

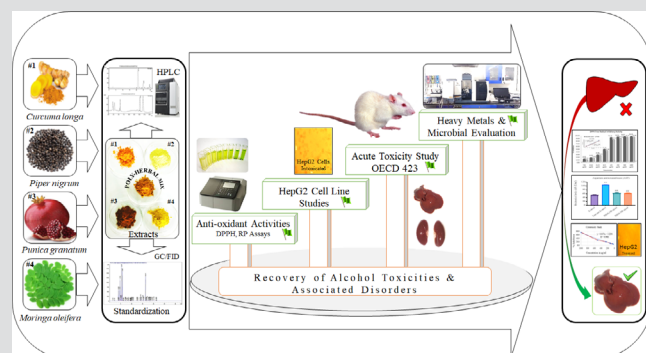
Research Article

Novel Health Supplement for Recovery of Alcohol Induced Adverse Effects and Associated Disorders: HepG2 Cells Study and Safety Dose Profiling on Rats

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Graphical abstract



Abstract

The present study objective was to design and develop novel health-supplement formula from plant extracts and was to evaluate the formula for high episodic alcohol toxicities, and associated disorders against alcohol intoxicated and oxidative damaged Human Hepatoma HepG2 cell line. The CC50 of the supplement was determined based on MTT assay. To get the safe dose concentration, an acute toxicity study was performed on Wistar rats as per OECD

guidelines 423. The phyto-constituents of the supplement e.g. curcuminoids, piperine, β -caryophyllene, punicalagin, ellagic acid and isoquercetins from respective plant extracts were also analyzed and confirmed via HPLC and GC-FID profiling, which recovered more than 70% HepG2 cells with >75% cell viability. Normalization of oxidative stressed cells were confirmed via biochemical parameter studies. The same was verified via ROS inhibiting properties of the formula to reinforce the claim. In this study, any abnormal clinical symptom was not observed in treated animals at 2000mg/kg BW, hence it was considered as a safe dose for oral consumption. The present studies conclude that this health supplement contains herbal ingredients has a preventing role against alcohol-induced adverse effects and associated disorders.

Keywords: Alcoholism; Alcohol disorder; Alcohol toxicity; Antihangover; Nutraceuticals

Introduction

Alcoholism is an extensive and serious Global Burden of Disease (GBD), interference to psycho-social lifestyle constantly [1,2]. Excessive consumption of alcohol makes undesirable and deleterious consequences to the human body and generates characteristic leading risk factors responsible for disability, morbidity and mortality [3,4]. More than 230 different types of diseases in 2016, where alcohol had a significant role directly or indirectly had affected 237 million men and 46 million women worldwide. The Americas, Europe and Western Pacific are the three regions as per WHO, where alcohol is consumed by more than half of the population. Acute or chronic ingestion of alcohol adversely affects human health especially leading to Alcoholic Liver Disease (ALD) e.g. inflammation, hepatocellular injury, oxidative stress, fat accumulation (steatohepatitis), fibrosis, cirrhosis or hepatocellular carcinoma and hangover [5]. Other secondary dysfunctions are also accompanied in association with some central nervous system complications and behavioral response [6,7].

Alcohol gets eliminated through its metabolic degradation products via multiple enzymatic and non-enzymatic pathways and affects specific organs or systems such as the brain, gastrointestinal tract, liver and immune system (Figure 1) resulting in characteristic symptoms like typical hangover, which may cause electrolyte imbalance, hypoglycemia, dehydration or gastrointestinal disturbances [8,9].

In order to reduce acute episodic alcohol and associated disorders, several therapeutic treatments and formulations are available and are already in practice. However, contemporary supporting agents focuses on an instant relief rather deep curing. Another side, synthetic agents may develop further complications after usage. Some of the marketed products, which may contain natural extracts either rapidly eliminate acetaldehyde from the blood by enhancing ALDH activity or they reduce the acetaldehyde formation in the body after the alcohol consumption [9]. Considering all the above mentioned facts and characteristics properties, this study was mainly designed to prepare a health supplement from turmeric, black pepper, pomegranate and moringa extracts based on preliminary screening of antioxidant

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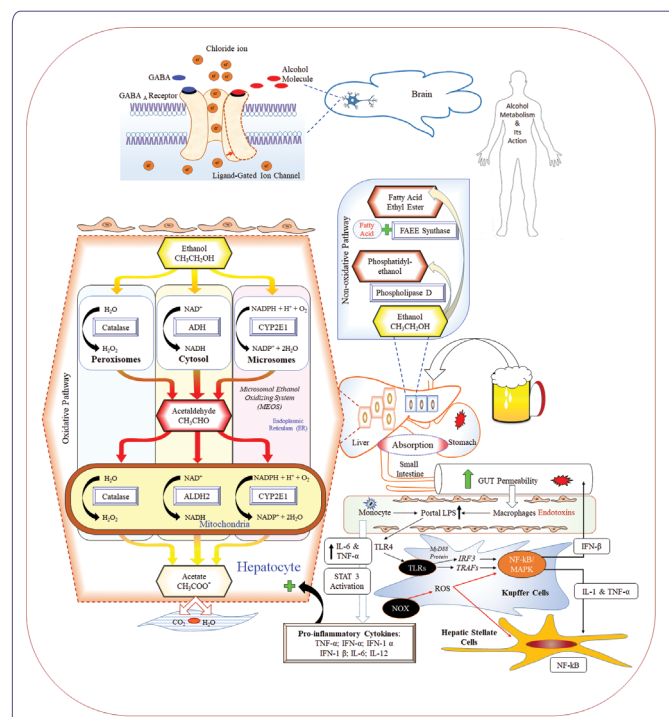


Figure 1: Alcoholic intoxication and its metabolism inside the body.

Alcohol Dehydrogenase (ADH), Cytochrome P450 2E1 (CYP2E1), and catalase are bio-transformed alcohol to acetaldehyde, which further transformed to acetate by the mitochondrial Aldehyde Dehydrogenase (ALDH) enzyme and coming out from the liver to circulates into peripheral tissues. Non-oxidative pathway responsible for the esterification of alcohol, where alcohol is metabolized to Fatty Acid Ethyl Esters (FAEE) through enzyme FAEE synthase. Alcohol is also catalyzed by phosphatidylcholine-specific enzyme phospholipase D and form Phosphatidylethanol product through non-oxidative pathway.

activities (DPPH and FRAP assays-Data not shown). The work was aimed to evaluate the supplement against alcohol-intoxicated and oxidative damaged HepG2 cell lines. An acute toxicity study was performed on rats to get the knowledge about a safe dose of supplement formula. To the best of our knowledge, this is a unique proprietary combination for treating alcohol-induced adverse effect and associated disorders.

Alcohol Dehydrogenase (ADH), Cytochrome P450 2E1 (CYP2E1), and catalase are bio-transformed alcohol to acetaldehyde, which further transformed to acetate by the mitochondrial Aldehyde Dehydrogenase (ALDH) enzyme and coming out from the liver to circulates into peripheral tissues. Non-oxidative pathway responsible for the esterification of alcohol, where alcohol is metabolized to Fatty Acid Ethyl Esters (FAEE) through enzyme FAEE synthase. Alcohol is also catalyzed by phosphatidylcholine-specific enzyme phospholipase D and form Phosphatidylethanol product through non-oxidative pathway.

Materials and Methods

Chemicals and reagents

MTT reagent 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide Phosphate-Buffered Saline (PBS), Trypsin and Antibiotics were purchased from Hi-media; Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG) were acquired from Gibco (New York, USA). 12-well cell culture cluster, 96-well cell culture cluster, T-25 cm² cell culture flask were purchased from GBO (Greiner Bio One, Austria). Standards e.g.

mixture of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin, ≥95.0%, Piperine and Isoquercetin were procured from Sigma Aldrich (Germany). Punicalagin α and β were procured from Phyto-comounds™, Natural Remedies Pvt. Ltd. (Bangalore, India). Phyto-constituents were standardized on fully automated latest Shimadzu HPLC system (Prominence-i, LC 2030C 3D Plus), equipped with variable wavelength PDA detector. All the chemicals, reagents, and buffers of analytical grade were employed in the.

Development of extracts and polyherbal mix

Health supplement Re-party™ further referred as “PHM”, which contains herbal ingredients was prepared by using four herbs with respective their five enriched extracts. Turmeric (*Curcuma longa* L.) rhizome extract and black pepper (*Piper nigrum* L.) fruit extracts (Powder and essential oil), were mixed with pomegranate (*Punica granatum* L.) fruit pericarp extract and moringa (*Moringa oleifera* Lam) leaves extract. The extracts were prepared using Synthite's trademarked Cold-Crafting technology. The individual extract was selected based on their screening potentials e.g. DPPH and FRAP assays (Data not shown). Extracts were homogenized in a Silverson L5M-A Laboratory mixer and were subjected to process ultra-fine grinding Vextrano™ to arrive at reduced particle size. The resultant solution was spray-dried using maltodextrin as a carrier to yield a fine dry powder “PHM, which was dissolved in methanol, DMSO and water according to biological and analytical evaluations.

HepG2 cell line and in vitro cells protective studies

Preparation of test solution for cytotoxicity studies: PHP (10mg) was dissolved separately and volume was made up with Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG) with 2% inactivated Fetal Bovine Serum (FBS) to obtain a stock solution of 1mg/mL concentration and it was sterilized through a 0.22 μm syringe filter. Six doses of test solutions from 1000-31.25 μg/mL were prepared by a two-fold serial dilutions method.

Cell line and culture medium: HepG2 (Human Hepatoma Cells) were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM-HG supplemented with 10% inactivated FBS, penicillin (100IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in a humidified atmosphere of 5% CO₂ at 37°C until confluent.

Determination of Cytotoxicity by MTT assay: The Microculture Tetrazolium Assays (MTT) assay was performed to determine cellular metabolic activity as it is an indicator of cell viability, proliferation, and cytotoxicity [10]. Briefly, the monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using DMEM (HG) containing 10% FBS. To each well of 96 well microtiter plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, once a partial monolayer formed, the supernatant was flicked off; monolayer was washed with medium and finally 100 μl of different concentrations of tested PHM was added individually into the partial monolayer of microtiter plates. The plates were incubated for 3 days at 37°C in a 5% CO₂ atmosphere, and microscopic observations were carried out at every 24h interval. At the end of incubation period (after 72h), solutions of PHM in the wells were discarded and 50 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) prepared in PBS (yellow colour) was added to each well. The plates were gently shook and incubated for 3 h at 37°C in a 5% CO₂ atmosphere. After 3h, the supernatant was removed and 100 μl of alcohol was added to solubilize the formed

formazan. Resultant plate was incubated for 30min at room temperature with constant shaking. The absorbance [Optical Density (OD)] was measured using a Microplate Auto-Reader (Bio-Tek Instruments, Inc., Winooski, VT) at a wavelength of 540nm. At the end of the period, cytotoxicity was assessed by estimating % growth inhibition of HepG2 cells [11].

$$\% \text{ Growth inhibition of cells} = (\text{Mean OD of normal control} - \text{Mean OD of test group} / \text{Mean OD of normal control}) \times 100$$

A dose-response curve was generated using % growth inhibition on Y-axis and the extract concentration ($\mu\text{g/ml}$) on X-axis. The dose-response curve was plotted to generate concentration of test sample, needed to inhibit 50% cell growth (CC_{50} value).

Cells protective activity against alcohol-intoxicated HepG2 cells

The protective activity of PHM was evaluated by using well-maintained HepG2 cells. Alcohol was used as toxicant with stressed toxic concentration $250\mu\text{M}$. The choice of concentrations of PHM were based on the results of the MTT assay. The experiment was performed as per the standard protocol [11,12]. Different non-toxic concentrations of PHM, substances (250 and $500\mu\text{g/ml}$) prepared in DMEM and FBS except control one. The plates were incubated for 24h at 37°C with an atmosphere of $5\% \text{CO}_2$. After incubation, cell supernatants were collected into micro tubes and were stored at -20°C for biochemicals estimation (AST, ALT, and LDH) by using standard kits. The groups were assigned as follows:

Group I (Control): HepG2 Cells + $100\mu\text{L}$ culture media as vehicle control.

Group II (Toxicant Control): HepG2 Cells + $100\mu\text{L}$ culture media containing $250\mu\text{mol}$ alcohol for induction of toxicity (24h).

Group III (PHM $250\mu\text{g/mL}$): HepG2 Cells + $100\mu\text{L}$ culture media containing $250\mu\text{mol}$ alcohol + PHM $250\mu\text{g/mL}$ ($100\mu\text{L}$).

Group IV (PHM $500\mu\text{g/mL}$): HepG2 Cells + $100\mu\text{L}$ culture media containing $250\mu\text{mol}$ alcohol + PHM $500\mu\text{g/mL}$ ($100\mu\text{L}$).

Antioxidant studies

DPPH free radical scavenging assay: The DPPH free radical scavenging assay was performed in triplicate as per earlier reported method [13]. Ascorbic acid was kept as standard [14].

Finally scavenging activity was determined by using the following equation:

$$\% \text{ Inhibition of DPPH radicals} = [(\text{Absorbance of Control} - \text{Absorbance of Test}) / \text{Absorbance of Control}] \times 100.$$

Reducing Power (RP) assay: The assay was performed as per the method described previously [15,16].

The reducing power activity of the blend of plant extracts “PHM” was calculated as follows:

$$\% \text{ Reducing Potential} = [(\text{OD of Control} - \text{OD of Test}) / \text{OD of Control}] \times 100$$

In vivo safety dose study

Experimental animals: An experiment was designed according to the standard protocol OECD guidelines 423 [17]. Healthy young

adult female (nullifiers and non-pregnant) female albino rats (Wistar strain-total nine) were selected for the study. Three animals were used for initial step. Animals were housed under temperature $22\pm 3^{\circ}\text{C}$, relative humidity 50-70%, and 12h light and 12h dark cycle. The diet and drinking water were free from any contaminant and provided ad libitum. All the experimental procedures and protocols used in this study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC- RR/IAEC/65-2019) of Radiant Research Services Pvt. Ltd., Bangalore (Regd no: 1803/PO/RcBi/S/2015/CPCSEA) constituted in accordance with the guidelines of the CPCSEA, Government of India.

Dose levels and acute toxicity study: The test substance PHM was administered in a single dose by oral gavage. Animals were fasted prior to dosing. After administering test substance, food was withheld for 3-4h. The dose level to be used as the starting dose is selected from one of four fixed levels, 5, 50, 300 and 2000mg/kg body weight. Mortality was unlikely at the highest starting dose level (2000mg/kg body weight), and then a limit test was conducted [one dose level of 2000mg/kg body weight carried out with extra six animals (three animals per step)]. All observations were systematically recorded for individual data. Based on overall observation, results were reported.

Statistical analysis

Data were expressed as Mean \pm SD of each group ($n=3$) for antioxidant and acute toxicity analysis using Window-10 (Microsoft Corporation, Redmond, WA, USA). Statistical analysis for hepatoprotective activity was done using software GraphPad Prism latest version 8.3.1 with one-way Analysis of Variance (ANOVA).

Phytochemical analysis

HPLC analysis: Individual botanical extracts of PHM were standardized through HPLC analysis performed on the Shimadzu HPLC system (Prominence-i, LC 2030C 3D Plus) and analyzed by LabSolutions™ software version 5.96.

Turmeric (*Curcuma longa* L.) rhizome extract: Chromatographic separations of curcuminoidscurcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were achieved isocratically on Capcell PAK C18, type MG-II column ($250\text{mm} \times 4.6\text{mm}$ i.d., $5\mu\text{m}$ particle size) referring USP-NF method [18], with slight modification. Reference standard and samples for analysis (1mg/ml) were prepared separately and further diluted with mobile phase to prepare 100ppm solution for the injection. The targeted curcuminoids were identified by observing specific peaks at suited retention times at detection wavelength 420nm .

Black pepper (*Piper nigrum* L.) fruit extracts: Content of piperine in Black pepper extract was standardized and confirmed through HPLC method described by International Standard Organization [19]. Working standard: 25mg of Piperine was weighed accurately into a 50ml volumetric flask with 5ml of acetonitrile to dissolve it completely. The volume was adjusted with mobile phase. 5ml of this solution was transferred to a 50ml volumetric flask, added acetonitrile, diluted to make up the volume. Sample preparation was done same as like standard with 1g of black pepper extract. The targeted Piperine present in sample was identified by observing specific peaks on retention times 15.13min at detection wavelength 343nm .

Pomegranate (*Punica granatum* L.) fruit pericarp extract: Punicalagin and ellagic acid were selected for the standardization of punica extract. Chromatographic separations and quantification were

achieved by gradient elution on Shimpack C18 column (250mm × 4.6mm i.d., 5µm particle size). Solvent A was prepared with 1mM of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) adjusted to pH 3 by 88% orthophosphoric acid. Solvent B was acetonitrile. The both solutions were filtered through 0.45µm membrane filter and sonicated them for 3 minutes. Clear separation of peaks were achieved by using the following gradient elution program (time in min, B in %; 0.01, 10%; 10, 15%; 20, 30%; 25, 50%; 30, 80%; 35, 40%; 40, 10% 45, 10%). Flow rate of the mobile phase was 1ml/min at 25°C. Aqueous and methanol solution of punicalagin and ellagic acid were injected (20µl) in to the system to get the standards chromatograms at the concentration of 100 and 50PPM respectively. Sample solutions (2000PPM) were also prepared similarly, and injected separately to obtained extract chromatograms. Sample peak areas were compared with standards peak areas and quantified at specific detection wavelength $\lambda=254\text{nm}$.

Moringa (*Moringa oleifera* Lam) leaves extract: Standardization of moringa leaf extract was accomplished by considering isoquercetin (3-O-glucoside of quercetin) as standard compound. Quantification was performed by gradient elution on a same column as mentioned. Mobile phase and other conditions were also the same except the elution program, which was as follows (time in min, B in %; 0.01, 5%; 8, 5%; 12, 20%; 15, 20%; 18, 40%; 21, 40%; 25, 70% 30, 70%; 35, 5%; 40, 5%). The flow rate of the mobile phase was set as 1ml/min at 25°C. Separations was carried out in standard conditions and peak was detected in $\lambda=254\text{nm}$. Standard chromatogram was obtained by injecting (10µl) Isoquercetin in methanol (500PPM). Sample was also prepared (2000PPM) in methanol and injected to get specific peaks area for comparison and quantification against standard chromatogram.

GC analysis of black pepper essential oil: The GC/FID analysis of the black pepper essential oil was analyzed using gas chromatograph 7890 B (Agilent Technologies, USA) equipped with a Flame Ionization Detector (FID). The separation was achieved by using column, HP-5 (5% phenyl methyl siloxane), 30m × 320µm ID, i.e. 0.25µm film thickness. The GC oven temperature was programmed as follows: 80°C for 3min, increased from 80°C to 180°C at 5°C/min for 10min; Run time 33min. The carrier gas was Nitrogen (99.99%) at a flow rate of 1.0ml/min; the injector and detector temperatures were 180°C and 200°C, respectively. Detector gas flows hydrogen, air were 30, and 300ml/min respectively to get makeup flow 13ml/min. The samples 0.2µl was injected using the split ratio 100:1. Identification of components was done based on retention index and by comparison with reference standard.

Macro and Micronutrient analysis: Essential elements including macro and micro elements are present in the human body at very low concentration but they play specific roles and clinical influences significantly on all body functions. Six essential elements were selected for the study. Macro elements namely, Calcium (Ca), Magnesium (Mg), were analysed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) instrument (iCAP 6300 Duo; Thermo Scientific; Mass., United States), whereas micro elements such as Copper (Cu), Iron (Fe), Zinc (Zn), and Selenium (Se) were analysed by using inductively coupled plasma-mass spectrometry (ICP-MS; NexIon 1000; Perkin Elmer; Mass., United States). Presence of essential elements in the PHM were detected according to the standard AOAC Official method 985.01 [20].

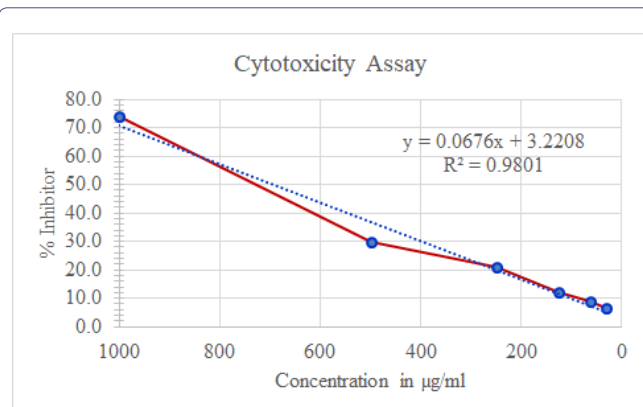


Figure 2: Cytotoxic effect of the PHM on HepG2 Cells.

Note: A dose-response curve-% growth inhibition on Y-axis and the extract concentration (µg/ml) on X-axis. Cytotoxicity concentration CC_{50} of PHM was recorded as $691.89 \pm 2.13 \mu\text{g/ml}$ from linear equation and regression analysis.

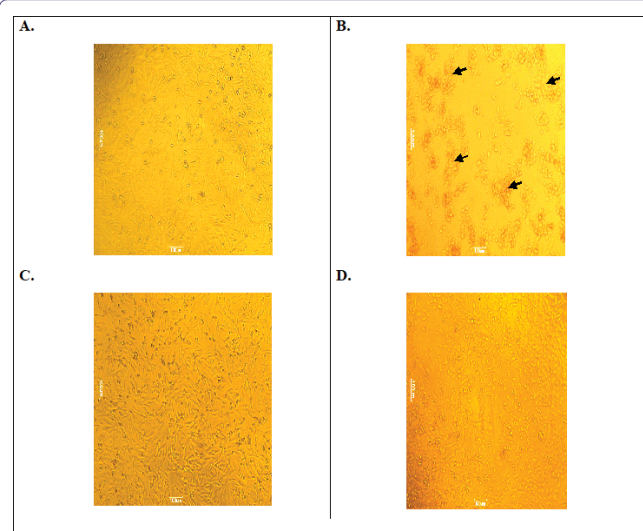


Figure 3: HepG2 cells under an inverted tissue culture microscope (40x). A: Control cell; B: Alcohol toxicant cells-accumulated cells highlighted by black arrow; C: PHM treated (250µg/ml) cells; D: PHM treated (500µg/mL) cells.

Results

Cytotoxicity level of HepG2 Cells

Active metabolites of viable HepG2 Cells convert MTT into a purple colored formazan product with an absorbance maximum near 540nm. After the experiment, absorbance were recorded against different concentrations of test samples. Cytotoxicity concentration CC_{50} of PHM was recorded as $691.89 \pm 2.13 \mu\text{g/mL}$ from linear equation $y=0.0676x+3.2208$ and regression analysis $R^2=0.9801$ (Figure 2). It indicated that at a lower concentration, 50% cells were viable than control cells and hence, two different nontoxic concentrations (250 and 500µg/mL) were considered for further studies. Cytotoxicity concentration of alcohol (CC_{50}) was found to be $44.86 \pm 2.7\%$ at a low concentration level (250µmol) and considered as a toxicant control for the experiments.

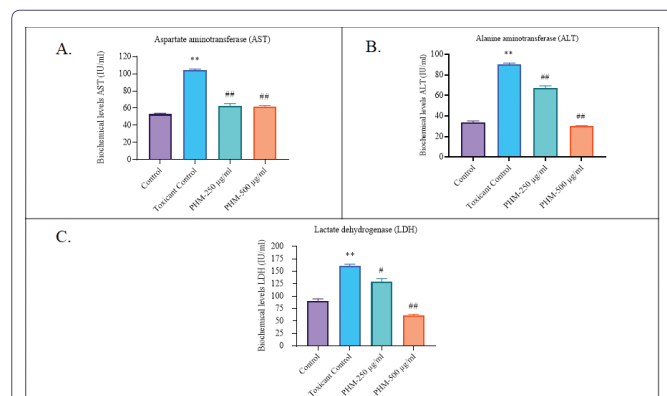


Figure 4: Biochemical levels. (A): Aspartate aminotransferase (AST); (B): Alanine aminotransferase (ALT); (C): Lactate dehydrogenase (LDH).

Note: One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test using Prism software (V. 8.3.1; GraphPad Prism, San Diego, CA, USA). Differences between means were considered significant at $p < 0.01$.

*Toxicant control group compared with control group.

##Treated groups compared with toxicant control group.

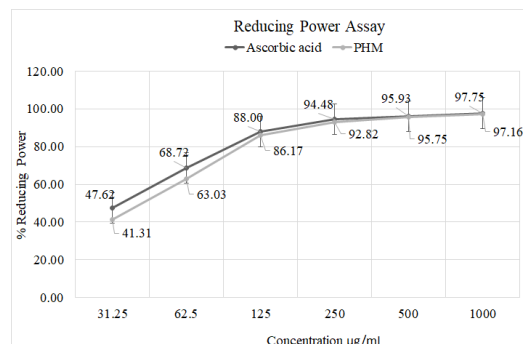


Figure 6: Antioxidant assays.

Note: Reducing Power (RP) activity of ascorbic acid and PHM; Independent variable concentration on X-axis and the percentage of reducing power (RP assay) as the dependent variable on Y-axis.

An- imal Group	Dose	Treatment				
		Before	After			
		Day 0	Day 3	Day 7	Day 14	Day 14
RA 01	2000 mg/ kg	189.3 ± 0.9	192.0 ± 0.6	195.7 ± 0.7	204.7 ± 0.7	No macroscopic alteration occurred
RA 02						
RA 03						
RA 04	Lim- it test					No macroscopic alteration occurred
RA 05						
RA 06						
RA 07	2000 mg/ kg	186.3 ± 0.8	188.8 ± 1.0	192.7 ± 1.1	201.8 ± 1.0	No macroscopic alteration occurred
RA 08						
RA 09						

Table 1: Body weight of Rats during the study.

Note: Values were expressed as Mean ± SEM.

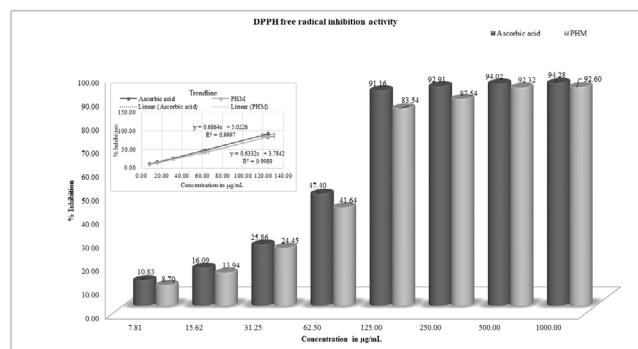


Figure 5: DPPH inhibition activity of Ascorbic acid and PHM at different concentrations.

Note: Independent variable concentration (7.81 to 1000 µg/ml) on X-axis and the percentage of scavenging effect as the dependent variable on Y-axis.

Protective activities against alcohol-intoxicated HepG2 Cell lines

Treatment of test substances were offered dose-dependent HepG2 cells protection $27.95 \pm 1.4\%$ and $70.63 \pm 8.7\%$ at 250 and 500 µg/mL respectively. Viability (%) of cells over the control were found to be $57.4 \pm 0.61\%$ and $76.54 \pm 3.9\%$ at the concentration of 250 and 500 µg/mL respectively. Percent viability over the control of alcohol was found to be $44.86 \pm 2.7\%$. Morphology of control, toxicant, and PHM treated HepG2 cells were examined under an inverted tissue culture microscope at magnifying power 40x and are presented in figure 3.

It's reported that hepatocytes cells are damaged by toxicants by collapsing the cells structure and thus allowing leakage of cellular enzymes/biomarkers into the serum [21]. Treatment with test substance at two different dose levels normalized the biochemicals release (AST, ALT, and LDH) significantly (Figure 4); 500 µg/mL of PHM was selectively more adequate as compared to 250 µg/mL.

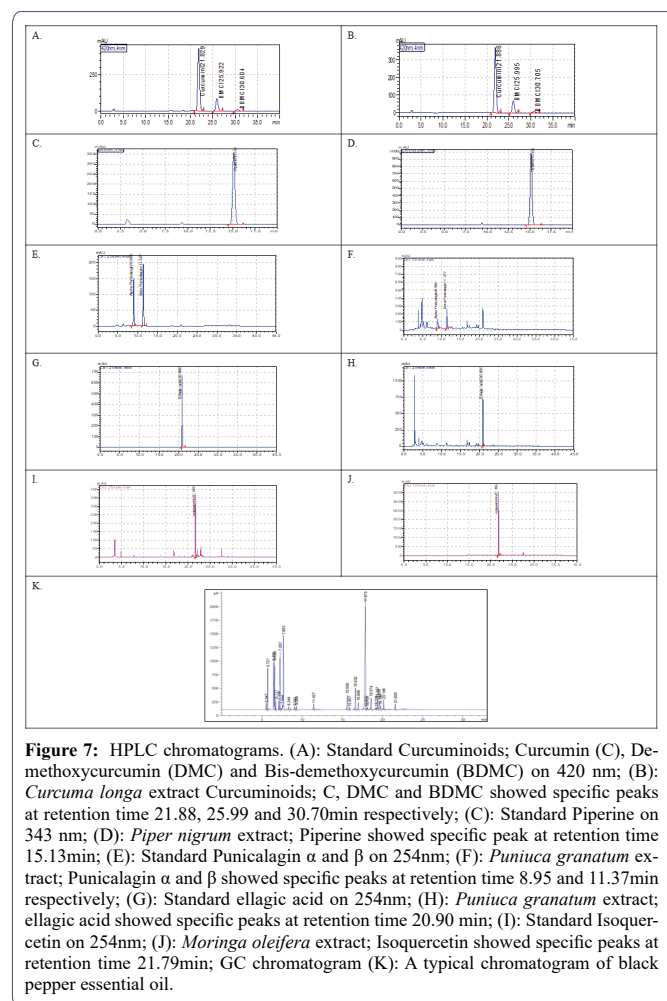
Antioxidant activities

In the present study of DPPH assay, linear equation $y = 6864x + 5.0226$ $R^2 = 0.9997$ and $y = 6332x + 3.7842$ $R^2 = 0.9989$ were drawn through best-fitting straight line to calculate IC_{50} value for ascorbic acid and PHM respectively. PHM was exhibited dose-dependent DPPH scavenging activity with a 50% inhibition (IC_{50}), at a concentration of 65.52 ± 1.32 µg/ml, which was observed very close to IC_{50} value of standard ascorbic acid (72.98 ± 2.46 µg/ml) (Figure 5).

Similarly, in reducing power assay, the yellow color changed to pale green and/or blue color (Fe^{3+} to Fe^{2+}) depending on the concentration of antioxidants, present in the sample. The antioxidants such as phenolic acids and flavonoids are presented in considerable amount in the blend therefore PHM was shown good reducing potential percentage (86.17%) at low dose 125 µg/ml. PHM was followed the same pattern as like ascorbic acid. At very high concentration e.g. 1000 µg/ml, both ascorbic acid and PHM were shown almost similar activities 97.75 and 97.16% inhibition respectively, whereas at very low concentration e.g. 31.25 µg/ml (5th diluted solution), ascorbic acid was shown 47.62% inhibition in compared to PHM, which was found to be 41.31% inhibition (Figure 6).

Safety dose activity

The safety dose study revealed that the test substance did not produce any mortality throughout the study period of 14 days even when the limit dose was maintained at 2000 mg/kg body weight with extra



Type	Name	Symbol	Unit of Measurement in	
			PPM	%
Macro-Elements	Calcium	Ca	6190	0.619
	Magnesium	Mg	2782	0.278
Micro-Elements	Copper	Cu	<10	<0.001
	Iron	Fe	<40	<0.004
	Zinc	Zn	<10	<0.001
	Selenium	Se	<2	<0.0002

Table 2: Essential metal contents in PHM.

Note: *AHPA-The American Herbal Products Association.

**FSSAI-Food Safety and Standards Authority of India; (Contaminants, toxins and residue Regulation).

six animals. All the animals were appeared normal throughout the experimental period. No groups at a single oral dose of 2000mg/kg body weight were caused any death or clinical symptoms over a period of 14 days treatment (Table 1).

Phytochemical analysis

HPLC Standardization: *Curcuma longa*: Total curcuminoids, i.e. curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin were found to be 76.7%, 16.53% and 2.32% respectively (Figure 7(A and B)). Piperine shown a specific peak at retention time 15.13 min and

found to be 93.12% w/w in the extract (Figure 7(C and D)). Compared to standard, total “punicalgins” (α and β) in the punica extract were found to be 5.88% with specific peaks at retention time 8.95 and 11.37min respectively (Figure 7(E and F)). Similarly compared to standard, ellagic acid was showed specific peaks at 20.90min and was found to be 2.72% (Figure 7(G and H)). In moringa extract, isoquercetin was shown specific peaks at 21.79min and was found to be 2.25% in the extract (Figure 7(I and J)).

GC chromatogram: GC-FID analysis of black pepper essential oil was revealed the existence of main components β-caryophyllene retention time 17.87min with Area% 31.85 (Figure 7K). Other components were also recognized with their Area % e.g. d-limonene (13.18%), δ-3-carene (8.53%) sabinene (7.51) β-pinene (6.71%) and α-pinene (6.00%).

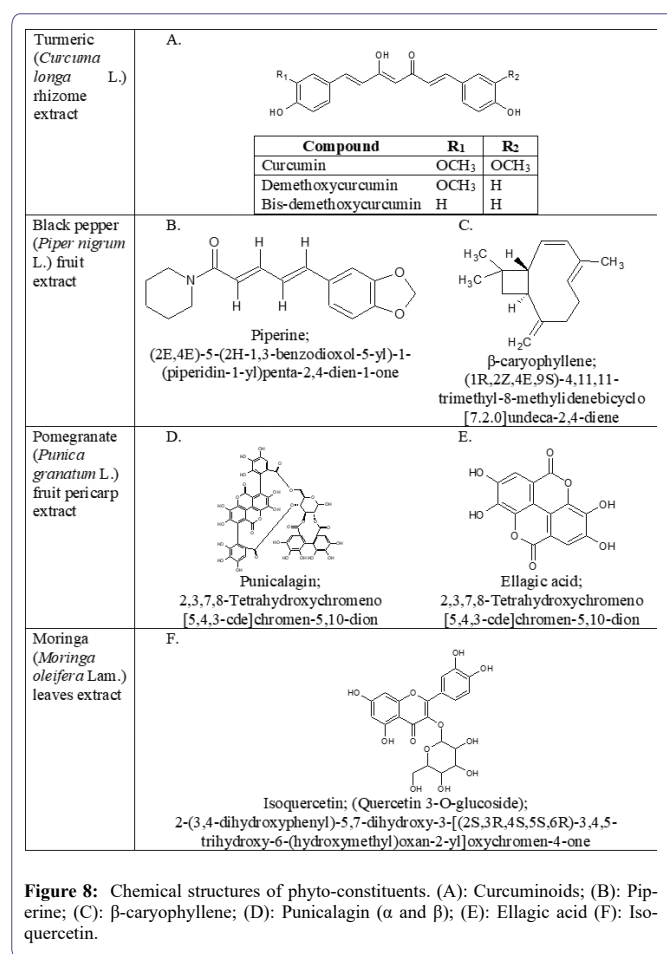
Micronutrient detection

The quantitative determinations were carried out using standard calibration curve, drawn by the AAS data obtained from the standard solution of metals having optimal detectable concentration ranges. Concentration of the metals were expressed in terms of parts per million; ppm [22,23] and presented in table 2.

Discussion

In the designed MTT assay, CC_{50} was found to be $691.89 \pm 2.13 \mu\text{g/mL}$ safe for the studies and hence two different doses were selected. $500 \mu\text{g/mL}$ dose was shown >75% hepatoprotection and the same dose was potent enough to normalize the biochemicals release (AST, ALT, and LDH). It specified that the PHM has potency to protect liver cells. All these findings strongly were supported that PHM can alter morphological changes and reversing the structure of affected HepG2 cells. PHM was exhibited dose-dependent DPPH inhibition activity. The value was very close to that of ascorbic acid, which indicated that the PHM would be a good formula for the proposed claim. More than 90% of DPPH inhibition activities was observed after the concentration of $125 \mu\text{g/mL}$. Likewise, PHM was also displayed significant reducing potential at a low dose of $125 \mu\text{g/mL}$. All these promising results were substantiated the use of PHM for treating ROS induced cells toxicity. Acute toxicity study revealed a safe dose of PHM. All the phytochemicals present in PHM were standardized and were quantified using HPLC system and oil was quantified by using GC-FID system.

Phytochemical point of view, Turmeric is one among, the most popular and explored herb, used as a spice, food colouring agent, and traditional medicine with a wide range of pharmacological activities due to its potential efficacy and to act on a number of biological targets with no major side effects. The therapeutic attributes of turmeric are mainly due to the presence of curcuminoids, a group of phenolic compounds namely curcumin, demethoxycurcumin and bis-demethoxycurcumin (Figure 8A). These molecules are well recognized for their anti-inflammatory activities by altering the mechanism NF-kB and MAPK pathways. Black pepper, the Indian spice has been used as a culinary ingredient from ancient times and is extensively used in the traditional practice of medicines [24]. The major phytochemical, piperine an alkaloid (Figure 8B) is proven to improve the serum bioavailability of therapeutic agents by inhibiting intestinal and hepatic glucuronidation [25]. Another effective molecule β-Caryophyllene (Figure 8C) is having high potency to treat or prevent hepatic injuries, associated with oxidative stress, inflammation and steatosis. It is having the ability to act on CB_2 receptors and thereby relieving



the physiological and mental stress like anxiety and depressive disorders, which are closely associated with alcohol intoxication and accompanying complications [26]. Pomegranate peels extract, a novel source of bioactive phytochemicals was considered in the blend since it is having a significant amount of phenolic contents compared to other parts of the fruit. Peel is a rich source of ellagitannins and derivatives such as punicalagin, ellagic acid (Figure 8(D and E)), etc. All these phytochemicals are identified for its anti-inflammatory, and gastro-protective activities [27]. Miracle tree “Moringa” leaves are universally known for its micronutrient properties and potential to maintain electrolyte concentration. Supplementing with micronutrients from moringa extract are keys to maintain a balanced serum osmolarity. It was considered in our formula to replenish and to prevent excess loss of micronutrients due to alcohol-induced diuresis [28]. Moringa leaves are having polyphenol isoquercetin (Figure 8F), which also possess liver protective potential [29]. The results from all these studies are supported physio-pharmacological characteristics of the phytochemicals present in our proprietary formula.

Conclusion

The outcome of our studies elucidates that herbal supplement, comprising five standardized extracts has immense protective potential on alcohol-intoxicated HepG2 cells. In addition, its free radical scavenging activities along with a considerable amount of reducing potential as well as the ability to normalize hepatic biochemicals make the supplement a suited solution for treating alcohol-induced

toxicity and associated disorders like hepatotoxicity, hangover, and nutrient balance. Promising results from acute toxicity studies on animals ensure that the developed polyherbal formula Re-party™ is a safe formulation for oral consumption. However, alcohol and aldehyde dehydrogenase activities and clinical investigation to be needed to support and endorse a complete mechanism of action to prove the above in the near future.

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Author Disclosure Statement

The authors declare that there was no conflict of interest with any commercial associations.

Ethical Statements

All the experimental procedures and protocols of this study were reviewed and were approved by the Institutional Animal Ethics Committee (IAEC- RR/IAEC/65-2019) of Radiant Research Services Pvt. Ltd., Bangalore (Regd. no: 1803/PO/RcBi/S/2015/CPCSEA) constituted in accordance with the guidelines of the CPCSEA, Government of India. The experiment procedures were followed in accordance with the OECD guideline 423 published in 2002.

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