

BIOLOGICAL VERIFICATION AND QUALITY ASSESSMENT OF A NATURAL HEPATOPROTECTIVE RECIPE

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Received: 30 May, 2014 Revised and Accepted: 05 Jun 2014

ABSTRACT

Objective: Biological verification of an eight-ingredient hepatoprotective recipe available in the Egyptian market was carried out followed by biologically guided reduction of non-essential ingredients aiming determination of the most potent formula containing the least number of ingredients to facilitate quality assessment procedure.

Methods: Investigation of hepatoprotective effect afforded by the marketed and the other reduced four recipes using carbon tetrachloride as hepatic toxicant in rats with reference to Silymarin as standard where values of certain hepatic and antioxidant enzymes were estimated. The chosen recipe was subjected to quality assessment procedure including microscopical identification of diagnostic elements; determination of pharmacopoeial constants; detection of heavy metals, mycotoxins' and microbial contamination, detection of pesticides' and radioactive residues and quantitative HPLC estimation of certain biochemical marker compounds.

Results: The most potent hepatoprotective recipe formed of three ingredients namely, Milk- thistle or Silybum (*Silybum marianum* Gaertn. fruits), Curcuma (*Curcuma longa* L. rhizomes) and Liquorice (*Glycyrrhiza glabra* L. roots and rhizomes) in its ethanol and aqueous extracts which reduced the elevated levels of liver and antioxidant enzymes to 69.43 ± 2.26 , 163.26 ± 2.16 , 40.85 ± 2.18 and 280.39 ± 8.45 & 70.50 ± 2.40 , 164.05 ± 2.20 , 40.65 ± 1.80 and 279.03 ± 7.90 for ALT, AST, GSH and SOD respectively while had non-significant effect on AP levels. Quality assessment procedure revealed that it is formed of genuine medicinal plants, devoid of hazardous heavy metals, mycotoxins, pathogenic organisms and radioactive residues while screening of pesticide residues revealed the existence of acetamiprid, carbendazim, chlorpyrifos, cyper-methrin, lambda-cyhalothrin and malathion at levels of 0.01, 0.09, 0.04, 0.01, 0.01 and 0.02 mg kg⁻¹ respectively which considered to be harmless. Quantitative HPLC investigation revealed that ethanol and aqueous extracts contain Silymarin, Curcumin and Glycyrrhetic acid at levels of (75, 120 and 40 mg ml⁻¹) and (100, 80 and 45 mg ml⁻¹) respectively.

Conclusion: The gained results proved that the three-ingredient recipe is the best among the screened ones as both ethanol and aqueous extracts, in addition, it meets the quality standards required by WHO, moreover, quantitative HPLC investigation revealed that both extracts contain comparable quantities of biochemical marker compounds leading to adequate hepatoprotective and so, the recipe fitted the basic requirements for developing natural hepatoprotective agents.

Keywords: Biological verification, Hepatoprotective, Silybum, Curcuma, Liquorice, Quality assessment and standardization.

INTRODUCTION

Medicinal plants and their preparations have been used since the earliest history of humanity and have formed one of the foundations for healthcare in different cultures throughout the world [1]. The World Health Organization (WHO) estimates that 80% of the world populations rely on natural medicines of plant origin for their primary healthcare [2], so the demand of such drugs is becoming universal as people became more cognisant about the benefits of natural medicines and affordability compared to the expensive modern pharmaceuticals [2, 3], most of traditional medicines are effective but just needed to be validated to assess the quality, quantity and purity of the drugs [4].

Liver diseases are worldwide problem which is much more exaggerated in Egypt [5], ranging from drug-induced toxicity, viral hepatitis to liver damage. Viral hepatitis accounts for about 75% of liver diseases are regarded a major threat to public health worldwide [6] they can lead to long lasting chronic effects as fibrosis resulting in cirrhosis and/or hepatocellular carcinoma [7].

Depending on the fact that the liver is one of the largest human body organs which is the chief site for metabolism and detoxification [8] plays surprising roles in several biochemical pathways responsible for growth, immunity, energy provision as well as reproduction [9] and in spite of the tremendous advances in modern medicine, no effective drugs are available to overcome some liver problems [9, 10] put challenges for scientists to explore the potential of hepatoprotective activity of plants based on traditional use where large number of natural medicinal preparations are recommended

for the treatment of liver disorders [10-13] keeping in consideration the importance of the conditions for proper and appropriate use of natural drugs to fulfill the criteria of safety, efficacy and quality [14], where safety in the meaning of assuring complete absence or at least presence of the least acceptable limits of toxic heavy metals, microorganisms, mycotoxins, pesticides and radioactive compounds in the drug; efficacy means that the drug must be verified biologically and described to be efficient in a given dose, while quality means evaluation of the identity, purity, content, chemical, physical and biological properties of the drug [14-16].

So, this work was carried out to biologically verify a hepatoprotective herbal recipe available in the Egyptian market followed by biologically guided reduction of some of ingredients aiming determination of the most biologically potent formula containing the least number of ingredients derived by the fact that each herbal extract represents complex chemical and biological mixture and so achieving reproducible quality can be a challenging task in its quality assessment [17].

MATERIAL AND METHODS

Plant material

The marketed herbal recipe and its individual components were purchased from a local indigenous herbal shop (Harraz herbal shop, Bab El-Khalk, Cairo) on April 2013, they included; dried fruits of *Silybum marianum* Gaertn. (Asteraceae); dried rhizomes of *Curcuma longa* L. (Zingiberaceae); dried roots and rhizomes of *Glycyrrhiza glabra* L. (Fabaceae); dried leaves of *Cassia acutifolia* L. (Fabaceae);

dried leaves of *Rosmarinus officinalis* L.(Lamiaceae); dried seeds of *Nigella sativa* L. (Ranunculaceae); dried leaves of *Cichorium intybus* L. (Asteraceae) and dried fruits of *Pimpinella anisum* L. (Lamiaceae); their identities were established by Professor Dr. Mohamed El-Sayed Tantawy, Professor of Botany, Faculty of Science, Ain Shams University.

Voucher specimens are kept in a herbarium in Pharmacognosy Department, Faculty of Pharmacy Al-Azhar University, Cairo, Egypt; the plant material were powdered and kept in tightly closed amber coloured glass containers protected from light at low temperature.

Animals

Adult albino mice and rats of either sex weighing 20-25 and 150-200 g obtained from The Animal House Laboratory, Faculty of Pharmacy, Al-Azhar University, Cairo were used for acute toxicity and hepatoprotective effect studies respectively, they were housed under standard conditions and received a standard diet and water *ad libitum*.

Diagnostic Kits

Pre-made kits for evaluation of glutamic oxaloacetic transaminase (ALT), glutamic pyruvic transaminase (AST), alkaline phosphatase in serum and reduced glutathione (GSH) and superoxide dismutase (SOD) RBCs (Randox Laboratories, Crumlin, England) suitable for automatic biochemical analyzers.

Authentic reference material

Mycotoxins

The authentic samples for B₁, B₂, M₁, G₁, G₂, Sterigmatosytin and Ochratoxin were supplied through The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Microorganisms

Bacterial strains; *Escherichia coli*, *Enterobacter*, *Clostridium perfringens*, *Coliforms*, thermotolerant coliforms, *Salmonella spp.*, *Micrococcus spp.* and fungi; *Candida albicans*, *Asperigillus parasiticus*, *A. flavus* were supplied through The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Pesticides

Authentic samples for pesticide analysis were supplied through Central Laboratory of Residues Analysis of Pesticides and Heavy Metals from Food - Agriculture Research Center, Giza, Egypt.

Radioactive material

Uranium-238, Radium-226, Thorium-232, Cesium-137 and Potassium-40 were supplied through The Central Laboratory for Radiological Environmental Measurements, Comparison and Training, The Nuclear and Radiological Regulation Authority, Nasr City, Cairo, Egypt.

HPLC

Silymarin (Legalon®70mg tablets, Chemical Industries Development Co. (CID), Giza, Egypt), Curcumin was kindly provided by Professor Dr. Mohamed Hosny hussien, Professor of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University and Glycyrrhetic acid supplied through The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Solvents and chemicals

Ethanol, tween-80, *n*-hexane, methanol, phosphoric acid (El-Nasr Chemicals Co., Egypt), chloroform, *n*-hexane, methanol, trifluoroacetic acid, acetonitrile, phosphoric acid, acetic acid, formic acid (analytical and HPLC grade), anhydrous sodium acetate, magnesium sulfate, sodium sulfate (E. Merck, Darmstadt, Germany) and de-ionized water from mille Q unit (Mille Pore).

Preparation of extracts

For each recipe, 50 g were extracted separately with 500 ml of hot distilled water and 70% (v/v) aqueous ethanol for 15 min, vacuum

filtered through Whatmann No.1 filter paper and vacuum dried at 40°C to yield aqueous and ethanol extracts respectively.

Acute toxicity studies [18]

adult albino mice of either sex were fasted overnight, divided into twelve groups - six animals each (equal number of males and females) - were fed with increasing doses of tested extracts at doses equivalent to 0.5, 1, 2 and 3 g / kg b.wt., animals were observed for any toxic symptoms and mortalities during the first 24-72 hours post treatment.

Evaluation of hepatoprotective effect

one hundred and fifty animals were divided into fifteen groups - ten animals each (equal number of males and females) - as follows; group1, normal control, received nothing except *ad libitum*, water and i.p. dose of 200 µl/kg b.wt. paraffin oil every three days; group2, received i.p. carbon tetrachloride in paraffin oil 200 µl/kg b.wt. every three days; group3, received daily oral dose of Silymarin 5 mg/kg b.wt; group4, received daily oral dose of market recipe aqueous extract 300 mg/kg b.wt; group 5, received daily oral dose of recipe-1 aqueous extract 300 mg/kg b.wt; group 6, received daily oral dose of recipe-2 aqueous extract 300 mg/kg b.wt; group 7, received daily oral dose of recipe-3 aqueous extract 300 mg/kg b.wt; group 8, received daily oral dose of recipe-4 aqueous extract 300 mg/kg b.wt; group 9, received daily oral dose of recipe-5 aqueous extract 300 mg/kg b.wt; group 10, received daily oral dose of market recipe ethanol extract 300 mg/kg b.wt; group 11, received daily oral dose of recipe-1 ethanol extract 300 mg/kg b.wt; group 12, received daily oral dose of recipe-2 ethanol extract 300 mg/kg b.wt; group 13, received daily oral dose of recipe-3 ethanol extract 300 mg/kg b.wt; group 14, received daily oral dose of recipe-4 ethanol extract 300 mg/kg b.wt; group 15, received daily oral dose of recipe-5 ethanol extract 300 mg/kg b.wt., meanwhile all the treated groups (4-15) received the extracts simultaneously with i.p. injection of carbon tetrachloride in paraffin oil at dose level of 200 µl/ kg b.wt. every three days for two weeks, blood samples were collected divided into two aliquots, the first received on EDTA in dry clean tubes for GSH [19] and SOD [20] in RBCs using tissue homogenizer (Omni Tissue Homogenizer (TH) while, the second was received in dry clean tubes, allowed to clot at 37°C for one hour, centrifuged at 3000 rpm for 15 minutes where serum was separated, divided into aliquots for evaluation of ALT [21], AST [21] and AP [22] activities spectrophotometrically using Genesys Spectrophotometer (Milton Roy, INC., Rochester, NY).

Determination of physicochemical parameters [23]

ash values i.e. total ash, water-soluble ash, alkalinity of water soluble ash and acid-insoluble ash values, moisture content and solvent extractives' values were determined for the chosen recipe.

Determination of microbial contaminants [24-28]

one g of the chosen recipe was mixed with 9 ml sterile peptone water and then 10 fold serial dilutions were made, five level-spacing single logarithmic unit were investigated by pipetting 1ml from each level in a plate, 15 ml of nutrient (agar for bacterial count, Sabouraud dextrose agar for fungal count and Mackonky agar for pathogenic coliform count), were added. The contents were allowed to be solidified and inverted plates were incubated at 37°C, examined after 2 days for both bacterial and coliform count and after 7 days for fungal count and suitable dilution were counted.

HPTLC/HPLC quantitative determination of mycotoxins [29, 30]

The herbal samples were extracted three times with chloroform, the chloroform extract was defatted using *n*-hexane and evaporated under nitrogen at room temperature, residue was dissolved in 50% methanol/ water (1:1 v/v). Trifluoroacetic acid (TFA; 50 µl) were added to 200µl of the methanol solution of the toxin extract where the mixture was allowed to react for 15 min. at room temperature and then was evaporated to dryness. The residue was dissolved in 2,000 µl of water-acetonitrile (75:25 v/v) for both HPTLC and HPLC.

HPTLC qualitative high pressure thin layer chromatography was carried out using silica gel 60F₂₅₄ aluminum sheets by Camag

Linomat-5 applicator, with mobile phase acetone: chloroform (1:9); analysis of the samples was achieved through co-chromatography using different authentic mycotoxins and was carried using HPTLC-Chroma Dex® system.

HPLC quantitative high pressure liquid chromatography investigation of the samples was carried out using system equipped with a binary pump (LC 1110; GBC scientific Equipment, Melbourne, Australia) and C18 column (hypersil Elite C18, Phenomenex, Torrance, CA, USA; 5µm, 250X6.4 mm) where the injection volume was 10 µl, the mobile phase was deionized water-methanol-acetonitrile (55:22.5:22.5, v/v/v) and the flow rate 1.0 ml/min. A fluorescence detector (LC 1255; GBC Scientific Equipment) with the excitation wavelength set at 365 nm and the emission wavelength 440 nm was used to detect and quantitate mycotoxins where individual standard curves for different mycotoxins (sigma chemicals Co.) were prepared using different concentrations ranging from 1µg/ml to 1ng/ml were used for calculation of each mycotoxins.

Determination of heavy metal contamination [31]

the herbal sample was subjected to screening for the existence of heavy metals contamination and elemental analysis where it was mounted onto electron prop X-ray microanalyzer (Module Oxford 6587 INCA x-sight) attached to JEOL JSM 5500 LV scanning electron microscopy at 20 Kev after gold coating using SPI- Module sputter equipped with a Gresham light element detector and a Quartz Xone energy dispersive X-ray (EDX) analysis system to quantify the elemental content in samples and to show spatial distribution of elements in the substrate. The significance differences in element concentrations between substrate and standards were evaluated by one-way analysis of variance and Tukey's pairwise comparisons (MINITAB software).

LC-ESI-MS/MS determination of pesticide residues [32]

LC- MS/MS was performed with an Agilent 1200 Series HPLC instrument coupled to an API 4000 Qtrap MS/MS from Applied Biosystems with an electrospray ionization (ESI) interface, separation was performed on an Agilent ZORBAX Eclipse XDB C18 column 4.6x150 mm, 5µm particle size and an Agilent Chromatograph system 7890A equipped with tandem mass spectrometer 7000B series; Column Phenomenex 50 mm column Aqua C18 with internal diameter: 0.32 mm and film thickness 5 µm, Flow rate; 0.2 ml min⁻¹, Mobile phase; Solvent A: deionized water-0.1 formic acid, Solvent B: acetonitrile - 0.1% formic acid with Mass Spectrometer Electrospray Ionization, Multiple Reaction and monitoring (MRM) mode were used where 50 g of sample under investigation was comminuted in a disintegrator for 1 min., 5 g was transferred into 50 ml centrifuge tube having 10 ml deionized water and 10 ml acetonitrile (containing 1% acetic acid), vortexed for 3min., set for 1 h, 5 g anhydrous sodium acetate and 4 g anhydrous magnesium sulfate were added, vortexed for 1 min., the tubes were immediately cooled in ice bath for 5 min., centrifuged for 5 min. at 5000 rpm, samples were then subjected to dispersive solid phase extraction (SPE) clean-up [(SPE column was conditioned with 10 ml acetonitrile-toluene 3:1 (containing 1% acetic acid)], 1 ml of the extract was subjected to CC eluted with 20 ml acetonitrile-toluene 3:1 (containing 1% acetic acid). The effluent was concentrated to 1 ml by evaporating with weak nitrogen stream at 40°C and the residue was reconstituted in 1ml acetonitrile (1% acetic acid), filtered with 0.2 acetonitrile (1% acetic acid) and with 0.2 µm organic filter and then injected into LC-MS/MS for analysis. Reference standard curves were prepared where 1000 µg/ml reference standard solution of each pesticide in methanol prepared as stock solution to be kept at -18±2°C were used for preparing intermediate solutions by diluting 10µg/ml of each standard pesticide solution with methanol to yield calibration solutions of 0.005, 0.01, 0.05, 0.1 and 0.5µg/ml.

Determination of radioactive residues [33]

the gamma ray spectrum of the prepared sample was measured using a typical high resolution gamma spectrometer based on coaxial type shielded HpGe detector, with relative photo peak

efficiency of 35% and energy resolution of 1.9 keV FWHM for the 1332 keV gamma ray line of ⁶⁰Co. The spectrum was analyzed using computer software Genie 2000 software where the activity of ⁴⁰K was measured directly via its 1461 (10.7%) keV peak of the gamma ray spectrum, to determine the activity concentration of ²²⁶Ra, gamma ray lines 295.1 (19.2%) and 351.9 (37.1%) keV from ²¹⁴Pb and 609.3 (46.1%) and 1764.5 (15.9%) keV gamma rays from ²¹⁴Bi were used. Activity concentration of ²³²Th was determined using gamma ray lines 238.6 (43.6%) keV from ²¹²Pb, 338.4 (12%), 911.1(29%) and 968.9 (17.4%) keV from ²²⁸Ac, 583.1 (86%) and 2614 keV from ²⁰⁸Tl. The spectrometer was calibrated for energy and efficiency over the photon energy range 186-2450 keV using two-stage method described in literature (Farouk and Al- Soraya, 1982).

HPLC quantitative estimation of biochemical marker compounds

Preparation of extracts

the aqueous extract prepared as follows; 5.0 g of powder were mixed with 50 ml boiling water in a 100 ml conical flask and soaked for 10 min. filtered, the filtrate cooled, transferred to 50 ml volumetric flask and the volume completed with water to the mark, while ethanol extract preparation involved two-step procedure; defatting of 0.5 g of powder with 20 ml *n*-hexane in a soxhlet apparatus for 1 h, then *n*-hexane was discarded and the residue was re-extracted again in a soxhlet apparatus for 1 h, filtered and evaporated to dryness under reduced pressure at 40°C where 10 mg of each residue were dissolved separately in 1 ml methanol and 10 µl of each prepared solution was subjected to HPLC.

HPLC for quantitative determination

HPLC- high performance liquid chromatography, Waters Millennium 32 system with GBC U.V/Vis detector, GBC LC 1110 pump, Win Chrome chromatography software version 1.3 and kromacil (5µm, 100X4.6 mm) column, flow rate 1 ml min⁻¹, samples injector (20 µl) was used where isocratic procedure was first used using water with 0.1% formic acid mobile phase followed by gradient procedure held at 35% methanol-water (v/v) for 3 min; 35 to 45% methanol (v/v) 10 min; 45% methanol (v/v) for 2 min; 45 to 100% methanol (v/v) in 5 min with UV detection at 254 nm.

Statistical analysis

data were presented as mean ± standard error while the differences between groups were statistically analyzed for significance by Student's t-test using the software Graph Pad Prism, version-3 for windows, Graph Pad Software (San Diego, CA,USA).

RESULTS AND DISCUSSION

Biological studies

The calculated LD₅₀ of the tested extracts ranged from 3-3.5 g/kg b.wt. this made all of them safe drugs and hence 300 mg/kg b.wt. was selected as the therapeutic dose.

Biological verification and biologically guided reduction of the hepatoprotective market recipe revealed that their administration for two weeks parallel to regular exposure to the hepatic toxicant carbon tetrachloride exhibited very highly significant to highly significant reduction in the activities of serum ALT (68.02-51.81% & 69.57-51.70%) AST (72.76-55.48% & 73.23-56.88%), non-significant effect on AP and achieving very highly significant to highly significant elevation of the antioxidant enzymes GSH (22.55-30.47% & 24.61-31.15%) and SOD (31.73- 37.04% & 31.13- 30.50 %) for aqueous and ethanol extracts respectively which suggests them to be potent hepatoprotective recipes (table 1).

The hepatoprotective effect of the recipes can be attributed to the main constituting medicinal plant ingredients viz. silybum, curcuma and liquorice [34-36] and derived by the fact that pure chemicals are not effective as the collective herbal extracts and may exhibit undesirable side effects European Economic Council (EEC) advice by the use of the total extracts rather than the individual chemical constituents [37-39] and supported by the fact that the larger the

number of the constituting plants, the more probable the interactions between their constituents which may lead to undesired effects upon prolonged administration, so our work was

directed towards choosing the recipe achieving adequate biological activity that consists of the least number of medicinal plants[40] which was recipe-5 consisting of curcuma, silybum and liquorice.

Table 1: Effect of aqueous and ethanol extracts of different natural recipes on serum ALT, AST, AP activities and GSH, SOD levels on carbon tetrachloride-induced hepatotoxicity in rats:

Parameters Groups	Liver Enzymes			Antioxidant Enzymes		
	ALT	AST	AP	GSH	SOD	
Normal Control	70.56±2.38	170.39±2.45	41.50±1.63	44.33±1.92	297.30±9.80	
CCL ₄	118.50±3.01	288.04±4.60	46.90±2.05	27.14±1.24	168.90±5.35	
CCL ₄ +Silymarin	65.44±2.89***	185.65±3.85***	44.35±1.95 ^{ns}	40.95±1.85***	289.30±6.20***	
Aqueous Extracts	CCL ₄ + Recipe-M	81.95±2.71**	193.50±2.98**	46.90±2.05 ^{ns}	37.15±1.95**	260.25±6.65**
	CCL ₄ +Recipe-1	82.94±2.60**	194.35±3.26**	47.45±2.10 ^{ns}	38.15±1.67**	262.30±6.88**
	CCL ₄ +Recipe-2	82.13±2.55**	185.45±2.85**	47.05±2.18 ^{ns}	38.42±1.79**	264.15±7.15***
	CCL ₄ +Recipe-3	79.16±2.45***	182.70±2.79***	46.49±2.05 ^{ns}	39.76±1.83***	266.93±7.20***
	CCL ₄ +Recipe-4	74.19±2.39***	170.64±2.35***	44.65±1.99 ^{ns}	39.75±1.85***	273.96±7.81***
Ethanol Extracts	CCL ₄ +Recipe-5	70.50±2.40***	164.05±2.20***	43.10±1.97 ^{ns}	40.65±1.80***	279.03±7.90***
	CCL ₄ +Recipe-M	82.02±2.63**	191.14±2.88**	47.10±1.95 ^{ns}	38.05±1.69**	261.45±6.58**
	CCL ₄ +Recipe-1	81.50±2.53**	190.18±3.05**	47.20±2.11 ^{ns}	38.29±1.55**	263.25±6.92**
	CCL ₄ +Recipe-2	78.25±2.48**	178.09±2.59***	45.95±2.00 ^{ns}	39.11±1.71***	268.74±7.29***
	CCL ₄ +Recipe-3	78.15±2.38***	179.75±2.40***	45.89±1.98 ^{ns}	39.52±1.95***	296.48±7.58***
Ethanol Extracts	CCL ₄ +Recipe-4	71.94±2.36***	164.74±2.21**	43.55±1.89 ^{ns}	40.38±2.05***	278.26±8.05***
	CCL ₄ +Recipe-5	69.43±2.26***	163.26±2.16***	42.58±1.85 ^{ns}	40.95±2.18***	280.39±8.45***

Where: Results representing means ± standard error, CCL₄: Carbon tetrachloride; *P < 0.05 (significant), **P < 0.01 (highly significant); ***P < 0.05 (very highly significant); ns: non-significant; Recipe-M: market recipe containing Curcuma, Silybum, Liquorice, Senna, Rosemary, Nigella, Chicory and Anise; Recipe-1: prepared standard-recipe containing Curcuma, Silybum, Liquorice, Senna, Rosemary, Nigella, Chicory and Anise; Recipe-2: Curcuma, Silybum, Liquorice, Rosemary, Nigella and Anise; Recipe-3: Curcuma, Silybum, Liquorice and Chicory; Recipe-4: Curcuma, Silybum, Liquorice and Senna; Recipe-5: Curcuma, Silybum and Liquorice.

Microscopical investigation

Microscopical examination of the three- ingredient recipe confirmed the existence of their diagnostic elements with reference to the reported data [41, 42] which are; compositae glandular hair with short biseriate stalk composed of two or four cells and biseriate glandular head, multicellular uniseriate non-glandular hair with enlarged apical cell, from two to three small basal cells and the spheroidal pollen grain with three germ pores and three germ furrows having spiny and warty exines of silybum; numerous masses of gelatinized starch grains with bright yellow colour, characteristic shiny oil cells, unicellular non-glandular hairs which are elongated, conical and bluntly pointed with moderately thickened walls and non-lignified vessels of curcuma and the characteristic crystal sheath which is formed of groups of lignified fibers surrounded by calcium oxalate prism sheath; the orange brown cork cells, bordered-pitted lignified fragments of large xylem vessels and numerous small spherical to ovoid starch grains of liquorice (figure 1).

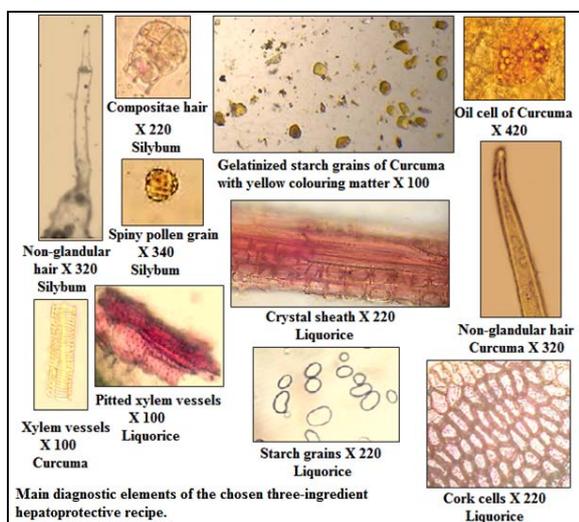


Fig. 1: Microscopical diagnostic elements of the chosen three-ingredient recipe

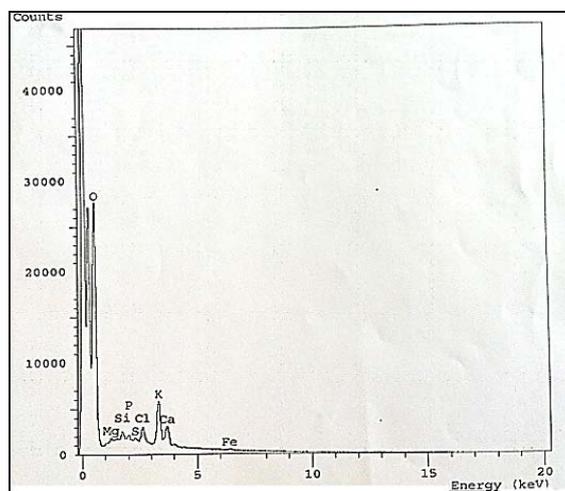


Fig. 2: Elemental analysis of the chosen three-ingredient recipe

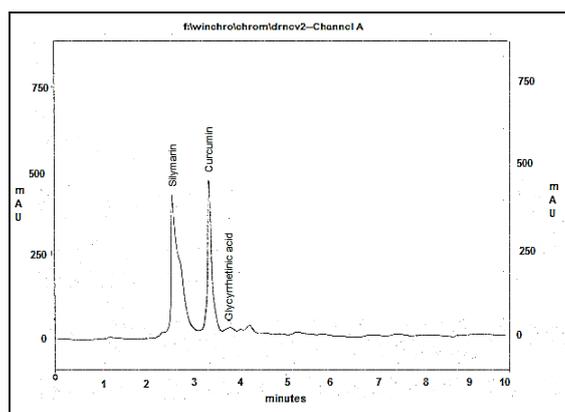


Fig. 3: HPLC chromatogram of the aqueous extract of the chosen three-ingredient recipe

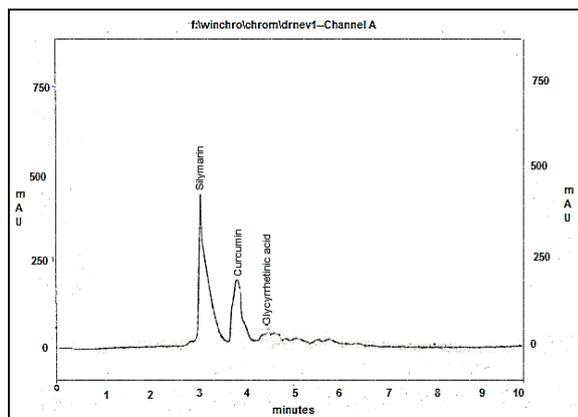


Fig. 4: ingredient recipe HPLC chromatogram of the ethanol extract of the chosen three

Table 2: Some physicochemical constants estimated for the chosen three-ingredient recipe:

Physicochemical parameter	Mean (%W/W)	
Moisture content	Loss on drying at 110 °C	17.59±2.25
Ash values	Total ash	7.35±1.96
	Water soluble ash	2.79±1.75
	Water insoluble ash	0.80±0.36
Extractives by cold maceration	Petroleum ether	5.19±0.95
	Ethyl acetate	6.92±1.50
	Ethanol	17.10±1.82
	Aqueous	17.90±1.79

Results listed are the means of three successive readings ± standard error.

Pharmacopoeial constants

The guidelines of WHO, 2007 stated that certain specific parameters and values must be determined during quantitative and qualitative estimations of any herbal drug either in single or in mixture form so moisture content, ash and extractives' values were determined for the chosen three-ingredient hepatoprotective recipe [14], the gained results are compiled in table; 2.

Microbial contaminants

Herbal preparations are not subjected to aseptic conditions during preparation, storage and transport, so, many plant materials may carry bacteria and fungi originating in soil, also, animal manure generally have pathogenic organisms, hence causing increase in contamination leading to deterioration of the preparations [43], in addition, such preparations consumption have been associated with several diseases, moreover, they have always been hurdle in quality approval of products and standardization [44]. The gained results of investigation of microbial contaminants of the chosen three-ingredient recipe compiled in table 3 revealed that the total aerobic bacterial count, total yeast count and total fungal count are within the acceptable of WHO requirements which stated that their count in dried and instant products should not exceed 10^3 CFU/g, moreover it is devoid of pathogenic organisms as *Salmonella spp.* and *E. coli* and aflatoxin producing fungi while other organisms either absent or existed in acceptable limits ≤ 100 CFU/g [14, 16].

Mycotoxins' contaminants

Mycotoxins are by-product of fungal growth occurring naturally in a range of plant products, among them aflatoxins are considered to be the most dangerous as they are mutagenic and carcinogenic even when given in very low doses [45], so their identification and quantification has to be carried out to allow effective control of their possible contamination of food and feed commodities [14, 16]. The gained results of investigation of mycotoxins contaminants of the

chosen three-ingredient recipe compiled in table 4 revealed their absence which agreed with the complete absence of aflatoxins producing fungi table 3 and confirming its safety.

Table 3: Microbial contaminants in the chosen three-ingredient recipe

Item	Microbial Count in Sample *C.F.U. (g/100ml)
Aerobic total plate count **	$2.40 \times 10^2 \pm 0.58$
Total yeast count (unicellular fungi)	$1.90 \times 10^2 \pm 0.58$
Total fungal count (filamentous fungi)	$1.20 \times 10^2 \pm 1.20$
Bacteria	
<i>Escherichia coli</i> **	Absent
<i>Enterobacter</i>	< 100
<i>Clostridium perfringens</i>	< 100
Coliforms	Absent
<i>Fecal thermotolerant coliforms</i>	Absent
<i>Enterococci</i>	< 100
<i>Salmonella spp.</i> **	Absent
Aflatoxin producing fungi	
<i>A. flavus</i>	Absent
<i>A. parasiticus</i>	Absent

Where; data expressed as mean count ± S.E., *C.F.U. (Colony Forming Unit) and ** microorganisms included in the accredited methods.

Heavy metal contaminants

Metals are widely used in various industries and considered as one of the most common water pollutants affecting human health as their accumulation can cause serious chronic toxicity problems i.e. arsenic and chromium induce cellular damages and carcinogenesis by increasing free radical generation and lowering cellular antioxidative defense capacity [46]; lead causes anorexia, constipation, abdominal cramps, peripheral neuritis, encephalopathy, renal dysfunction, anemia, psychological disorder and olegospermia; cadmium causes renal injury, liver damage, anemia, yellow teeth and distributes calcium metabolism, hypercalcinuria and renal stones [37], thus elemental analysis was carried out adopting X-ray micro-analytical technique [47]. The results in table 5, figure 2 revealed that the sample is free from toxic heavy metal contaminants.

Table 4: Mycotoxins' contaminants in the chosen three-ingredient recipe

Compound	Result
Aflatoxin B ₁	<LQD
Aflatoxin B ₂	<LQD
Aflatoxin M ₁	<LQD
Aflatoxin G ₁	<LQD
Aflatoxin G ₂	<LQD
Sterigmatocystin	<LQD
Ochratoxin	<LQD

The limits of qualification of mycotoxins detected are 0.001 mg/kg.

The estimated relative standard deviation of this method is < 10%.

Table 5: Elemental analysis of the chosen three-ingredient recipe

Element	%
Oxygen	83.557
Magnesium	0.777
Silicon	0.987
Phosphorous	0.823
Sulfur	0.533
Chlorine	1.613
Potassium	8.097
Calcium	3.170
Iron	0.443

Pesticide residues

Pesticides are widely used in agricultural production to prevent or control pests, diseases, and other plant pathogens to reduce or eliminate yield losses and maintain high product quality [48]. The chronic ingestion of pesticides residues can cause chronic illnesses even if the amounts taken up are small as many commonly used pesticides are directly correlated to different cancers as sarcomas, multiple myelomas, cancer of the prostate, pancreas, lungs, ovaries,

breasts, testicles, liver, kidneys, and intestines as well as brain tumors [49, 50]. The results of pesticides' residues estimation in chosen three-ingredient recipe table 6 revealed the existence of six pesticides, among them acetamiprid, cypermethrin and lambda-cyhalothrin are within the limits of qualifications detected (0.01 mg kg⁻¹) while carbendazim (0.09 mg kg⁻¹), chlorpyrifos (0.04 mg kg⁻¹) and malathion (0.02 mg kg⁻¹) are exceeding these limits but found to be significantly lower than the maximum residual limits and the calculated accepted daily intake for 70 kg person [51] confirming its safety.

Table 6: Pesticide residues' concentrations of the chosen three-ingredient recipe compared to maximum residual limits (MRLs) and accepted daily intake (ADI) [51]

Pesticide detected	Concentration (mg kg ⁻¹)	MRL (mg kg ⁻¹)	ADI (mg kg ⁻¹)
Acetamiprid	< 0.01	0.20	14.00
Carbendazim	0.09	0.10	7.00
Chlorpyrifos	0.04	0.05	3.50
Cypermethrin	0.01	0.07	4.90
Lambda-Cyhalothrin	0.01	0.30	21.00
Malathion	0.02	2.00	140.00

Radioactive contaminants

Nuclear accidents usually associated with environmental contamination with airborne radioactive material which may deposit on the plants [14] meanwhile, direct exposure to ionizing radiation is unavoidable because many sources, including of

radionuclides occur naturally in the ground and the atmosphere [52]. The results of radioactive contaminants estimation in chosen three-ingredient recipe table 7 revealed the existence of ⁴⁰K 620.41±20.64 Bq kg⁻¹ which is natural radionuclide while ²³⁸U, ²²⁶Ra, ²³²Th and ¹³⁷Cs are below their acceptable detectable limits confirming its safety.

Table 7: Radioactive contaminants of the chosen three-ingredient recipe

Radioactive element	²³⁸ U	²²⁶ Ra	²³² Th	⁴⁰ K	¹³⁷ Cs
Concentration (Bq/kg)	< D.L.	< D.L.	< D.L.	620.41±20.64	< D.L.

Radioactivity detection limits D.L.; Uranium-238 < 0.7 Bq kg⁻¹, Radium-226 < 0.7 Bq kg⁻¹, Thorium-232

< 0.6 Bq kg⁻¹ and Cesium-137 < 0.04 Bq kg⁻¹.

Quantitative HPLC of the biochemical markers

HPLC was carried out for quantitative estimation of the main biochemical markers of the constituting medicinal plants (silymarin, curcumin and glycyrrhetic acid) for standardization of silybum, curcuma and liquorice respectively. The gained results showed that

the concentration of silymarin in aqueous extract 100 mg ml⁻¹ exceeds that in ethanol extract 75 mg ml⁻¹, curcumin concentration in aqueous extract was 80 mg ml⁻¹ much lower than in ethanol extract 120 mg ml⁻¹ while glycyrrhetic acid has comparable in both aqueous and ethanol extracts notably these concentration variations exhibited no significant biological differences between them.

Table 8: HPLC results for the aqueous and ethanol extracts of the chosen three-ingredient recipe

Item Biochemical marker	Aqueous Extract			Ethanol Extract				
	RT (min)	Peak area	Peak height	Conc. (mg ml ⁻¹)	RT (min)	Peak area	Peak height	Conc. (mg ml ⁻¹)
Silymarin	2.53	530910	424554	100.00	2.55	5921346	424756	75.00
Curcumin	3.32	2904292	184973	80.00	3.37	3507011	465665	120.00
Glycyrrhetic acid	4.00	1243867	36863	45.00	3.82	489880	29268	40.00

CONCLUSION

Traditional herbal formulations can be biologically verified where preparation with better efficacy has to be selected, quality and purity assessed and standardized schematically to formulate medicaments using raw materials collected from different localities and conditions looking forward to formulate naturally originated medicaments based on traditional medicines.

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