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Research Article

An Appraisal And Evaluation Of Frap Antioxidant Power And Hepatoprotective Activity Of A Herbal Blend Comprising Two Cold Macerated Methanol Extracts Of *Litsea Polyantha* Linn And *Angelica Sinensis* In Hepg$_2$ Cells

Pavan Kumar Padarthi[1], Miss. Komal Kriti[2], Uppuluri Varuna Naga Venkata Arjun[3*], Touseef Begum[4], Karuna Priyachitra[5], Shikha Atteri[6], Saish Pawar[7], Arghya Paria[8],

[1]Departement of Physiology and Pharmacology, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur-522019.
[2]Dhanbad College of Pharmacy, Shaktinagar,Dhanbad, Jharkhand, Pin Code:-826001.
[3*]Department of Pharmaceutics, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), PV Vaithiyalingam Rd, Velan Nagar, Krishnapuram, Pallavaram, Chennai, Tamil Nadu 600117.
[4]Department of Pharmaceutical Sciences, Ibn Sina National College for Medical Studies,
P.O. Box 31906, Jeddah 21418, Kingdom of Saudi Arabia.
[5]Department of Pharmacology, Tagore College of Pharmacy Vandalur kelambakkam road, Rathinamangalam, Chennai-600127.
[6]Department of Pharmacology, Chandigarh Group of Colleges Jhanjeri, Pin Code:-140307.
[7]Department of Pharmaceutical Sciences, School of Health Sciences and Technology, Dr. Vishwanath Karad MIT World Peace University, Kothrud, Pune, Maharashtra 411038.
[8]Sanaka Educational Trust's Group of Institutions, Malandighi, Durgapur, West Bengal 713212.

Corresponding Author: Uppuluri Varuna Naga Venkata Arjun
*Department of Pharmaceutics, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), PV Vaithiyalingam Rd, Velan Nagar, Krishnapuram, Pallavaram, Chennai, Tamil Nadu 600117.

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ABSTRACT

The present study aimed to investigate the antioxidant and hepatoprotective activity of an herbal blend consisting of two medicinal plant extracts viz, methanol leaf extract of *Litsea polyantha* Linn and *Angelica sinensis*. The total phenolic contents were assayed for the extracts and for the herbal blend using Folin-Ciocalteu technique. In vitro antioxidant activity was screened and evaluated using the ferric reducing/antioxidant power (FRAP) method. In vitro hepatoprotective activity of the herbal blend was investigated against CCl4-caused hepatic cell injury model in HepG$_2$ cell lines. In the culture supernatant layer, the hepatic marker enzymes viz., ALT, AST, and LDH were estimated and also estimated the levels of GSH and MDA in lysates of cell. The results demonstrated the significant synergistic activity of the herbal blend against ccl4 induced hepatic injury in HepG$_2$ cells. The herbal blend also indicated significant antioxidant activity in terms FRAP assay.

Keywords: Litsea polyantha, Angelica sinensis, antioxidant, Total antioxidant activity, hepatoprotective, carbon tetrachloride.

INTRODUCTION

People have searched for medicines in nature for the treatment of various illnesses since ancient times. The usage of therapeutic plants was first instinctual, just like with animals. In actuality, everything back then was dependent on experience and there was insufficient knowledge about the causes of the ailments or the best plant to use as a preventive measure. As the use of certain medicinal plants for the treatment of particular ailments became known, the empirical framework grew and the medicinal plants' uses evolved into explicative facts. Prior to the development of iatrochemistry in the sixteenth century, plants were the source of both prevention and therapy. Since need is the mother of innovation, people have looked for cures for a variety of illnesses since prehistoric times [1]. Throughout history, humans have looked to nature to provide two basic needs: food for sustenance and medicinal plants for illnesses and pains. The ancient societies employed plants

or concoctions of them known as *corpus therapeuticum* to cure a wide range of illnesses. Ebers's papyrus was one of these parts. The Egyptian papyrus, written in 1550 BC, provided an explanation of the therapeutic plants utilized in Egyptian society. Dr. George Ebers, a German Egyptologist, examined the papyrus's components after buying it in Thebes in 1872 and declared it to be of exceptional worth. Known as the "science and knowledge of life," Indian Ayurveda was discovered and has been associated with India for thousands of years [1, 2].

In India, medicinal plants have been utilized for 5000 years as part of the Ayurvedic medical system. This approach combines herbal medicines and food tailored to the body, mind, and spirit to prevent and treat illness. Customers see herbal items as being more "natural" than "synthetic," and they also consider that they are more likely to be safe than synthetic pharmaceuticals. These attitudes are shared by consumers around the globe. They started avoiding touch with traditional "western" medicine and began to think about leading a healthy lifestyle. It has been noted that herbal medications and the ingredients in them are beneficial to long-term health and are effective in treating human illnesses. Since the beginning of time, people have experimented with using herbal remedies to treat a variety of illnesses and relieve pain [1]. Herbal remedies are being widely utilised in both developed and developing nations for medical purposes. Herbal remedies, sometimes referred to as combinations of chemical compounds made from plants, are not very efficient when taken orally because of low absorption. About 80% of people worldwide use herbs and other traditional medicines for basic healthcare, according to a World Health Organization (WHO) survey. Three types of herbal medicines have been established: raw plant material, processed plant material, and herbal products. (www.who.int/research/en). Herbal medications are undoubtedly natural items that have been shown to be safe since they have fewer adverse effects when used to treat illnesses and also function as dietary supplements to shield the body against illnesses. In response to consumer demand, herbal products are now offered in the forms of fresh or dried plants, pills, capsules, powder, and tea extracts. Because herbal remedies are generally thought to be safe, individuals use them without a prescription. However, some are ineffective and interfere with other medications, while others could result in health issues. In the current situation, standardising herbal goods and evaluating the quality of pharmaceuticals appear to be essential for determining the concentration of their active constituents [1, 3].

One of the most important organs in the human body, the liver is in charge of both endogenous and external chemical detoxification. Toxic substances, medications, and viral infiltration by ingestion or infection can all result in hepatic damage[4-8]. Interestingly, synthetic medications for hepatic damage have few therapeutic benefits and may have negative side effects.[9]. Hence, it is essential to investigate alternative treatments options for treatment and prevention of hepatic or liver injury. Acute liver injury is common and can be easily triggered by various toxicants [10]. One well-known environmental biohazard that is frequently used to artificially induce acute liver damage in experiments is carbon tetrachloride (CCl_4). The cytochrome P450, phase I metabolic enzyme system viz., CYP2E1 catalyzes the accumulation of CCl_4 mainly in hepatic parenchymal cells, where it produces unstable free trichloromethyl radicals (CCl_3•). Reactive oxygen species and trichloromethyl-peroxyl (CCl_3O•2) radicals are created when these radicals combine with oxygen (ROS) [11, 12].. An antioxidant mechanism found in hepatocytes aids the liver in getting rid of too much ROS. On the other hand, after a spike in ROS production, further ROS buildup promotes oxidative stress. Overexposure to reactive oxygen species (ROS) can attach to proteins or lipids, causing lipid peroxidation and oxidative damage. [10]. Consequently, it is thought that antioxidant substances reduce oxidative stress during acute liver damage caused by CCl_4.[12].

The complex system of enzymatic and non-enzymatic natural antioxidant defenses in the body functions to lessen the harmful effects of oxidants, such free radicals. Numerous illnesses, including cancer, are brought on by free radicals.[13]., cardiovascular disease [14], neural disorders [15]., Alzheimer's disease [12], mild cognitive impairment, Parkinson's disease, alcohol induced liver disease, ulcerative colitis, aging [16-18] and atherosclerosis [19, 20]. A diet rich in antioxidants can help protect against free radical damage. There is a lot of evidence to suggest that eating foods high in antioxidants, and maybe especially the antioxidant nutrients, can play a significant role in preventing illness. [21-28]. However, a rising body of research suggests that a mixture of antioxidants rather than individual ones may be more beneficial in the long run.[11, 12]. Antioxidants have the potential to significantly enhance life expectancy by delaying or avoiding the onset of degenerative illnesses. Furthermore, they might result in significant cost reductions in the provision of healthcare. [7, 8, 29-31].

The aim of this present work was to examine the antioxidant and hepatoprotective actions of *Litsea polyantha Linn* and *Angelica sinensis* leaf methanolic fraction. The methanolic leaf fraction of *Litsea polyantha Linn* and *Angelica sinensis* were subjected to initial screening for major phytochemicals and also assayed the total phenolic content. Further the study aimed to investigate and evaluate the hepatoprotective effect of the herbal blend in HepG2 cells.

MATERIALS AND METHODS

Plant
The medicinal plant crude drugs of *Litsea polyantha* Linn and *Angelica sinensis* were procured from Angel herbs from Khari Bauri Area of New Delhi, India. Fresh plants were also collected from Kullu District of Himachal Pradesh, India. The fresh plant were also identified and authenticated by a Botanist from Pharmacognosy department and herbariums were prepared and kept for future reference.

Drugs and chemicals
Gallic acid, Silymarin, Penicillin and Streptomycin were procured from Himedia Laboratories, Mumbai, India. Folin-Ciocalteu reagent and all other biochemical kits were purchased from Sigma Aldrich, Mumbai, India and R&D systems, India respectively. All other chemicals and reagents were of good quality and of analytical grade arranged from reputed and pre-verified vendors only.

Preparation of extracts
The leaves of *Litsea polyantha* and *Angelica sinensis* Linn. Were dried in shade for at least a month and then made to crude powder using a mechanical grinder. Cold maceration extraction techniques were applied to extract the plants individually using methanol as solvent. Once extracted the extracts were dried and concentrated in a vacuum. A 100 gram od each plant was extracted and yielded 6.4 and 7.2 gram of extract for *Litsea polyantha* and Angelica sinensis Linn., respectively (Yield, 6.4 and 7.2 percent, respectively).

Preliminary phytochemical screening
The extracts and the herbal blend were subjected a preliminary phytochemical screening by employing a array of standard tests described elsewhere [32].

Measuring and calculating the total phenolic content
The Folin-Ciocalteu technique was utilised to estimate the total phenolic content of the extracts.[33]. Two duplicates of the 200 µL sample were placed in test tubes, and then 0.8 mL of 7.5% sodium carbonate and 1.0 mL of Folin-reagent Ciocalteu's were added. After mixing, the tubes were let to stand for thirty minutes. Using a Perkin-Elmer λ15 UV-vis spectrophotometer located in Norwalk, CT, the absorption at 765 nm was measured. Gallic acid equivalents (GAE) were used to express the overall phenolic content in milligrams per gramme of dry material.

$Y = 0.0023x + 0.0944$, $r^2 = 0.9136$,
Where, y = absorbance and x = concentration.

In vitro antioxidant activity
Determination of Antioxidant Activity Using the Ferric Reducing/Antioxidant Power (FRAP) Method
The FRAP test was carried out using the previously mentioned protocol [34]. Aliquots containing 0.2 mL of methanolic extract were treated with 3.8 mL of FRAP reagent (at four different concentrations: 0.1, 0.5, 1, and 2 mg/mL; two duplicates per sample and concentration). This reagent was previously made by combining one part of 10 mM TPZT, one part of 20 mM FeCl3 hexahydrate, and ten portions of buffer solution of pH 3.6 of sodium acetate at 300 mM strength. (Alfa Aesar, Kandel, Germany). The resultant mixture was incubated at 37 °C for 30 minutes. The rise in absorbance was evaluated using a UV-VIS spectrophotometer at 593 nm (GB-BOR SRL, Italy). The blank was made by adding methanol in the same volume as diluted extract. The findings were presented as milligrams of $FeSO_4$ equivalent per milligrams of dry weight. The amounts of $FeSO_4$ that were used to construct the calibration line were 0.003, 0.006, 0.012, and 0.025 mg/mL.

Evaluation of hepatoprotective activity *In vitro*
Cell line
HepG2 cells were cultured at 37 °C in a controlled humidified atmosphere with 6% CO_2 in DMEM containing 100 IU/mL penicillin, 10% FBS, 0.1 µg/ml streptomycin, and non-essential amino acids. Using trypsinization, the cells were made to pass [35, 36].

Assessment of Hepatoprotective activity of UDLE-M using hepatic ($HepG_2$) Cell Line
The MTT test was used to assess the cell viability.[37, 38]. Following the cell harvesting process during the exponential growth phase, 100 µl of seeded cells (HepG2) per individual well in 96-well plate system were then nurtured for a full day. CCl_4, TFs, and Silymarin were applied to test cells at specific doses. 0.1 percent DMSO (v/v) in serum-free DMEM was used to produce these solutions. Following a 48-hour period, each well's media was supplemented with 20 µl of MTT (5 mg/ml), and the plates were incubated for three hours at 37 °C. After the solution was extracted, 100 µl of DMSO was added each well. Using a 96-well plate reader, the absorbance

in each well was measured at 492 nm. The following formula was used to determine the % viability: Cell viability (%) = (Abscontrol – Abssample) × 100 / Abscontrol [38].

Experimental design

Plates were chosen and HepG2 cells were plated at a density of 3 × 105 cells/ml. Following a 24-hour cell culture process, the HepG2 were primed and arranged for the investigation and grouped as follows:
Group 1 (Normal control): After removing the media, the cells were incubated for two hours in 100 µl of serum-free medium. For the next 24 hours, another 100 µl of the serum-free media was added.
Group 2 (Positive control): After removing the media, the cells were incubated for two hours in 100 µl of medium free from serum containing 1.5% percent CCl4. For the following 24 hours, additional 100 µl of the media added with 1.5% percent CCl4.
Groups 3 (Silymarin control): Following the removal of the media, the cells were incubated for two hours in 100 µl of medium free from serum and the media had been supplemented with CCl4 and Silymarin at 0.5 percent and 400 µg/ml, respectively. Next, 24 hours later, 100 µl more of the serum-free medium containing 1.5 percent CCl4 was added, and it was left for another 24 hours.
Groups 4 (HB-AL treatment): After removing the media, the cells were incubated for two hours in 100 µl of medium free from serum with different HB-AL concentrations (60, 120, 240, 500 and 1000 µg/ml). Also added a 1.5% percent CCl4 to the culture system. In next 24 hours, another 100 µl of the medium free from serum containing 1.5 percent of CCl4 had been added.
In the long run, the MTT assay was employed to measure the cell vitality of the HepG2 cells. The supernatant layer from the cell culture had been taken out and collected. Then, estimation of ALT, AST and LDH were performed using biochemical jots as mentioned as per the instruction provided by the manufacturer. The cells were scraped, frozen, and thawed three times and then, the cell lysates had been prepared and the level of MDA and GSH had been estimated.

AST, ALT, and LDH leakage measurements

Using commercial kits obtained from R & D systems (India), AST, ALT and LDH were detected and estimated in the supernatant layer obtained from the cell culture.

MDA and GSH levels in cell lysates

Commercial kits from R & D systems (India) were utilised for the estimation and measurement of GSH and MDA levels in the lysates of cell in the supernatant layer from the cell culture.

Statistical analysis

Using Graph Pad Prism software, the findings are presented as mean ± SD and subjected to one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. A significance level of $p < 0.05$ was determined.

RESULTS

Preliminary phytochemical screening

Preliminary phytochemical screening was performed on the herbal blend (HB-AL) and the results were tabulated and presented in table 1.

Table 1. Results of preliminary phytochemical screening with the extract (HB-AL).

Phytochemical Compound Group	HB-AL
Flavanoids	+
Phenols	+
Alkaloids	+
Saponins	+
Phytosterols	+
Borntrager test	-

+: Presence of moderate active constituents, **-:** Absence of active constituents

Estimation of total phenolic content

The concentration of total phenolic contents in the herbal blend (HB-AL) was expressed as gram of Gallic acid equivalent (GA) using an equation attained from the equation of regression line of standard Gallic acid graph [33]:
$Y = 0.0023x + 0.0944$, $r^2 = 0.9136$ (HB-AL)
Where, y = absorbance and x = concentration.

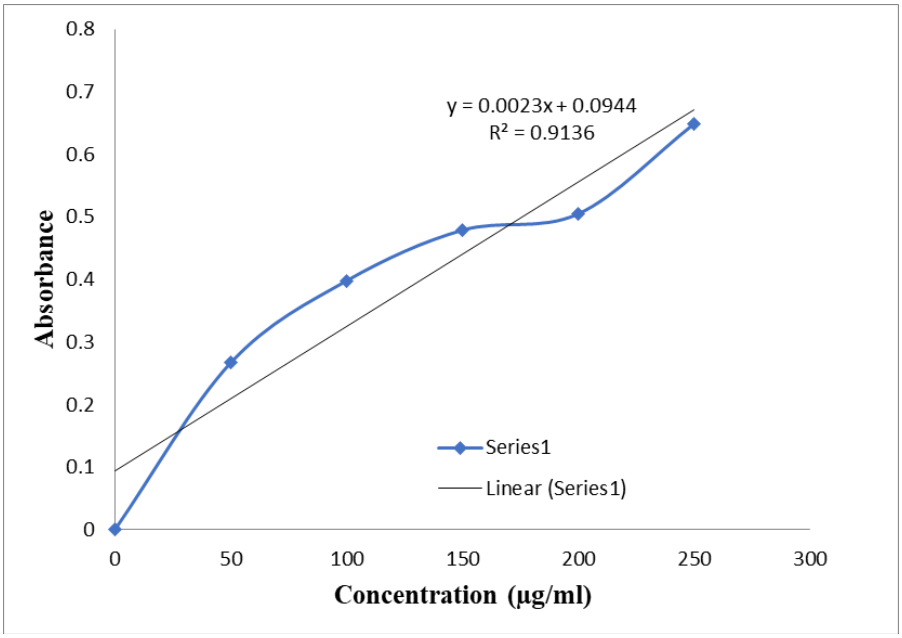

Figure 1. The estimation of total phenolic content in HB-AL.

In vitro antioxidant action

Evaluation of antioxidant activity in the Ferric Reducing system using FRAP technique

The results of the FRAP assay demonstrated the dose-dependent antioxidant activity of HB-AL, with the maximum antioxidant activity being associated with a concentration of 2 mg/mL of HB-AL corresponding to 0.071 FeSO4 E mg/mg dw respectively.

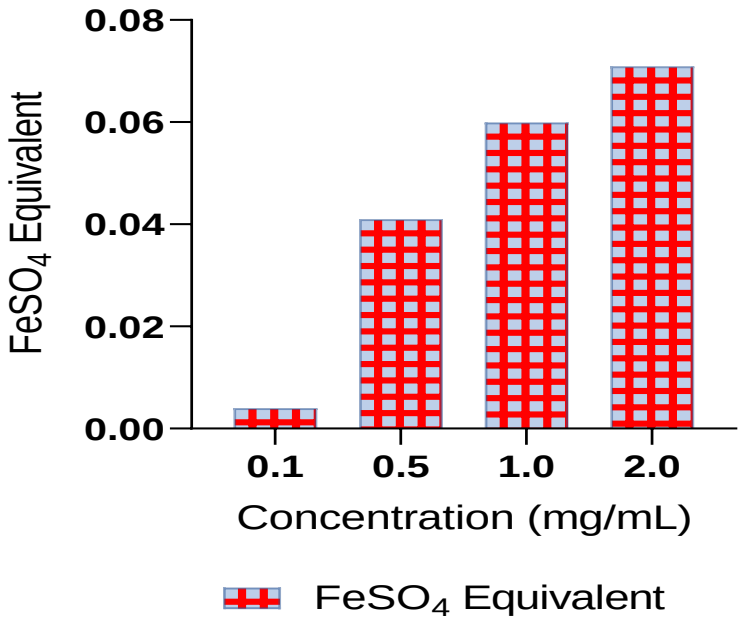

Figure 2. The results of antioxidant activity using the ferric reducing/antioxidant power (FRAP) of HB-AL.

In vitro evaluation of hepatoprotective action

Hepatoprotective action assessment of HB-AL using HepG2 Cell Line

The MTT assay had been performed to evaluated and measure the cytotoxicity in terms of cell viability in percentage. The percent cell viability had been measured in all the groups including CCl4 group, Silymarin group, and HB-AL groups on HepG2 cell. As indicated in literature, hepatotoxicity induced by CCl4 in HepG2 cells were preferred as an in vitro cell model to appraise the hepatoprotective effect of HB-AL. A 1.5 percent CCl4, 400 µg/ml Silymarin, and various concentrations of HB-AL (60, 120, 240, 500, and 1000 µg/ml) were applied to the cells. In comparison to a normal control, Table 1 demonstrates that after 48 hours of exposure to 1.5 percent CCl4 (12.82 ± 2.18) cell viability (%) was nearly 12 percent. Significant improvements in cell

viability were seen following treatment with high quantities of HB-AL (1000 µg/ml) and Silymarin (400 µg/ml). These findings would supply HB-AL and silymarin at safe dosages for the next HepG2 cell study.

Table 1. Estimation of hepatoprotective effect of HB-AL in CCl_4 induced injury in $HepG_2$ cells.

Sample (Treatment Groups)	Concentration (µg/ml)	% Cell Viability
Control (Normal Group)	-	100
Control (Positive Group, CCl_4 induced)	-	12.82 ± 2.18*
Silymarin Treated + CCl_4 (1.5 %) induced (Standard Group)	400	94.87 ± 4.12 #
HB-AL treated + CCl_4 (1.5 %) (Test Group)	1000	95.01 ± 3.27 #
	500	92.75 ± 3.06 #
	240	90.96 ± 3.87 #
	120	83.98 ± 3.27 #
	60	77.89 ± 3.11 #
Data were represented as Mean ± SD for 3 replicate determinations as an average. * indicated significant differences compared to normal group (p< 0.001); and # indicated significant as compared to Toxicant group (Positive control) (p< 0.01).		

Effect of HB-AL on oxidative stress and hepatotoxicity biomarkers in HepG2 cells caused by CCl4

The findings of cell viability, GSH and MDA in lysates of cell following treatment, and the amount of ALT, LDH, and AST in the supernatant layer from the cell culture are shown in Table 2. When compared to the normal control group, the cells exposed to CCl4 showed a substantial loss in cell viability ($p < 0.001$) and a considerable rise in the ALT, AST, LDH and MDA levels ($p < 0.01$ or $p < 0.001$). The cell viability of the HepG2 cells pre-treated with various HB-AL doses (60, 240, and 1000 µg/ml) and Silymarin (400 µg/ml) was not significantly affected by exposure to CCl4. The outcomes demonstrated that pretreatment with HB-AL diminished the death of the HepG2 cells in a concentration-dependent manner ($p < 0.001$). HB-AL considerably ($p < 0.01$) brought the biochemical indicators back to almost normal levels as compared to the CCl4-exposed group. According to the outcomes of the earlier studies conducted in HepG2 cells, TFs exhibited notable hepatoprotective properties against cytotoxicity generated by CCl4. Liver damage indicators were nearly back to baseline.

Table 2. Effect of HB-AL on the hallmark biological markers of hepatotoxicity in $HepG_2$ cells.

Experimental Groups	AST (Unit/L)	ALT (Unit/L)	LDH (Unit/L)	MDA (nM/mg protein)	GSH (nM/mg protein)
Normal Control	2.89±0.47	3.13±0.30	178.64±5.66	4.13±0.20	95.20±1.89
Positive Control (CCl4 intoxicated)	8.89±0.75**	5.77±0.61**	398.29±12.20***	5.82±0.19**	28.27±2.74**
CCl4 (1%) intoxicated + Treated with Standard Silymarin	3.56±0.36##	3.86±0.45##	220.92±4.89###	5.35±0.15#	52.29±2.89##
CCl4 (1%) intoxicated + Treated with [UDLE-M + Silymarin]	3.91±0.44#	3.67±0.77##	323.22±8.16##	4.84±0.17#	45.97±1.42##
	5.02±0.52#	4.06±0.32#	344.73±5.58#	5.08±0.12#	43.32±0.47#
	5.65±0.34#	4.59±0.45	379.89±2.47	5.41±0.12#	38.80±1.09#

DISCUSSION

Liver diseases are a major worldwide health problem that are frequently caused by alcohol intake, chemical damage, and viral infections. Over the past few decades, discovering potent liver protecting medicines has become an extremely difficult task. More and more people are realising that natural remedies, especially those enhanced with flavonoid components from food or medicinal plants, are safe and helpful in preventing hepatotoxicity [39]. The purpose of this study was to assess antioxidant potential HB-AL in FRAP assay. Total phenolic content was also estimated. The investigation focused on hepatoprotective actions and examined how they affected HepG2 cells. Furthermore, an investigation was conducted to examine the biomarkers of hepatoprotective effectiveness. Cell culture models of liver damage were used to provide insights into processes and possible therapies; CCl4 exposure was used as a popular model [40]. Although CCl4-induced liver damage is rare, viral infections and diseases associated to medications have common pathogenic causes. The liver damage caused by CCl4 is an essential model for researching natural antioxidants since it is linked to both

acute and chronic liver toxicity, which can lead to fibrosis, cirrhosis, cellular necrosis, and steatosis [41]. Cytochrome P450 is used by the liver to convert CCl_4 into the extremely reactive trichloromethyl free radical (CCl_3•). From there, deadly trichlorimethyl peroxyl radicals and trichloromethyl peroxyl radicals are formed (CCl_3O). According to studies, this cascade causes lipid membranes to peroxidatively degrade, which leads to the creation of lipid peroxides [42-44]. This suggests a direct connection between CCl_4-induced liver injury and lipid peroxidation. Antioxidants have drawn a lot of interest because of oxidative stress. Their primary means of action is scavenging free radicals and diminishing their strength. Through its protective function against oxidative damage, this mechanism reduces the likelihood of acquiring long-term ailments [41]. On the other hand, a range of antioxidants can specifically target peroxide radicals and reactive oxygen species (ROS). One of the popular and well studies model to study mechanism of antioxidant of natural origin is estimation of Lipid peroxidation (LPO) inhibition [45]. A plethora of research also suggested the estimation of LPO in plasma as well as estimation of other oxidative stress markers such as GSH, MDA etc. In this research, HB-AL treatment showed that it can protect HepG2 cells in a model where CCl_4 causes liver damage. The liver-protecting action of HB-AL, an herbal blend, also showed up when we looked into levels of ALT, AST, and LDH in fluids collected from the cultured cells. The levels of GSH and MDA in the HepG2 cells' breakdown product or lysates revealed solid evidence of the herbal blend's liver-protection and antioxidant effects.

CONCLUSIONS

In this present study, the combo of two distinctive herbal extracts, namely the methanolic leaf fractions from *Litsea polyantha* Linn and *Angelica sinensis*, exhibited both liver-saving and antioxidant qualities. The research points to a solid liver protective characteristic of this herbal mixture or blend, labelled as HB-AL. This has been asserted by an increase in percentage cell viability as evident by MTT assay results and a return to normal levels of key biological markers like AST, ALT, LDH, GSH, and MDA. Given these outcomes, this herbal blend, HB-AL, could be a top pick for safeguarding against and handling liver disorders. The possible high levels of phenols and related compounds could be driving its strong antioxidant and liver protective powers. Moving forward, deeper exploration is warranted, perhaps with different live model studies. Also, efforts should be made to isolate and assess the key plant-derived compounds in the herbal blend.

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