) increase in serum globulin in treatment group as compared to control group.

5

Effect of PHF on Histopathological studies

Effect of different doses of PHF on CCL₄ induced liver damage in Sprague Dawley rats. 40 X magnified photographs of liver from different treatment groups stained with haemotoxoline and eosin. The normal group shows a portal vein constriction and the control group shows a portal vein dilation and also there were completely alerted hepatic cells architecture, centrilobular necrosis, hepatic steatosis and macro vascular fatty changes noticed. But in treatment with 100 mg/kg, 200 mg/kg and 400 mg/kg of PHF, this showed almost normal architecture and absence of centrilobular necrosis and hepatic steatosis.

10

15

Table 25 illustrates the effect of PHF on Serum Biochemical parameters.

Table 25

Treatment Group	Globuline (% changes)	
	Day 28	Day 56
Normal	0.00	7.43
Control	-61.29	-51.21

Standard	-30.23	56.59
T1 (100mg/kg)	-9.24	47.90
T2 (200mg/kg)	-10.20	61.22
T3 (400mg/kg)	-34.31	31.75

CCl₄, a xenobiotic, is a chemical commonly used to generate experimental liver damage. CCl₄ is an uncolored, transparent, volatile liquid that is used in experiments to induce liver damage.

- 5 It is well established that CCl₄ induces hepatotoxicity by metabolic activation, therefore it selectively causes toxicity in liver cells maintaining semi normal metabolic function. The CCl₄ tissue damage mechanism begins with the formation of CCl₃ by CYP enzyme system. CCl₄ is biotransformed by cytochrome P450 system in the endoplasmic reticulum to produce trichloromethyl free radical (CCl₃).

10

Trichloromethyl free radicals when combined with cellular lipids and proteins in the presence of oxygen form trichloromethylperoxyl radicals, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radicals. Thus, trichloromethylperoxyl free radicals lead to elicit lipid peroxidation or leads to the degradation of the cell membrane by covalent bonding of lipids with proteins, thereby causing liver damage.

15

Liver damage activates Kupffer cells, resulting in the release of early inflammation mediators such as superoxide anions, which cause the formation of reactive oxygen species (ROS) and especially peroxynitrites and hydrogen peroxides (H₂O₂), leading to oxidative stress. The destruction of Ca²⁺ homeostasis finally results in cell death.

20

In the study, it was observed that the administration of CCl₄ decreased the levels of serum total proteins and increased the levels of serum marker enzymes significantly. CCl₄ administration caused significant increases ($p < 0.05$ to $p < 0.001$) in serum AST (SGPT), ALT (SGOT), total bilirubin and ALP levels and significant decreases ($p < 0.05$ to $p < 0.001$) in albumin, total protein and globulin. This may be due to elevated levels of cytosolic enzymes in serum levels such as ALT, AST and ALP, owing to the disturbance of the liver cell membrane caused by oxidative stress.

25

The results of the study showed that the serum levels of these enzymes were elevated after the hepatic injury induced by CCl₄. It was also shown that administration of PHF improves subsequent elevations in the levels of AST, ALT, total bilirubin and ALP by CCl₄ administration. The significant ($p < 0.05$ to $p < 0.001$) decrease in ALT, AST, total bilirubin and ALP levels and significant ($p < 0.05$ to $p < 0.001$) increase in albumin, total protein and globulin was a result of the PHF regulating the integrity of the hepatic cellular membrane owing to its strong antioxidant properties, which was clear evidence of the hepatoprotective effect observed after PHF administration.

In vivo evaluation of the effect of the test substance for Non-Alcoholic Fatty Liver Disease (NAFLD)

Effect of PHF on Serum Biochemical parameters

Tables 26 to 29 illustrate the effect of PHF on Serum Biochemical parameters. Table 30 illustrates the effect of PHF on Biochemical parameters of liver tissue.

Table 26 supports a significant ($p < 0.001$) decrease in serum ALP, SGOT and SGPT in treatment and standard groups as compared to control group.

Table 27 supports a significant ($p < 0.001$) decrease in serum total bilirubin, creatinine and urea in treatment and standard groups as compared to control group.

Table 28 supports a significant ($p < 0.001$) decrease in serum uric acid, glucose and cholesterol in treatment and standard groups as compared to control group.

Table 29 supports a significant ($p < 0.001$) decrease in triglyceride, LDL in treatment and standard groups as compared to control group whereas there is significant ($p < 0.001$) increase in HDL in treatment and standard groups as compared to control group.

Table 30 supports a significant ($p < 0.05$ to $p < 0.001$) decrease in total cholesterol, total protein, malondialdehyde (MDA) and triglyceride in treatment and standard groups as compared to control group.

Table 26

Treatment	ALP(IU/L) % change		SGOT(IU/L) % change		SGPT(IU/L) % change	
	Day 28	Day 56	Day 28	Day 56	Day 28	Day 56
Normal	-42.56	-75.55	110.83	-86.53	168.10	-86.97
Control	117.89	225.07	775.00	-32.61	2368.86	-7.35
Standard	187.62	-54.53	506.22	-78.89	2634.40	-15.01
PHF (100mg/kg)	153.37	-84.87	637.74	-76.09	2061.03	-61.29
PHF (200mg/kg)	139.88	-84.46	624.26	-86.24	3318.03	-64.27
PHF (400mg/kg)	164.08	-90.59	718.24	-79.33	3486.75	-74.90

5

Table 27

Treatment	Total Bilirubin (mg/dl) % change		Creatinine (mg/dl) % change		Urea (mg/dl) % change	
	Day 28	Day 56	Day 28	Day 56	Day 28	Day 56
Normal	32.88	34.66	-16.52	-43.27	4.55	30.45
Control	191.26	310.25	84.59	666.92	289.75	231.69
Standard	222.72	185.89	66.16	86.13	252.22	42.69
PHF (100mg/kg)	153.49	144.63	58.16	211.16	293.74	53.99
PHF (200mg/kg)	197.45	168.09	92.31	198.62	223.62	45.50
PHF (400mg/kg)	218.30	144.00	87.31	105.48	224.97	73.74

Table 28

Treatment	Uric acid (mg/dl) % change		Glucose (mg/dl) % change		Cholesterol (mg/dl) % change	
	Day 28	Day 56	Day 28	Day 56	Day 28	Day 56

Normal	-12.18	0.33	-9.43	-1.76	21.17	65.49
Control	179.94	306.33	29.66	42.82	159.84	222.85
Standard	229.06	186.24	27.56	-6.73	124.53	53.10
PHF (100mg/kg)	183.80	177.91	50.97	20.81	171.10	115.66
PHF (200mg/kg)	201.00	150.13	46.58	3.78	127.05	72.45
PHF (400mg/kg)	198.92	134.80	38.83	-13.51	145.19	58.36

Table 29

Treatment	Triglyceride (mg/dl) % change		HDL (mg/dl) % change		LDL (mg/dl) % change	
Group	Day 28	Day 56	Day 28	Day 56	Day 28	Day 56
Normal	7.57	-9.67	14.49	2.48	14.19	5.80
Control	31.93	13.44	0.90	-16.98	63.77	87.17
Standard	37.91	-26.52	-8.28	2.43	59.32	-43.45
PHF (100mg/kg)	48.00	-20.67	-0.65	10.20	104.82	-33.27
PHF (200mg/kg)	28.46	-35.64	-1.88	20.63	138.32	-23.33
PHF (400mg/kg)	18.96	-43.85	-4.12	16.27	87.09	-58.13

Table 30

Treatment Group	Triglyceride (mg/dl) % change	Total Cholesterol (mg/dl) % change	Total Protein (mg/dl) % change	Malondialdehyde (nmoles/mg of protein) % change
Normal	0	0	0	0
Control	18.78	131.90	-98.60	2302.39
Standard	24.42	26.00	-93.40	65.94
PHF (100mg/kg)	116.28	84.24	-97.04	1107.87
PHF (200mg/kg)	105.35	73.72	-96.30	483.71
PHF (400mg/kg)	75.5	33.39	-95.70	96.28

5

DISCUSSION & CONCLUSION

The therapeutic potential of the PHF of the invention in addressing liver fibrosis and cirrhosis has undergone comprehensive evaluation. Biochemical parameters in liver function tests for animals treated with PHF demonstrated a restoration to normal levels compared to untreated counterparts, emphasizing the drug's effectiveness in treating liver conditions, particularly fibrosis and cirrhosis. Histopathological analysis of treated animals' livers revealed a near-normal architectural structure, marked by the absence of centrilobular necrosis and hepatic steatosis.

15

Notably, consistent results were achieved with varying treatment durations, highlighting the adaptability and reliability of PHF. Fibrosis responded to PHF treatment within a brief period of 7 days, while addressing cirrhosis and fatty liver conditions required a more extended treatment duration of 56 days. These findings underscore the promising efficacy of PHF in mitigating liver fibrosis, cirrhosis, and fatty liver, offering potential therapeutic interventions in hepatic disorders. The safety profile of PHF further supports its use, making it a promising candidate for addressing various liver conditions effectively within different timeframes.

More specifically, the above results show that the PHF according to the application is active in treating NAFLD, which is a continuous spectrum of diseases characterized by excessive lipid accumulation in hepatocytes. It progresses from simple liver steatosis to non-alcoholic steatohepatitis (NASH) and in more severe cases, to liver fibrosis and cirrhosis.

The PHF according to the application is also active in fatty liver disease that is the most common chronic liver disease in Western society. It is not caused by alcohol or hepatitis virus, but by the accumulation of fatty acid in liver parenchymal cells in the form of triglycerides. In particular, histological fat accumulation in the liver is a feature of non-alcoholic fatty liver disease and manifests as various symptoms including simple steatosis, steatohepatitis and cirrhosis. Also, fatty liver disease increases insulin resistance, which promotes decomposition of adipose tissue. The resulting increased fatty acid supply to the liver promotes fat oxidation that induces fat accumulation in the liver cells.

The PHF according to the application also provides protection against these processes. Evaluation of hepatic content of MDA by lipid peroxidation can directly damage hepatocytes as well as increase hepatic inflammation and can mediate fibrogenesis.

The PHF according to the application is also helpful in treating hepatic steatosis that is the hallmark feature of NAFLD, whereby fat droplets accumulate in the form of triglycerides in hepatocytes. NAFLD is histologically diagnosed when accumulation occurs in >5% of hepatocytes.

Further to the above, the PHF according to the application is active in the following conditions described below. Triglycerides in the livers with NAFLD derive from esterification of glycerol and free fatty acids (FFAs). Triglyceride accumulation occurs when the rate of import or synthesis of FFAs by hepatocytes exceeds the rate of export or catabolism. Obesity and particularly insulin resistance (IR) are tightly associated with the genesis of NAFLD. Over expression of tumour necrosis factor (TNF)- α activates I κ B kinase β , which plays an important role in IR development by inhibiting the phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2. IR leads to an increase in the liver triglyceride level and ultimately liver steatosis through various mechanisms. Insulin fails to suppress adipose tissue lipolysis via hormone-sensitive lipase, resulting in increased efflux of FFAs into the circulation and consequent uptake by the liver.

The PHF according to the application is beneficial on the metabolic alterations in the lipid profile, glucose homeostasis and the functional and structural changes of hepatocytes in NAFLD induced by a high fat diet. Feeding the control group animals with a high fat diet resulted in dramatic increases in the total cholesterol, triglycerides, LDL-C but it decreased the HDL-C levels. This hyperlipidemia could be related to the enhanced de-esterification of the abundant FFAs and decreased lipoproteins. In addition, control group of animals showed enhanced oxidative stress, as evidenced by increased MDA levels which is related to beta oxidation of fatty acids in hepatic steatosis which stimulates reactive oxygen species generation, lipid peroxidation, hepatocyte necro-inflammation and apoptosis. The results showed that administration of PHF at different doses effectively alleviated NAFLD and abnormal lipid metabolism in rats fed a high-fat diet.

Toxicity of the PHF has been studied in *in vitro* and *in vivo*. The test animals have been fed with PHF for 60 days. All the 17 organs of the animals have been extracted and studied for acute and sub-acute toxicity. It is not toxic to the animals. Its result showed that PHF is safe to consume at the concentration of 2000 mg/kg/day.

Both DPPH and ABTS antioxidant analyses revealed that PHF capsule exhibits antioxidant property. The significant antioxidant activity of PHF capsule can potentially reduce inflammation in the liver. Moreover, PHF capsule has possibly stimulated the

secretion of IL2, IL4 and IFN γ cytokines in peripheral blood mononuclear cells (PBMCs).

5 The anti-HBV analysis of PHF has shown that the PHF of the invention has potentially inhibited the hepatitis B virus in *in vitro*. The potential of PHF to treat the liver fibrosis, cirrhosis and non-alcoholic fatty liver disease has been studied. The biochemical parameters for liver function test of PHF treated animals restored normal compared with non-treated animals. It shows the efficacy of PHF in treating the liver conditions such as fibrosis, cirrhosis and non-alcoholic fatty liver disease. The same result has been
10 observed in the tissues of treated groups. The histopathological study of treated animal livers showed almost normal architecture and absence of centrilobular necrosis and hepatic steatosis.

Therefore, the application reveals that PHF powder has significant multifactorial
15 effectiveness for hepatoprotective activity. This study claims the novel use of PHF powder that is effective in having an antioxidant property, immunomodulatory property, antiviral property, anti-HBV property, which is useful in treating liver fibrosis, liver cirrhosis and/or NAFLD.

20 In detail, the application i.e. the PHF shows the following effects:

The activity against NAFLD comprises within the range from liver steatosis to NASH. The biochemical parameters for liver function are restored to normal levels. Further, a liver of the subject restores to a normal architecture with substantially no observable
25 centrilobular necrosis and hepatic steatosis.

The activity against liver disease includes hepatotoxicity especially if is a result of one or more selected from the group comprising alcohol, environmental pollution, viral-induced hepatitis, hepatocellular carcinoma, or hepatotoxic drugs. Further to that, PHF
30 powder according to the application is active against viral-induced hepatitis caused by hepatitis B viruses. In particular, the PHF powder is very active if the oxidative injury is a result of oxidative stress or lipid peroxidation, especially if this is caused by reactive oxygen species. Typical reactive oxygen species comprises one or more

selected from the group comprising hydrogen peroxide, superoxide anion radical, or hydroxyl radical.

5 The PHF powder of the application also provides a hepatoprotective activity, together with its antioxidant properties. The hepatoprotective activity can be demonstrated by a decrease in the levels of any one or more selected from the group comprising: AST, ALT, total bilirubin and ALP. Further to that, the hepatoprotective activity is shown by an increase in the levels of any one or more selected from the group comprising: albumin, total protein and globulin. Further to that, the hepatoprotective activity is a
10 result of the polyherbal formulation regulating the integrity of the hepatic cellular membrane due to its antioxidant properties.

In addition to the above, the PHF according to the application reduces insulin resistance in a subject in need thereof. The PHF powder of the present application also
15 has shown that it does not significantly change the intake requirement of a subject. Also, it has been shown that the treatment with the PHF comprises no significant changes in blood parameters except lymphocytes. The PHF of the application also has antioxidant properties for use in a subject. A typical antioxidant property reduces inflammation in the liver of the subject. Further to that, the PHF has been
20 demonstrated to exhibit immunomodulatory properties, which simulate secretion of at least one of Interleukin-2, Interleukin-4, and Interferon Gamma cytokines in the PBMCs of the subject.

Finally, it has been shown that the PHF has antiviral properties, especially anti-HBV
25 properties and it further shows activity against liver fibrosis, liver cirrhosis and NAFLD. Further, the PHF increases secondary metabolites when the polyherbal formulation is increased when given into a subject. The secondary metabolites are responsible for an antioxidant and for anti-inflammatory properties in the subject. Further, the PHF can be used to inhibit hepatitis B virus in a subject. Finally, it can be
30 used to alleviate NAFLD, abnormal lipid metabolism, and/or excessive lipid accumulation in hepatocytes, in a subject in need thereof. Also, the PHF reduces lipid accumulation in hepatocytes of the liver of a subject, especially if the lipid comprises triglycerides.

While the above described *in-vivo* experiments were done with mice, the PHF volumes can be adapted for administration to humans based on the following formula:

< **Human equivalent dose (mg/kg) = Dose to be converted / (Human Km/Mouse Km)>.**

- 5 Here, Km factor is constant for a species with specific body weight – i.e. Km= body weight/ body surface area,

Using this, experiments with human beings have been carried out for the treatment of hepatitis B, with similar results as the *in-vivo* experiments with mice.

10

These results show that the PHF powder has wide application, demonstrating properties such as immunomodulatory property, antiviral viral property and an activity against non-alcoholic fatty liver disease, cirrhosis and fibrosis.

15 **GENERALISED DISCLOSURES IN FURTHER EXEMPLARY EMBODIMENTS:**

Example 1: Use of a polyherbal formulation for preparing a pharmaceutical composition for the treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need
20 thereof, the polyherbal formulation comprising plant material from two or more selected from the group comprising: Rauwolfia spp., Eclipta spp., and Phyllanthus spp.

Example 2: Use according to example 1, wherein the Rauwolfia spp. comprises Rauwolfia serpentina.

Example 3: Use according to anyone of the preceding examples, wherein the Eclipta
25 spp. comprises Eclipta alba.

Example 4: Use according to anyone of the preceding examples, wherein the Phyllanthus spp. comprises Phyllanthus amarus.

Example 5: Use according to anyone of the preceding examples, wherein the plant material in the polyherbal formulation comprises Rauwolfia serpentina, Eclipta alba, and
30 Phyllanthus amarus.

Example 6: Use according to anyone of the preceding examples, wherein the polyherbal formulation comprises approximately 40-50% Eclipta alba.

Example 7: Use according to anyone of the preceding examples, wherein the polyherbal formulation comprises approximately 20-30% Phyllanthus amarus.

Example 8: Use according to anyone of the preceding examples, wherein the polyherbal formulation comprises approximately 25-35% *Rauwolfia serpentina*.

Example 9: Use according to anyone of the preceding examples, wherein the polyherbal formulation comprises approximately 44-45% *Eclipta alba*, 24-25% *Phyllanthus amarus*, and 30-31% *Rauwolfia serpentina*.

Example 10: Use according to anyone of the preceding examples, wherein a 100 mg preparation of the polyherbal formulation comprises 44.75 mg of *Eclipta alba*, 24.58 mg of *Phyllanthus amarus* and 30.66 mg of *Rauwolfia serpentina*.

Example 11: Use according to anyone of the preceding examples, wherein the pH of the polyherbal formulation comprises approximately 4.5 to 6.5, more preferably 4.5 to 5.

Example 12: Use according to anyone of the preceding examples, wherein the polyherbal formulation is dissolved in one or more selected from the group comprising: water, chloroform, ethanol, and diethyl ether.

Example 13: Use according to anyone of the preceding examples, wherein the phytochemical content of the polyherbal formulation comprises greater than 35 mg/g alkaloids, and more preferably greater than 45 mg/g alkaloids.

Example 14: Use according to anyone of the preceding examples, wherein the phytochemical content of the polyherbal formulation comprises greater than 110 mg/g flavonoids, and more preferably greater than 120 mg/g flavonoids.

Example 15: Use according to anyone of the preceding examples, wherein the phytochemical content of the polyherbal formulation comprises greater than 40 mg/g total phenol, and more preferably greater than 50 mg/g total phenol.

Example 16: Use according to anyone of the preceding examples, wherein the phytochemical content of the polyherbal formulation comprises greater than 20 mg/g tannin, and more preferably greater than 25 mg/g tannin.

Example 17: Use according to anyone of the preceding examples, wherein the phytochemical content of the polyherbal formulation comprises greater than 60 mg/g saponin, and more preferably greater than 70 mg/g saponin.

Example 18: Use according to anyone of the preceding examples, wherein the phytochemical content of the polyherbal formulation comprises greater than 10 mg/g total protein, and more preferably greater than 15 mg/g total protein.

Example 19: Use according to anyone of the preceding examples, wherein the polyherbal formulation comprises secondary metabolites comprising at least one of flavonoids, saponins, coumestan, phenols, alkaloids, tannins and/or lignans.

Example 20: Use according to example 19, wherein the most abundant secondary metabolite in the polyherbal formulation comprises flavonoids.

Example 21: Use according to anyone of examples 19 and 20, wherein the flavonoids comprise one or more selected from the group comprising: Luteolin-7-glucoside,

5 Luteolin, Apigenin, Orobol (isoluteolin).

Example 22: Use according to anyone of examples 19 and 21, wherein the flavonoids providing hepatoprotectivity comprise one or more selected from the group comprising: Luteolin and Apigenin.

Example 23: Use according to anyone of examples 19 to 22, wherein the flavonoids
10 providing antioxidant activity comprise one or more selected from the group comprising: Luteolin-7-glucoside, Luteolin, Apigenin, and Orobol (isoluteolin).

Example 24: Use according to example 19, wherein the coumestans providing hepatoprotectivity comprise one or more selected from the group comprising: Wedelolactone, Demethylwedelolactone, Demethylwedelolactone-7-glucoside.

15 Example 25: Use according to example 19, wherein the tannins comprise one or more selected from the group comprising: Geraniin, Corilagin, Geraniinic acid, Amarinic acid, and Elaeocarpusin.

Example 26: Use according to anyone of examples 19 and 25, wherein the tannins providing hepatoprotectivity comprise one or more selected from the group comprising:

20 Corilagin, Gallic acid, and Amariin.

Example 27: Use according to anyone of examples 19, 25, and 26, wherein the tannins providing antioxidant activity comprise one or more selected from the group comprising: Corilagin and Geraniin.

Example 28: Use according to example 19, wherein the lignans comprise one or more
25 selected from the group comprising: Phyllanthin, Hypophyllanthin, Lintetralin, Niranthin, Nirtetralin, Isolintetralin, and Isonirtetralin.

Example 29: Use according to anyone of examples 19 and 28, wherein the lignans providing hepatoprotectivity comprise one or more selected from the group comprising: Phyllanthin and Hypophyllanthin.

30 Example 30: Use according to anyone of examples 19, 28, and 29, wherein the lignans providing antioxidant activity comprise one or more selected from the group comprising: Phyllanthin and Hypophyllanthin.

Example 31: Use according to example 19, wherein the Alkaloids comprise one or more selected from the group comprising: Reserpine, Rescinnamine, and Serpentine.

Example 32: Use according to anyone of the preceding examples, wherein the polyherbal formulation is sterilized by microwave pasteurization and or ultraviolet radiation.

5 Example 33: Use according to anyone of the preceding examples, wherein the liver disease comprises NAFLD, cirrhosis, fibrosis or anti-HBV.

Example 34: Use according to example 33, wherein the NAFLD comprises within the range from liver steatosis to NASH.

Example 35: Use according to anyone of the preceding examples, wherein the biochemical parameters for liver function are restored to normal levels.

10 Example 36: Use according to anyone of the preceding examples, wherein the liver of the subject restores to a normal architecture with substantially no observable centrilobular necrosis and hepatic steatosis.

Example 37: Use according to anyone of the preceding examples, wherein the liver disease is caused by hepatotoxicity.

15 Example 38: Use according to anyone of the preceding examples, wherein the hepatotoxicity is as a result of one or more selected from the group comprising: alcohol, environmental pollution, viral-induced hepatitis, hepatocellular carcinoma, and hepatotoxic drugs.

20 Example 39: Use according to example 38, wherein the viral-induced hepatitis is caused by hepatitis A, B or C viruses.

Example 40: Use according to anyone of the preceding examples, wherein the hepatic injury is as a result of oxidative injury or hepatic inflammation.

Example 41: Use according to example 40, wherein the oxidative injury is as a result of oxidative stress or lipid peroxidation.

25 Example 42: Use according to example 41, wherein the oxidative stress and lipid peroxidation is caused by reactive oxygen species.

Example 43: Use according to example 42, wherein the reactive oxygen species comprises one or more selected from the group comprising: hydrogen peroxide, superoxide anion radical, or hydroxyl radical.

CLAIMS

1. A polyherbal formulation comprising, or consisting of:
 - a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
 - b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
 - c) plant or plant parts of the *Rauwolfia* family, especially *R serpentina*, in an amount of at least 25 %, preferably at least 27 %.
2. A polyherbal formulation according to claim 1 comprising not more than 70%, and preferably not more than 60%, plant or plant parts of the *Eclipta* family.
3. A polyherbal formulation according to claim 1 or 2 comprising not more than 30 % plant or plant parts of the *Phyllanthus* family.
4. A polyherbal formulation according to any one of claims 1 to 3 comprising not more than 36 % plant or plant parts of the *Rauwolfia* family.
5. A polyherbal formulation according to any one of claims 1 to 4 comprising 43% to 46% plant or plant parts of the *Eclipta* family, 23% to 26% plant or plant parts of the *Phyllanthus* family and 29% to 32% plant or plant parts of the *Rauwolfia* family.
6. A polyherbal formulation according to any one of claims 1 to 6 wherein 100 mg the PHF comprises 44.5 mg to 44.85 mg of plant or plant parts of the *Eclipta* family, 24.5 mg to 24.6 mg of plant or plant parts of the *Phyllanthus* family and 30.6 mg to 30.7 mg of plant or plant parts of the *Rauwolfia* family.
7. A polyherbal formulation according to any one of claims 1 to 6 wherein the plants or plant parts are of *Eclipta alba*, *Phyllanthus amarus* and *Rauwolfia serpentine*.
8. A polyherbal formulation according to any one of claims 1 to 7 wherein plant parts are selected from one or more of fruit, seeds, rhizome, buds, leaves, husk, bark, petals and petal extracts, fruit cover, seed cover and stems.

9. A polyherbal formulation according to any one of claims 1 to 8, further comprising one or more secondary metabolites selected from flavonoids, saponins, coumestan, phenols, alkaloids, tannins and lignans.

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10. A polyherbal formulation according to any one of claims 1 to 9 wherein the phytochemical content of the polyherbal formulation comprises greater than 35 mg/g alkaloids, greater than 110 mg/g flavonoids, greater than 40 mg/g total phenol, greater than 20 mg/g tannin, greater than 60 mg/g saponin, and greater than 10 mg/g total protein.

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11. Use of a polyherbal formulation according to any one of claims 1 to 10 in the preparation of a medicament for the treatment of a liver disease, hepatic injury, or hepatotoxicity in a subject in need thereof.

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12. Use according to claim 11, wherein the liver disease comprises non-alcoholic fatty liver disease (NAFLD), cirrhosis, fibrosis or infection by HBV.

13. Use according to claim 12, wherein the liver disease is caused by hepatotoxicity, and wherein the hepatotoxicity is as a result of one or more selected from the group comprising: alcohol, environmental pollution, viral-induced hepatitis, hepatocellular carcinoma, and hepatotoxic drugs.

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14. Use according to claim 13, wherein the viral-induced hepatitis is caused by hepatitis A, B or C viruses.

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15. A liver specific polyherbal formulation comprising, or consisting of:

- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
- b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*, in an amount of at least 15%, preferably at least 20%, and at most 40%; and
- c) plant or plant parts of the *Rauwolfia* family, especially *R serpentina*, in an amount of at least 25 %, preferably at least 27 %.

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16. A hepatic polyherbal formulation comprising, or consisting of:
- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
 - b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*,
5 in an amount of at least 15%, preferably at least 20%, and at most 40%; and
 - c) plant or plant parts of the *Rauwolfia* family, especially *R serpentina*, in an amount of at least 25 %, preferably at least 27 %.
17. Use of a polyherbal formulation in the preparation of a medicament for the
10 treatment of Hepatitis B Virus (HBV) infection, in a subject in need thereof, wherein the polyherbal formulation of the medicament comprises, or consists of:
- a) plant or plant parts of the *Eclipta* family, in particular *Eclipta alba*, in an amount of at least 30%, preferably at least 35 or 40%; and
 - b) plant or plant parts of the *Phyllanthus* family, in particular *Phyllanthus amarus*,
15 in an amount of at least 15%, preferably at least 20%, and at most 40%; and
 - c) plant or plant parts of the *Rauwolfia* family, especially *Rauwolfia serpentina*, in an amount of at least 25 %, preferably at least 27 %.