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Acute oral toxicity study of novel polyherbal formulations by using wistar rats and Swiss albino mice as per OECD 425 TG

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ABSTRACT

Background: Hydaljss08 is a new semisolid fermented hydroalcoholic polyherbal formulation containing crude drug substances. Immunoboost Tab is a novel polyherbal dosage form that is incorporated isolated, and fractionated phytochemical substances. Acute oral toxicity is a primary safety profile of the novel polyherbal formulations. As a part of our study, we estimated acute toxicity safety profile of novel dosage forms by a Swiss albino mice and Wistar rats.

Aim: According to the OECD 425 test guideline., the study aims to estimate acute toxicity tests of the "Hydaljss08, and Immunoboost Tab" dosage forms in rats and mice to assess the safety profile. Mice and rats are normally used in safety studies because of their susceptibility to toxic challenges, tumor induction, and carcinogenicity.

Material and Methods: 1 % CMC, Hydaljss08, Immunoboost-Tab polyherbal formulations, female rats, and albino mice. Animals were divided into the control groups, and treated groups with test dosage forms. At the end of the study, animals were euthanized by using the CO_2 asphyxiation technique and subjected to gross pathological examinations.

Results: According to the assessment of the experimental data, the single-dose acute toxicity study of Hydaljss08 and Immunoboost-Tab formulations in mice and rats was fine. No signs of toxicity were observed in the animals treated at (2000 mg/kg.b. wt, Po), and revealed dullness, causing lethargy, and piloerections, which only persisted on the 1st day. At the end of the study, mild congestion in the liver, and lungs was observed in animals, treated with "Hydaljss08 formulation" compared to the vehicle control groups.

Conclusions: The estimated LD_{50} of Hydaljss08, and Immunoboost-Tab polyherbal formulations in rats and mice by oral route was found >2000 mg/kg, b.wt.

Abbreviations: A/G Ratio, Albumin and Globulin Ratio; ACE-2, Angiotensin converting enzyme 2; ALT, Alanine aminotransferase; ANOVA, Analysis of Variance; APCs, Antigen-presenting cells; AP-1, Activating protein-1; AST, Aspartate aminotransferase; ASU, Ayurveda Siddha Unani Drugs; B.wt, Body weight; CAM, Complementary and Alternative medicine; CMC, Carboxymethyl cellulose; CCRAS, Central Council for Research in Ayurvedic Sciences; 3 CL^{Pro}, 3-chymotrypsin like protease inhibitors; CD4±T, Cluster of differentiation 4; CD8±T, Cluster of differentiation 8; CCSEA, The Committee for Control and Supervision of Experiments on Animals; COX-2, Cyclooxygenase-2; CO_2, Carbon dioxide; 1% CMC, Carboxymethylcellulose; CG, Vehicle Control group; EDTA, Ethylene diamine tetra acetic acid; FDA, Food and Drug Administration; HG, Immunoboost-Tab and Hydaljss08 formulations treated groups; Hb, Hemoglobin; IAEC, Institutional Animal Ethics Committee; IFN-γ, Interferon; IL, Interleukin; KSK, Kabusura kudineer churna; LD_{50}, Median Lethal Dose; LFT, Liver function Test; MPV, Mean Platelet Volume; MHC, Major histocompatibility Complex; NK, Natural killer cells; NF-κB, Nuclear factor Kappa B; OECD, Organisation for Economic Co-operation and Development; PCV, Packed cell Volume; Po, (Orally) per os- given by mouth alone or in food or water; PNo, Indian patent filing Application number; RBC, Red blood cells; RDW, Red cell distribution width; RFT, Renal function Test; SEM, Standard error of the mean; STAT-1, Signal transducer and activator of transcription 1; SGPT, Serum Glutamic Pyruvic Transaminase; SGOT, Serum Glutamic Oxaloacetic Transaminase; TLC, Total leukocyte count; TNF-α, Tumor necrosis factor alpha; USEPA, United States Environmental Protection Agency; WHO, World Health Organisation; WBC, White Blood Cells.

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Introduction

Traditional medicine is receiving attention as it is the source of phytopharmaceuticals for the development of pharmacological agents that can yield the desired results (Hamid et al., 2021). The immune system involves many diseases etiology and pathophysiological mechanisms (Ghule et al., 2006). Immunomodulation can be immunostimulating in drug-induced and immunosuppression in an experimental hyper-reactivity model by the same preparation. Immunosuppression is a major drawback in radiotherapy, because of escalated severe late toxicity. Both methods have side effects such as hypertoxicity, nausea, vomiting, mucosal ulceration, alopecia, pulmonary fibrosis, and cardiac arrest. Drugs that could reduce these adverse effects and stimulate immunity will be a great help in improving cancer treatment strategies (Sonawane et al., 2022). The immune system is designed to protect against the antigens and to eliminate the disease. Immunity has been scientifically divided into two types, innate and adaptive (Cruvinel et al., 2010). Plants are an essential and integral part of complementary and alternative medicine (CAM), and this is due to their ability to develop and generate secondary metabolite compounds, called as phenolic substances, flavonoids, alkaloids, glycosides, terpenoids, steroids, and proteins. These secondary metabolites are used to promote the health and treat many diseases i.e. Congestive heart failure (Digoxin, digitoxin, Scillaren-A), Anti-cancer agents (Vincristine, Vinblastine, etoposide (Etoposide), Taxol, and Teniposide), Anti-malarial drugs (Quinine, and Artemisinin), Laxatives (Sennosides-A, B, C, D, and Isabgol), Immunomodulatory drugs (Kabusura kudineer churna(KSK), Triphala churna, Piperine, Curcumin, and Apigenin), Anti-viral Drugs (Quercetin, 6-Gingerol, and Lupeol), Cardiac depressants (Quinidine), Microbial tumor agents (Daunorubicin, bleomycin (Bleomycin) A_2, bleomycin (Bleomycin) B_2, and Actinomycin-D). Herbal drugs are believed to enhance the body's natural resistance against infection, and many immunomodulatory effects have been reported in a various plant (Kajaria et al., 2012). In the 19th century, herbal treatment was the most preferred and available method when the medicinal therapy era began. Many compounds of herbal origin have achieved widespread use as medicinal agents, e.g., Taxol from *Taxus baccata* (English yew), Vincristine, and vinblastine from *Vinca rosea* as anticancer agents, Silibinin from *Silybum marianum* (milk thistle) as a liver tonic, Andrographolides from *Andrographis paniculata*, and 6-Gingerol from *Zingiber officinalis* as an Immunomodulatory and Anti-viral agent, etc. (Ishtiaq et al., 2017). Polyherbal formulations reveal high effectiveness in various diseases because of the presence of phytopharmaceuticals and their therapeutic effects are further potentiated when compatible herbs are formulated together in a dosage forms (Bisht and Ram, 2017). There has been wide concern about the toxicity of herbal medicine, and several side effects (such as allergic reactions, hepatotoxicity, nephrotoxicity, and cardiac toxicity) of herbal medicines have been reported in recent years (Chen et al., 2018). Plants with medicinal effects should have low toxicity because of their long-term use in humans. However, various medicinal plants used in folklore medicines have been reported to exhibit toxic effects. According to the father of toxicology "Paracelsus" all the substances that exist in the universe are poisons. That dose decides substances, drugs, and medicines from the poison (Saleema et al., 2017). Toxicology is the study of unwanted effects of substances, drugs, medicines, plant-based dietary products, and nutrients. It is the interaction between toxicants of particular substances and cells of the animals and human body. These adverse effects may occur between the toxicants, and the whole human, animal body, and particular organs skin, ovaries, testes, and vital organs such as the heart, kidney, liver, lungs, brains, and spleen. Hence, evaluating the toxicity of substances is mandatory when considering the public health protection, because exposure to chemical substances may cause severe adverse effects in human beings, animals, and living organisms. An example of the case study was the Bhopal gas tragedy i.e. methyl isocyanate (MIC) gas leaked from the union carbide India (Pvt) Ltd (UCIL) plant in December 3rd,1984. A recent good example of drugs, and medicine toxicity according to the World Health Organisation (WHO) linked to cough, and cold syrups manufactured by the Indian drugmaker Maiden Pharmaceuticals Pvt ltd, products are (Promethazine oral solution, Kofexmalin baby cough syrup, Makoff baby Cough Syrup, and Magrip N Cold Syrup) contaminated with diethylene glycol (DEG), and ethylene glycol (EG). The consumption of contaminated syrups about 70 children died between the ages of 5 at the Gambia, because of the severe acute kidney damage and its due to the diethylene glycol (DEG), and ethylene glycol (EG) are used in syrups preparation (Jothy et al., 2011). Administering toxicity tests in appropriate animal models is key to ensuring drug safety and it is the fundamental science of poison. According to the Organization of economic co-operation and development (OECD) guidelines, acute toxicity study was an advanced technique for controlling single, and multiple-dose substances to report the primary safety profile of particular substances (Majumdar et al., 2021). The British pharmacologist J. W. Trevan created the first acute oral toxicity test in 1927. It used to involve up to 150 animals, until the OECD test guideline TG 401 standardized, and it was revised 45 animals in the year 1981 (Luechtefeld et al., 2016). The safety of phytopharmaceuticals in isolated, fractions, extracts, and herbs are well documented, but the adverse effects of these developed herbal products in a combined dosage form, and compounds in combination are unclear, not reported and it was mandatory to study the acute toxicity before proceeding of the efficacy, repeated dose, and developmental toxicity of the polyherbal formulations and Pharmaceutical based products. Thus, the safety and toxicity for the combination of herbs and polyherbal formulations were evaluated before their use of the human consumption, and approval required from the regulatory bodies (Pandhare, 2020). The safety of herbal medicines remains a major concern. According to the report published by the United States Food and Drug Administration (USFDA), and it was estimated that over 50, 000 unwanted effects are produced by phytopharmaceutical and plant-based dietary nutrients. In addition, the efficacy is not proven for most of the herbal drugs, and the quality is not assured. The vision of 2014–2023 was for the WHO to promote the Ayurveda, Siddha, Unani Drugs (ASU) and Traditional medicine (TM) by increasing awareness, and providing guidance on regulatory, and quality assurance standards (Bhatt, 2016; Strickland et al., 2018). Kabusura kudineer churna (KKR) is a marketed siddha-based polyherbal formulation widely used in the immunomodulatory, anti-viral effects, and strongly recommended by the Ministry of Ayush govt of India during the pandemic condition of COVID-19 (Parameswaran et al., 2021, Judy, Strickland et al., 2023). The principle of the Indian traditional and the allopathic system of medicine was optimized to developed a polyherbal formulations for the immunostimulant, anti-viral effects and treatment of COVID-19 called as "Hydaljss08" and Immunoboost-Tab dosage forms. "Hydaljss08″ is a hydroalcoholic semisolid fermented polyherbal formulation composed of dried powdered and fresh forms of crude drugs. "Immunoboost-Tab" is a solid tablet formulation containing pure isolated phytopharmaceuticals and fractionated substances. Both developed dosage forms (Hydaljss08, and Immunoboost-Tab) were experimentally proven to have potential immunomodulatory and anti-viral effects by using the neutrophil adhesion and cyclophosphamide-induced immunosuppression Test. The present study was intended to investigate the acute toxicity profile of "Hydaljss08" and "Immunoboost-Tab" polyherbal formulation in the female nulliparous, non-pregnant, Swiss albino mice, and Wistar rats by the OECD guidelines-425 TG (http://www.ccras.nic.in/sites/default/files/viewpdf/Publication/CCRAS_Guideline%%20of%20Safety_Toxicity.pdfoxicity.pdf).

Materials and methods

Collection of plant materials

The Authenticated dried crude drugs were purchased from the "KRC Crude Drugs" Chennai, Tamil Nadu, India (13.0827°N, and 80.2707°E).

The fresh crude drugs and materials were purchased from the local municipal market at the Udhagamandalam, Tamil Nadu, India.

Collection of phytopharmaceutical standards

All the phytopharmaceutical, isolated, fractionated, and marker standards were used in the "Immunoboost-Tab (PNo:202,341,030,833)" solid formulation was purchased from "Yucca Enterprises," in Mumbai, Maharashtra, India, "Natural Remedies Pvt Ltd," Bengaluru, Karnataka, India, Wuhan Chemfaces Biochemical Pvt Ltd, Wuhan, China, and Biosynth-Carbosynth, Pvt Ltd, United Kingdom (Lee et al., 2024).

Preparation of method of Hydaljss08 (P.No.202341030827) polyherbal formulation

Weigh 100 gm of each powdered, dried, and pasted form of crude drugs, which were transferred to a 10,000 ml of standard flask and mixed well, and additionally 2 kg of Jaggery, 3000 ml of boiled, and cooled water was added. About 1 gm of *Cinnamomum cassia* bark, *Elettaria cardamomum* dried fruits were added as flavoring agents, and 1 gm of Yeast (*Saccharomyces cerevisiae*) was added for the fermentation process. The resulting solution was mixed well, and the bottom of the flask was tightly covered with aluminum foils and a rubber band. The flask was kept in a dark, and warm place for up to 21 days for the fermentation process. Shaking and stirring occasionally when required. After 21 days, collect the fermentation solution by the filtration process. Fermented products were evaporated in an open condition at room temperature for up to one week and stored in a well-closed amber color container ,till further use (http://ccras.nic.in/sites/default/files/viewpdf/Publication/CCRAS_Guideline%%20of%20Drug%20Development.pdf).

Preparation method of "Immunoboost-Tab (P. no: 202341030833)" polyherbal formulation

Weigh the required amount of the phytopharmaceutical ingredients, transfer them to a mortar, mix well, until it forms an amorphous powder. Add the required amount of lactose and acacia powder, and mix with a pestle until all the ingredients attain a uniform. Prepare 2 % aqueous solution of the *acacia* powder and boil the mixture. Add dropwise boiled *acacia* solution in a mortar and mix with a pestle up to moist. Pass the above mixture through Sieve no.16. Dry the obtained granule in a hot air oven at 40 °C until the granules are completely dried. Store in a well-closed, and air-tight container till further use. Before punching the tablets, add the required quantity of magnesium stearate and talc in a granule for lubrication, and finally tablet's hardness was evaluated by the hardness tester (Mishra and Mishra, 2019).

Table 1
Effect of the "Hydaljss08", and "Immunoboost-Tab" Polyherbal formulations on body weight of Rats and Mice in acute toxicity study.

S. No	Groups	Animals	1st Day Body Weight (gm)	7th Day Body Weight (gm)	14th Day Body Weight (gm)
1	Control	Rats	156 ± 0.714	176.5 ± 0.804	185 ± 0.869
		Mice	32.11 ± 0.395	33 ± 0.325	34 ± 0.415
2	Hydaljss08	Rats	156 ± 0.701	173.6 ± 0.762*	196 ± 0.876*
		Mice	32 ± 0.325*	33 ± 0.345*	34 ± 0.375*
3	Control	Rats	146 ± 0.501	166.7 ± 0.421	189 ± 0.642
		Mice	29 ± 0.128	30 ± 0.132	33 ± 0.138
4	Immuboost-Tab	Rats	147 ± 0.048*	170.3 ± 0.462*	190 ± 0.989*
		Mice	29 ± 0.24*	32 ± 0.28*	32 ± 0.43*

Values are presented as mean ± SEM., N = 5; CMC.1 % gel = 1 % Carboxymethyl cellulose gel, Hydaljss08, Immunoboost-Tab polyherbal formulations treated groups; organ-to-body weight index = (organ weight × 100)/body weight, Po =given by mouth alone. * $p < 0.05$ when compared with the vehicle control groups.

Animals and the animal ethics committee

Swiss albino mice (29–32 gm weight) and Wistar Rats (146–156 gm weight) female, nulliparous and non-pregnant, were used in the acute toxicity study. The experimental study was conducted at Mass Biotech, Hila Nagar, Kanthalur, Chengalpet, Tamil Nadu, India, after the approval process from the Institutional Animal Ethics Committee (IAEC), and IAEC approval number was MB/IAEC/2022/03/01 and 02.

Acute toxicity assay

To study the acute toxicity in animals, OECD 425 test guidelines (TG), were followed, and 1 % CMC gel was used as vehicle control. A single dose of Hydaljss08, and Immunoboost-Tab polyherbal formulations was induced in Swiss albino mice and Wistar rats orally at 48 hour intervals. As no toxicity information of the test substance was available, for that (175 mg/kg body weight, Po), initial dose was selected and administered to the first animal. Subsequent animals were given higher amounts of doses (550, 2000 mg/kg, b. wt, Po) depending on the survival of the previous animals. The remaining animals were treated with the same method after reaching the limit dose (2000 mg/kg, b. wt, Po). Clinical observations were performed during pretreatment at 30 min, 1st, 2nd, 3rd, and 6th hours post dosing and at least once a day, up to the end of the day to assess survival, and maintain the general condition. Body weights were recorded before dose administration, on day 7th, and just before the necroscopy. Animals were euthanized on day 14th and subjected to gross pathological examination was conducted. The results were processed using the AOT425 software (AOT425 Stat program, USEPA, 2002), and the median LD_{50} was estimated (Shivaraj et al., 2021). The Scarified animals and their organs are disposed by the carcass disposal methods (Stull, 2013).

Biochemical analysis

The biochemical analysis was carried out to assess the safety profile. The parameters included in the biochemical studies are direct bilirubin, creatinine, bilirubin, total protein, total bilirubin, albumin, globulin, albumin globulin ratio, alanine aminotransferase (ALT), aspartate aminotransferase (ASAT), and alkaline phosphate.

Hematological analysis

The blood samples from the treated and vehicle control groups were collected in the EDTA containing tubes. The parameters to estimate blood reports were WBC Count, total leukocyte count (TLC), packed cell volume (PCV), RBC count, hemoglobin (Hb), Red cell distribution width (RDW) %, and Platelets count. The above parameters are estimated with a Sysmex pocH-100i™ Haematology Analyzer and CBC line kits.

Histopathological study

The heart, kidney, and liver obtained from the experimental studies are kept in a 10 % formalin solution. Then, after being immersed with 5 mm paraffin wax and treated with the stained reagents called hematoxylin and eosin. The stained slides were kept at 40X objective in light microscopes and magnified images of tissue structure was captured.

Statistical analysis

The statistical analysis was conducted on the experimental data from the animal's results and presented as mean ± SEM and it was analyzed

Table 2
Behavioral patterns of Mice[a], and Rats[b] in "Hydaljss08" Polyherbal formulation treated (2000 mg/kg.b.wt. p.o.) and Vehicle-treated groups.

S. No	Observations of vehicle control and "Hydaljss08" Polyherbal Formulation treated group.											
	Parameters	**30 mins**		**4 hrs**		**24 hrs**		**48 hrs**		**07 days**		**14 days**	
		CG	**HG**	**CG**	**HG**	**CG**	**HG**	**CG**	**HP**	**CG**	**HG**	**CG**	**HG**
1	Fur & Skin	N	N	N	N	N	N	N	N	N	N	N	N
2	Eyes	N	N	N	N	N	N	N	N	N	N	N	N
3	Piloerection	N	N	N	P [b]	N	P [b]	N	N	N	N	N	N
4	Salivation	N	N	N	N	N	N	N	N	N	N	N	N
5	Respiration and Abdominal breathing.	N	N	N	P [a, b]	N	N	N	N	N	N	N	N
6	Lethargy	N	N	N	P [a, b]	N	P [a, b]	N	N	N	N	N	N
7	Urination (color)	N	N	N	N	N	N	N	N	N	N	N	N
8	Faeces consistency	N	N	N	N	N	N	N	N	N	N	N	N
9	Somatomotor activity and behavior pattern	N	ND	ND	N	N	N	N	N	N	N	N	N
10	Sleep	N	N	N	N	N	N	N	N	N	N	N	N
11	Mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N
12	Convulsions & tremors	N.F	N	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F
13	Itching	N	N	N	N	N	N	N.F	N.F	N.F	N.F	N.F	N.F
14	Coma	N	N	N	N	N	N	N.F	N.F	N.F	N.F	N.F	N.F
15	Mortality	N	N	N	N	N	N	N.F	N.F	N.F	N.F	N.F	N.F

Key: CG = Vehicle Control group, HG = Hydaljss08 Polyherbal formulation treated groups, N = Normal, P = Present, ND= Not detected, N.F = Not found- Mice[a] and Rats[b], Po=given by mouth alone.

Table 3
Behavioral patterns of Rats[a], and Mice[b] in "Immunoboost-Tab" Polyherbal formulation treated (2000 mg/kg.b.wt. p.o.) and Vehicle-treated groups.

S. No	Observations of vehicle control and "Immunoboost-Tab" Polyherbal formulation treated group.												
	Parameters	**30 mins**		**4 hrs**		**24 hrs**		**48 hrs**		**07 days**		**14 days**	
		CG	**HG**	**CG**	**HG**	**CG**	**HG**	**CG**	**HP**	**CG**	**HG**	**CG**	**HG**
1	Fur & Skin	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**
2	Eyes	N	N	N	N	N	N	N	N	N	N	N	N
3	Salivation	N	N	N	N	N	N	N	N	N	N	N	N
4	Piloerection	N	N	N	P [a]	N	N	N	N	N	N	N	N
5	Lethargy	N	N	N	P [a]	N	P [a, b]	N	N	N	N	N	N
6	Respiration and abdominal breathing	N	N	N	P [a, b]	N	N	N	N	N	N	N	N
7	Urination (color)	N	N	N	N	N	N	N	N	N	N	N	N
8	Faeces consistency	N	N	N	N	N	N	N	N	N	N	N	N
9	Somatomotor activity & Behavior pattern	N	ND	ND	N	N	N	N	N	N	N	N	N
10	Sleep	N	N	N	N	N	N	N	N	N	N	N	N
11	Mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N
12	Convulsions & tremors	N.F	N	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F
13	Itching	N	N	N	N	N	N	N.F	N.F	N.F	N.F	N.F	N.F
14	Coma	N	N	N	N	N	N	N.F	N.F	N.F	N.F	N.F	N.F
15	Mortality	N	N	N	N	N	N	N.F	N.F	N.F	N.F	N.F	N.F

Key: CG = Vehicle Control group, HG = Immunoboost-Tab Polyherbal formulation treated groups, N = Normal, P = Present, ND = Not detected, N.F = Not found-Rats[a] and Mice [b], Po=given by mouth alone.

by using one-way ANOVA with Dunnett's test. Statistical significance between the groups was found less than $p < 0.05$ (Roome et al., 2021; Brígido et al., 2021).

Results

When the experiments of acute toxicity limit test were conducted with the dose of (175, 500, and 2000 mg/kg, b. wt, Po) in a female, nulliparous, non-pregnant Wistar rats and Swiss albino mice in the developed polyherbal dosage forms called "Hydaljss08" and "Immunoboost-Tab", and the vehicle control is used as an 1 % CMC gel. All the test and control animals were observed for 30 mins, 1st, 2nd, 3rd, 4th, and 6 h, followed by 1st day, and 14th days. The obtained results are discussed in the following Sections (Park et al., 2023; Suvarna et al., 2023).

Behavioral pattern and body weight

Clinical observations were made in the test sample treated, and vehicle control groups. The body weight increase was observed gradually in test mice and rats throughout the study periods (Table 1). The behavioral observation of the animals treated with a polyherbal formulations called "Hydaljss08″ (Table 2), and Immunoboost-Tab" (Table 3), throughout the study periods was recorded. The symptoms of lethargy, abdominal breathing, piloerection, and dullness were identified from the first 30 min, and followed by up to 24 h in test treated animals. The remaining external parameters are normal and was found at the end of the study (Patil et al., 2009).

Organ and body weight index statistical analysis

The isolated organs, such as the liver, kidney, and hearts, recovered from the during and after acute toxicity studies was found without lesions. The organ-to-body weight index was estimated and measured, showing no significant difference in the organ-body weight index in test groups and compared to the vehicle control groups (Table 4).

Biochemical analysis

Biochemical analysis of "Hydaljss08" (Table 5) and "Immunoboost-Tab" (Table 6) polyherbal formulations were supervised in a female mice and rats. The parameters included were to assess the liver function test (LFT) called serum glutamic pyruvic transaminase (SGPT), serum

Table 4
Effect on the organ to body weight index in Rats, and Mice of "Hydaljss08", and "Immunoboost-Tab" Polyherbal formulations treated, and Vehicle control groups.

S. No	Animals	Organs	Vehicle control (1 % CMC)	Hydaljss08 2000 mg/kg. b.wt. Po.	Immunoboost-Tab 2000 mg/kg b.wt. Po.
1	Rats	Heart	0.45 ± 0.04	0.46 ± 0.08*	0.45 ± 0.08
		Kidney	0.29 ± 0.05	0.30 ± 0.02	0.28 ± 0.04
		Liver	3.30 ± 0.030	3.31 ± 0.31*	3.32 ± 0.31*
2	Mice	Heart	0.724 ± 0.021	0.690 ± 0.019	0.693 ± 0.023*
		Kidney	1.464 ± 0.010	1.562 ± 0.021*	1.572 ± 0.023
		Liver	6.540 ± 0.217	7.310 ± 0.201*	7.330 ± 0.221*

Values are presented as mean ± SEM., $N = 5$; CMC.1 % gel = 1 % Carboxymethyl cellulose gel, Hydaljss08, Immunoboost-Tab polyherbal formulations treated groups; organ-to-body weight index = (organ weight × 100)/body weight, Po =given by mouth alone. * $p < 0.05$ when compared with the vehicle control groups.

Table 5
Effect of "Hydaljss08" Polyherbal formulation and vehicle-treated groups on Liver function test in Mice and Rats.

S. No	Parameters	Unit	Vehicle control (1 % CMC), Rats.	2000 mg/kg. b.wt. Po, Rats.	Vehicle control (1 % CMC), Mice.	2000 mg/kg. b. wt, Po. Mice.
1	SGPT (ALT)	U/L	20 ± 0.124	24 ± 0.116*	21.2 ± 0.113	24.2 ± 1.415*
2	SGOT (AST)	U/L	125 ± 1.021	140 ± 1.028	14.1 ± 0.062	16.3 ± 0.325
3	Alkaline phosphatase	U/L	162 ± 0.424	207 ± 2.020*	89 ± 0.044	101.3 ± 0.485*
4	Bilirubin total	mg/dl	ND	ND	0.78 ± 0.046	0.97 ± 0.435*
5	Direct bilirubin	mg/dl	ND	ND	0.55 ± 0.010	0.61 ± 0.215
6	Total protein	G/dl	4.8 ± 0.019	6.0 ± 0.112*	6.1 ± 0.012	6.3 ± 0.015
7	Albumin	G/dl	1.6 ± 0.012	1.7 ± 0.130	3.1 ± 0.010	4.1 ± 0.065
8	Globulins	G/dl	4.5 ± 0.011	4.7 ± 0.120	1.4 ± 0.008	2.2 ± 0.125*
9	A/G Ratio		1.5 ± 0.028	0.36 ± 0.01	1.2 ± 0.002	1.9 ± 0.0.85

Hydaljss08 = Hydroalcohalic polyherbal formulation treated groups; CMC. 1 % gel = 1 % Carboxymethyl cellulose gel; Values are presented as mean ± SEM., N = 5. * $p < 0.05$ when compared with the vehicle control groups.

glutamic-oxaloacetic transaminase (SGOT), Alkaline phosphatase, Bilirubin, Total protein, Albumin, Globulin, and A/G Ratio, was slightly changed in test animal groups when compared to the control groups. All the biochemical parameters are significant, and found to be less than $P < 0.05$.

Hematological analysis

There are no particular changes that has been not occurred in the hematological parameter called as hemoglobin (Hb), Total RBC, Red cell distribution width (RDW), platelets count, WBC cells called as, monocyte, basophils, eosinophils, and total leukocyte count (TLC) during the acute toxicity study periods. A significant change has occurred and the total count % increased in neutrophils, lymphocytes, and packed cell volume (PCV) in formulations treated groups when compared to the vehicle control groups (Tables 7 and 8). The CBC blood parameter was significant and was found to be less than $P < 0.05$ (Srivastava et al., 2021, Singh and Ilango, 2024).

Table 6
Effect of "Immunoboost-Tab" Polyherbal formulation, and vehicle-treated groups on Liver function test in Mice, and Rats.

S. No	Parameters	Unit	Vehicle control (1 % CMC), Rats	2000 mg/kg. b. wt, Po. Rats Group	Vehicle control (1 % CMC), Mice	2000 mg/kg. b. wt, Po. mice Group
1	SGPT (ALT)	U/L	125 ± 1.101	136 ± 1.127*	16.4 ± 0.072	19.4 ± 0.123*
2	SGOT (AST)	U/L	45 ± 1.023	54 ± 1.021	12.5 ± 0.213	13.1 ± 0.064*
3	Alkaline phosphatase	U/L	225 ± 1.324	255 ± 2.120*	89.1 ± 0.324	91.7 ± 0.310
4	Bilirubin total	mg/dl	ND	ND	0.76 ± 0.001	0.89 ± 0.002
5	Direct bilirubin	mg/dl	ND	ND	0.43 ± 0.001	0.51 ±0.003
6	Total protein	G/dl	6.3 ± 0.32	7.3 ± 0.63*	5.4 ± 0.089	6.6 ± 0.067*
7	Albumin	G/dl	2.2 ± 0.21	3.2 ± 0.27	3.5 ± 0.045	4.2 ± 0.024*
8	Globulins	G/dl	3.3 ± 0.1.0	4.3 ± 0.2.0*	1.6 ± 0.025	2.4 ± 0.033
9	A/G Ratio		0.68 ± 0.056	0.74 ± 0.043	1.2 ± 0.084	1.8 ± 0.013

Immunoboost-Tab = Immunoboost-Tablets polyherbal formulation treated groups; CMC. 1 %gel = 1 % Carboxymethyl cellulose gel; Values are presented as mean ± SEM., N= 5. * $p < 0.05$ when compared with the vehicle control group.

Table 7
Effect of the "Hydaljss08" Polyherbal formulation and vehicle-treated groups on CBC in Rats and Mice.

S. No	Parameters	Unit	Vehicle control (1 % CMC), Rats	2000 mg/kg b. wt, Po. Rats Group	Vehicle control (1 % CMC), Mice	2000 mg/kg, b.wt. Po. Mice group
1	Hb	gm/dl	10.5 ± 1.21	11.1 ± 1.20*	11.8 ± 0.075	11.3 ± 0.015*
2	TLC	millions/mm^3	3.1 ± 0.231	3.6 ± 0.620	3.2 ± 0.115	10.6 ± 0.015
3	Total RBC	%	4.8 ± 0.401	5.9 ± 0.016*	7.45 ± 0.173	8.0 ± 0.112
4	Plate late count	10^3/mm^3	287 ± 2.012	337 ± 3.008*	245 ± 4.619	400 ± 4.012*
5	WBC cell count					
a	Neutrophils	%	18.1 ± 0.567	24 ± 0.201*	10 ± 0.058	19 ± 0.012*
b	Lymphocytes	%	61 ± 1.210	65 ± 1.012	86 ± 2.309	70 ± 2.102
c	Monocytes	%	0.8 ± 0.001	01 ± 0.001*	3 ± 0.144	00 ± 0.045*
d	Eosinophils	%	0.8 ± 0.001	01 ± 0.001	1 ± 0.115	01 ±0.012
6	PCV	%	28.5 ± 1.001	33.4 ± 1.034*	32–48 ±0.012	43.4 ± 0.010*
7	Red cell distribution width	%	10.5 ± 1.21	11.1 ± 1.20	18.7 ± 0.019	18.5 ± 0.018

Hydaljss08 = Hydroalcohalic polyherbal formulation treated groups; CMC.1 % gel = 1 % Carboxymethyl cellulose gel; Values are presented as mean ± SEM.* $p < 0.05$ compared to the vehicle control group.

Discussion

Acute oral toxicity is a primary safety profile of the novel polyherbal

Table 8
Effect of the "Immunoboost-Tab" Polyherbal formulation and vehicle-treated groups on CBC in Rats, and Mice.

S. No	Parameters	Unit	Vehicle control (1 % CMC) Rats	2000 mg/kg. b.wt. Po. Rats Group	Vehicle control (1 % CMC) Mice	2000 mg/kg, b. wt Po. Mice Group
1	Hb	gm/dl	12.1 ± 1.024	13.1 ± 1.030*	10.5 ± 0.081	12.1 ± 0.14*
2	Total RBC	millions/ mm^3	7.1 ± 0.21	7.8 ± 0.28	9.1 ± 0.062	9.8 ± 0.034
3	Red cell distribution width	%	00 ± 00	00 ± 00	15.2 ± 0.21	17.3 ± 0.32
4	Plate late count	10^3/ mm^3	423 ± 2.113	434 ± 2.008*	342 ± 1.014	409 ± 1.236*
5	WBC Cells count					
a	Neutrophils	%	22 ± 0.021	28 ± 0.016*	14 ± 0.043	18 ± 0.024*
b	Lymphocytes	%	65 ± 0.216	71 ± 0.219	64 ± 0.056	75 ± 0.032
c	Monocytes	%	02 ± 0.01	03 ± 0.010*	0.65 ± 0.006	01 ± 0.001
d	Eosinophils	%	01 ± 0.004	01 ± 0.002	0.58 ± 0.003	01 ± 0.001
6	TLC	10^3/ mm^3	4.8 ± 0.046	5.6 ± 0.061	5.4 ± 0.021	6.6 ± 0.905*
7	PCV	%	35.3 ± 0.986	38.4 ± 0.989*	40.1 ± 1.021	48.4 ± 0.916*

Immunoboost-Tab = Immunoboost-Tablets polyherbal formulation treated groups; CMC. 1 %gel = 1 % Carboxymethyl cellulose gel; Values are presented as mean ± SEM.* $p < 0.05$ when compared with the vehicle control group.

formulations. The novelty of our work is we developed a polyherbal dosage form (Hydaljss08 & Immunoboost-Tab) cause of the Immunostimulant and Anti-viral effects (COVID-19). As a part of our study, we estimated acute toxicity safety profile of dosage forms by a Swiss albino mice and Wistar rats. Polyherbal formulations and phytopharmaceutical are strongly recommended for the treatment, mitigation, and prevention of diseases called cancer (Taxol, etoposide (Etoposide), Teniposide), laxatives (Senna, and Castor oil), bronchodilators (Codeine phosphate, and Vasaka leaves), immunomodulators (KSK, Triphala churna), antimalarials (Artemisinin, Quinine), and congestive cardiac failure (Digitoxin, Strophanthus, Squills) (Syahmi et al., 2010; Niyomchan et al., 2023). The Ministry of Ayush, government of India, and the World Health Organization (WHO) was recommended and prompted the use of phytopharmaceuticals based medicine, drugs, and polyherbal formulations in the past and ongoing pandemic situation of COVID-19, due to the synergistic, additive effects, safety, and efficacy profile (Huang et al., 2023). The acute toxicity is necessary and mandatory of the test polyherbal formulations "Hydaljss08 and Immunoboost-Tab" to assess the preliminary safety profile (Nayak and Mengi, 2009). Therefore, the present work enhances to measure the toxicological safety profile of novel dosage forms by using a Wistar rats and Swiss albino mice was performed, and reported (Tarannum et al., 2014). The Fig. 1, represented the graphical abstract and acute toxicity profile mechanism of the polyherbal formulations. The Fig. 2, illustrated possible molecular mechanism of the novel polyherbal formulations (Hydaljss08, and Immunoboost-Tab) was to increase the white blood cells (WBC) count, inhibiting the angiotensin converting enzyme (ACE-2), transmembrane protease, serine 2 (TMPRSS2), Nuclear factor kappa B (NF-κB), the entry of virions into host cells, pro-inflammatory signals, viral replication, and inhibiting 3-chymotrypsin protease (3 CL^{Pro}) receptor. Inhibits the action of Cyclooxygenase (COX-2) by suppressing NF-κB, activating protein (AP-1), signal transducer and activator of transcription 1 (STAT-1), signaling cytokines, and viruses' entry through membrane fusion and endocytosis. These polyherbal formulations can modulate innate and adaptive immune responses by regulating the macrophage phenotypic polarization, antigen-specific antibody production, and increasing the number of CD4± and CD8±T cells. They can promote the production of

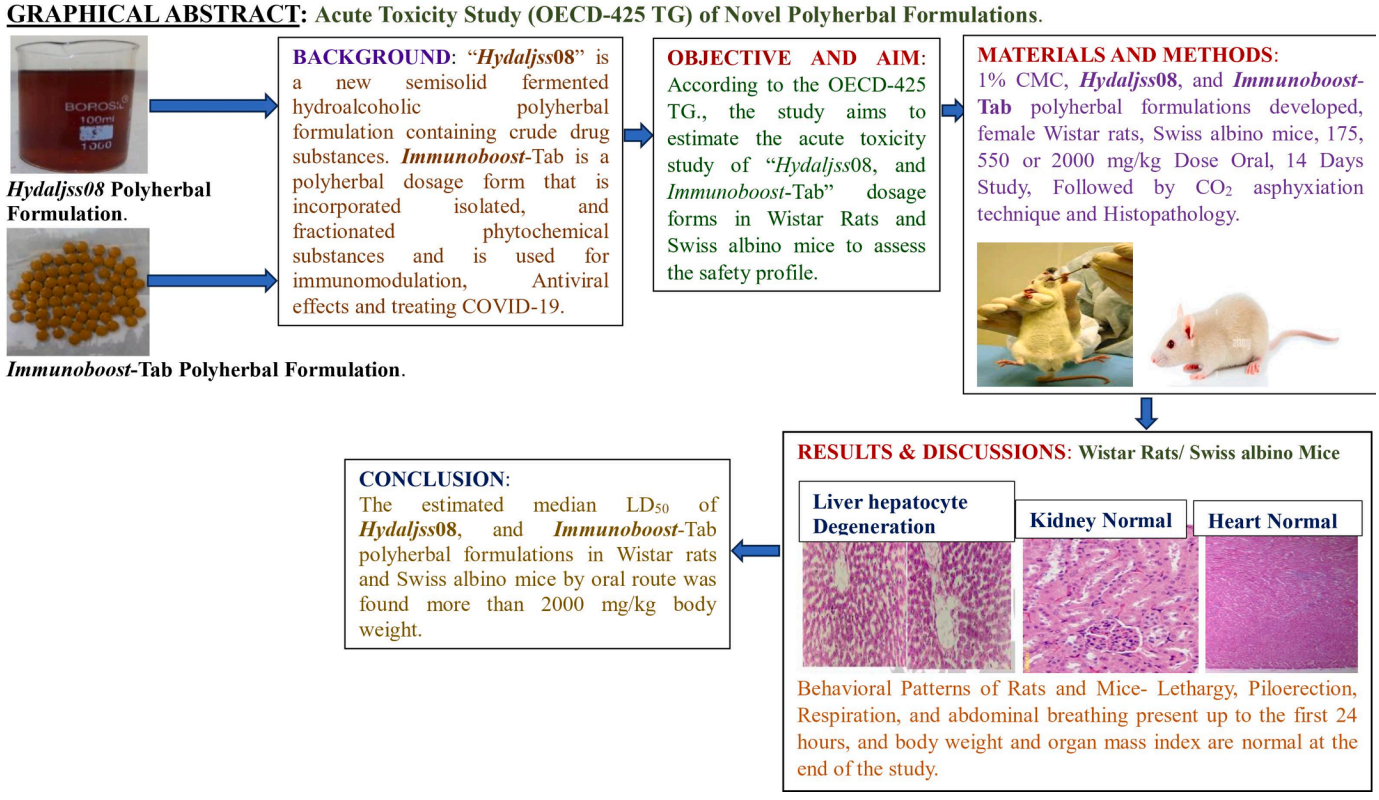

Fig. 1. Typical graphical abstract and mechanism of acute toxicity profile for the polyherbal formulations (Hydaljss08, & Immunoboost-Tab).

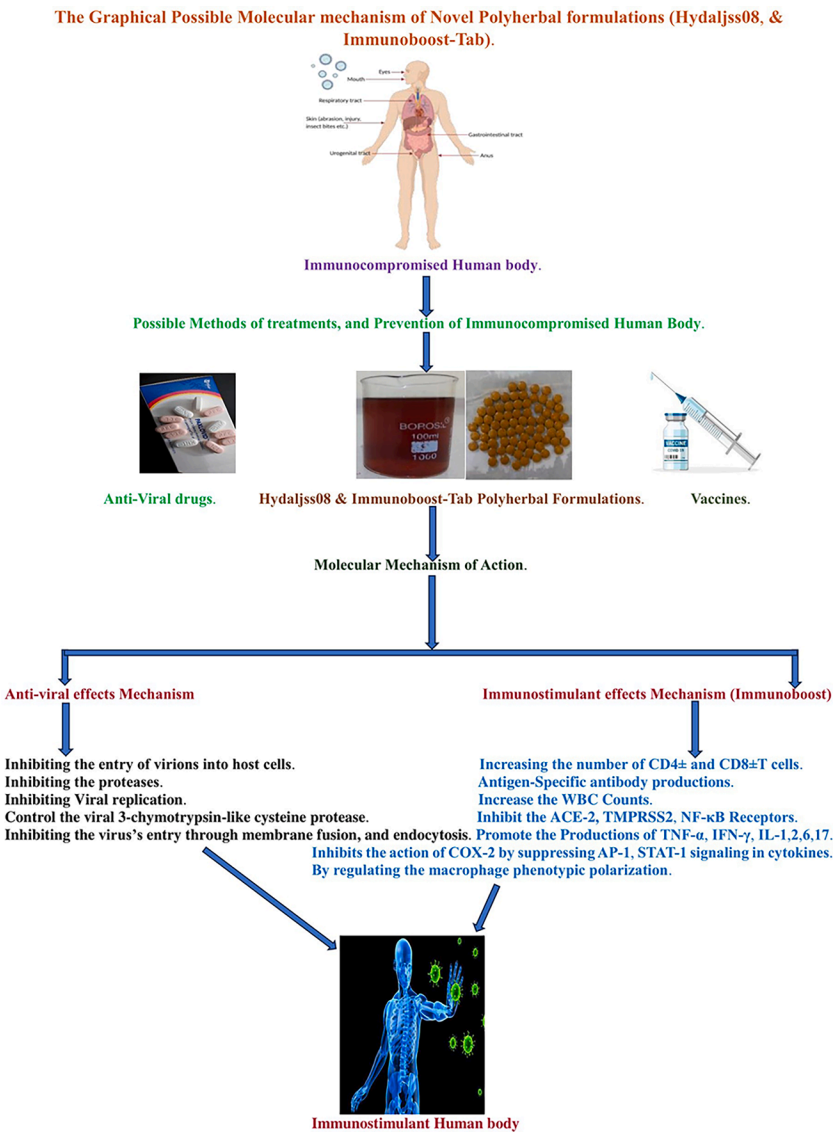

Fig. 2. Typical graphical possible (anti-viral and immunostimulatory effects) molecular mechanism of Polyherbal formulations (Hydaljss08, & Immunoboost-Tab).

cytokines called tumor necrosis factor (TNF-α), Interferons (IFN-γ), Interleukin (IL-1, IL-2, IL-6, IL-17), to induce the immune profile toward a type T-helper 1 (Th1) cell's response (Zebeaman et al., 2023; Alhazmi et al., 2021; Ratheesh et al., 2023).

The limit test of oral acute toxicity by using animals was rapid, simple, suitable, economical, and painless. Drugs absorption may be slow in the oral administration of polyherbal formulations compared to the other methods. The body mass and organ weight index of animals are the primary histopathological evidence of the test drugs. No alternation and injury were observed in the organ body weight index, and body weight increased gradually from the 1st day to the end of the study. It was indicated that no adverse effects and no animals was died throughout the study period, when compared to the test treated, and vehicle control groups. The toxicological reports of vital organs for the test sample treated animals strongly exposed in clinical signs and symptoms are normal, and which may be expected. The study is concluding that no mortality was observed in both animals treated test samples, and the lethargy, piloerection, dullness, respiration, abdominal breathing, tremors, and itching were monitored from the first 30 min to 6, 24 h of the dose administration. No other sign and symptoms were reported after 24 h and till the end of the study. Nutrients play a significant role in the physiological function of the animals, throughout the study period, food and water intake was standard. The liver, kidney, heart, and spleen of animals are the foremost vital organs in the histopathology study to assess the toxicity of the test substances. No lesions were found in the autopsy report of mice and rats in the microscopical examinations. The mild congestion and hepatocyte degeneration of the liver was found in the single mice treated "Hydaljss08" polyherbal formulation with a dose of (2000 mg/kg, b. wt, Po), and it may be due to the cardiomyopathy, or alcoholic content in the dosage forms. Mild congestion of the lungs occurred in a single rats treated "Hydaljss08" polyherbal formulation with a dose of (2000 mg/kg.b. wt, Po), and it may be due to the mucus accumulating in the bronchi and the lungs (Ijiom et al., 2021; Wang et al., 2024). Figs. 3 and 4, demonstrated histopathological images of rats and mice for the "Hydaljss08" polyherbal formulation treated animals.

Figs. 5 and 6, illustrated in the end of the study, no abnormal reports were found in the histopathology of the "Immunoboost -Tab," dosage form treated rats and mice. No significant variation was monitored in animal organs body weight index in the polyherbal formulation treated group compared to the vehicle control groups.

The SGPT, SGOT, and total protein quantity, and percentage increased in animals, and humans are the clinical sign, and symptoms of the hepatocellular injury, and it was not monitored animals treated with

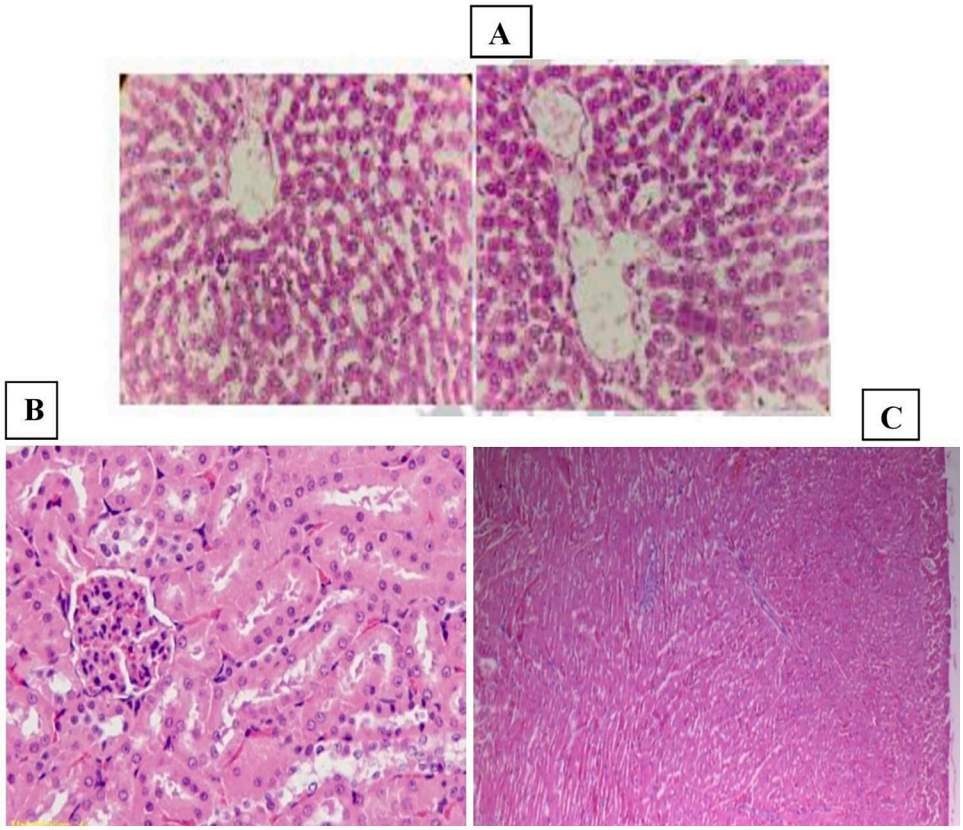

Fig. 3. Typical Histopathology images of Hydaljss08 polyherbal formulation treated Wistar Rat's major vital organs. **A**. A typical histopathology of hepatocyte degeneration characterized by karyolitic changes, loss of hepatocellular architecture, and focal area of atrophy in hepatocyte images of Hydaljss08 polyherbal formulation treated Rat' liver; **B**. A typical image of Hydaljss08 polyherbal formulation treated Rats normal Kidney; **C**. A typical image of Hydaljss08 polyherbal treated Heart normal myocardium of Rats.

the polyherbal formulations. All the treated animals passed the liver function test (LFT), and the test report data was found within the limit of reference standards when compared to the control groups. In the current study, there was no decreased level of SGPT was found in both dosage forms treated rodent species, and it was indicated that the hepatoprotective phytopharmaceuticals content in the test samples. Hematological parameters play a vital role in physiological alteration in animals by the external cause of the toxicity stress and environmental pollutants. At the termination of the toxicity study in mice and rats, there was no increased, and reduced level of the blood parameters that were not monitored. Lymphocyte and neutrophil counts were stable and slightly increased in treated animals and bone marrow restoration was observed. Therefore, we strongly support that the formulations containing immunostimulatory, and anti-viral effects phytopharmaceuticals. The present study strongly supports young budding researchers in the development of new phytopharmaceuticals and related drugs (Kaden et al., 2023).

Conclusion

The acute toxicity study of the novel polyherbal formulations in Wistar rats and Swiss albino mice safety profile was found fine with a monitored dose (2000 mg/kg, b. wt, Po) and the toxicity parameters, called as blood, renal function test (RFT), liver function test (LFT), body weight, and organ weight index, are standard in mice and rats treated groups. The study found no mortality was observed in animals, and the lethargy, piloerection, dullness, respiration, abdominal breathing, tremors, and itching was monitored from the first 30 min to 6, 24 h of the dose administration of the test samples. The mild congestion of the liver and lungs was found in the "Hydaljss08″ dosage forms (2000 mg/kg, b. wt, Po) treated animals. The polyherbal dosage forms (Hydaljss08, and Immunoboost-Tab) was developed for the prevention, and treatment of Immunostimulant, and Antiviral effects (COVID-19). The details, safety profile of the formulations was further recommended and to assess the chronic, genotoxicity, teratogenicity, and repeated dose toxicity study. Finally, it was estimated that the median lethal dose (LD_{50}) of Hydaljss08, and Immunoboost-Tab dosage form in Wistar rats and Swiss albino mice by oral route was found more than (2000 mg/kg, b. wt, Po).

Declaration of generative AI and AI-assisted technologies in the writing process

Not applicable.

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Ethical statement

The experimental study was conducted at Mass Biotech, Hila Nagar, Kanthalur, Chengalpet, Tamil Nadu, India, after the approval process from the Institutional Animal Ethics Committee (IAEC), and file numbers was MB/IAEC/2022/03/01 and 02. The care and handling of all the animals in this experiment was also in compliance with the recommendations given by the CCRAS and CCSEA. The bodies of the animals who were sacrificed were buried with respect in a place with little

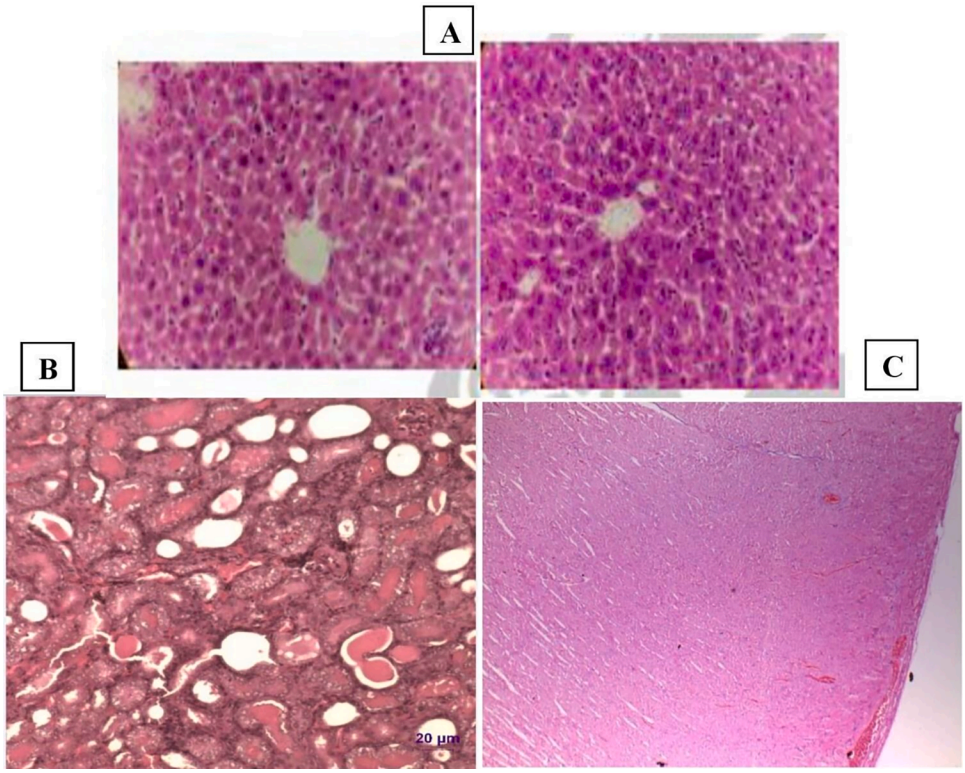

Fig. 4. Typical Histopathology images of Hydaljss08 polyherbal formulation treated Swiss albino mice major vital organs. **A**. A typical Histopathology of mild degenerative changes images of Hydaljss08 polyherbal formulation treated liver in Mice; **B**. A typical image of Hydaljss08 polyherbal formulation treated mice normal Kidney; **C**. A typical image of Hydaljss08 polyherbal formulation treated Heart normal myocardium of Mice.

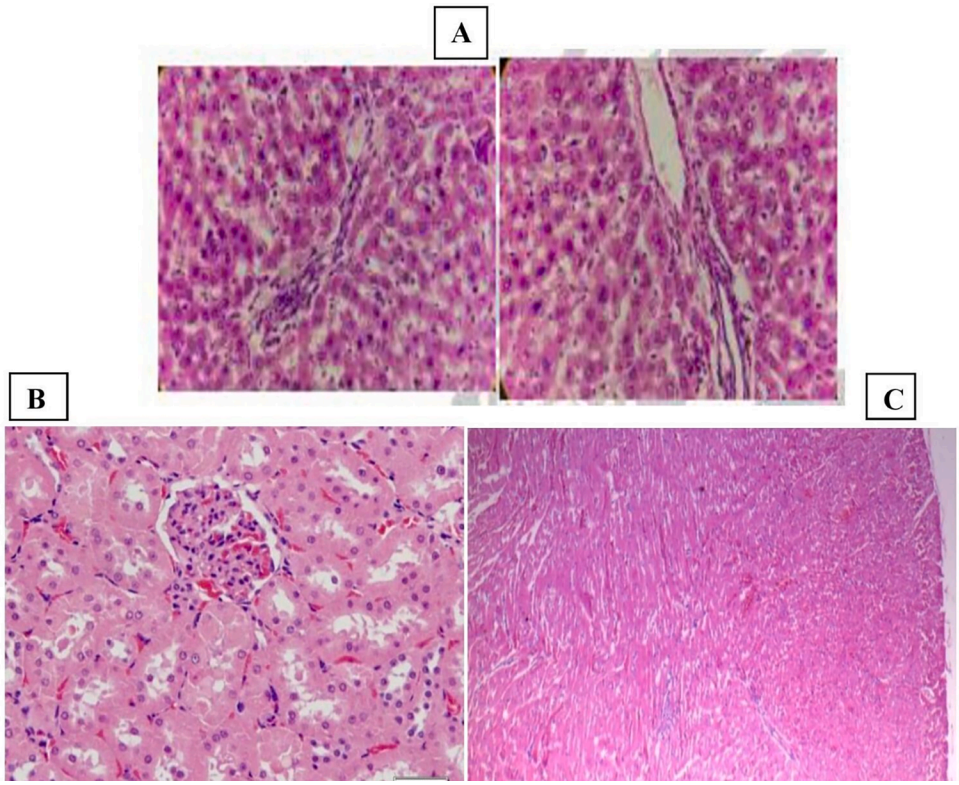

Fig. 5. A typical Histopathology image of Immunoboost-Tab polyherbal formulation treated Wistar Rats' major vital organs. **A**. A typical Histopathology of the multifocal area of Hepatocyte degeneration images of Immunoboost-Tab polyherbal formulation treated liver; **B**. A typical image of Immunoboost-Tab polyherbal formulation treated Rats normal Kidney; **C**. A typical image of Immunoboost-Tab polyherbal treated Heart normal myocardium of Rats.

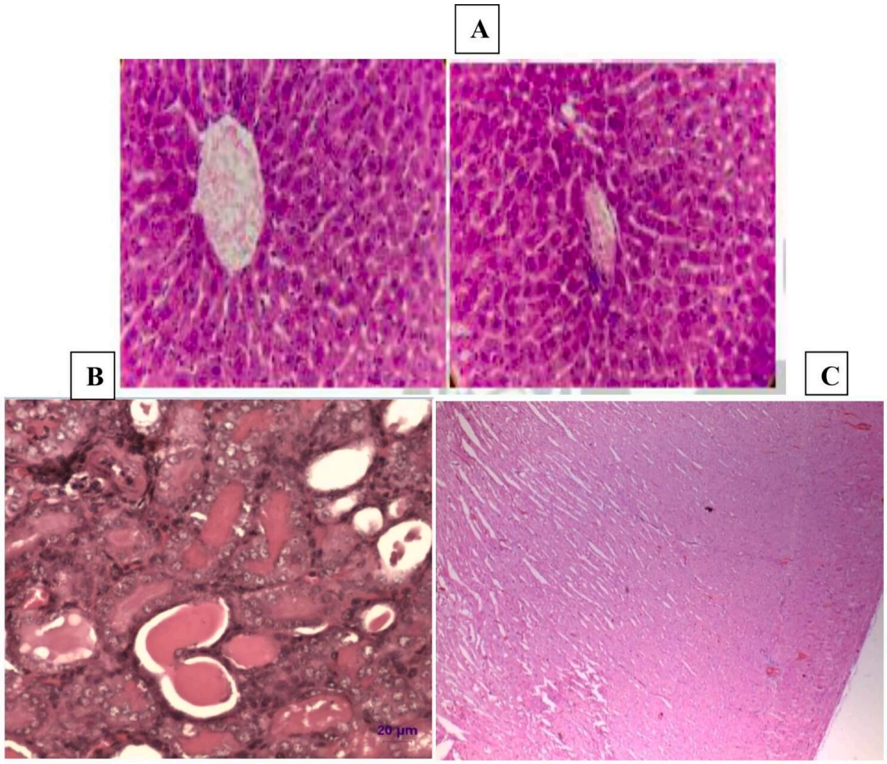

Fig. 6. A typical Histopathology image of Immunoboost-Tab polyherbal formulation treated Swiss albino mice major vital organs. **A**. A typical Histopathology of the normal architecture of hepatic parenchyma images of Immunoboost-Tab polyherbal formulation treated liver; **B**. A typical image of Immunoboost-Tab polyherbal formulation treated mice normal Kidney; **C**. A typical image of Immunoboost-Tab polyherbal treated Heart normal myocardium of Mice.

to no human interaction, and all of the animals participated in the experiment were handled humanely throughout the entire procedure (Jegnie et al., 2024).

CRediT authorship contribution statement

Ramkishan Jatoth: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Data curation. **S.P Dhanabal:** Supervision, Project administration, Conceptualization. **V. Senthil:** Supervision, Project administration, Conceptualization. **T. Ganesh:** Supervision, Resources, Project administration, Conceptualization. **Jubie Selvaraj:** Writing – review & editing, Supervision. **P.S. Venkatesan:** Software, Resources, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: RAMKISHAN JATOTH reports financial support was provided by JSS College of Pharmacy Ooty. RAMKISHAN JATOTH reports a relationship with CIRA HERBAL PVT LTD MUMBAI AND MASS BIOTECH CHENNAI that includes: paid expert testimony. RAMKISHAN JATOTH has patent pending to Application No: 202,341,030,833.Dated.29/06/2024. AND Application No: 202,341,030,827.Dated. 29/06/2024. Dhanabal S.P, V. Senthil, T. Ganesh, Jubie Selvaraj. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data used for the findings of this study are available from the corresponding author upon request.

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