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Potential of Bioactive Compounds in Hepatotoxicity Using Primary Cell Culture Method: A Systematic Review

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Abstract *– Hepatotoxicity is a condition characterized by liver cell damage caused by toxic chemicals. This article aims to explore the effects of compounds that contribute to mitigating hepatotoxicity, focusing on in vitro studies, particularly primary cell culture. In this systematic review, we conducted searches in the PubMed, Science Direct, and Google Scholar databases to find articles published between 2008 and 2022. Several active compounds were identified, including N-Benzylpiperazine (BZP), 1-(3-trifluoromethylphenyl) piperazine (TFMPP), antimycin A, coenzyme Q (CoQ) substrates, the antioxidant Vitamin C, L-glutamine (Gln), Nimesulide, Luteolin, glutathione, angelica sinensis polysaccharide (ASP) from Angelica sinensis, ammonium glycyrrhizin (CAG) from Glycyrrhiza glabra, L-arginine, silymarin from Silybum marianum, and glucurolactone (GA). However, only six compounds were shown to have remedial and treatment effects on hepatotoxicity and utilized primary cell culture with MTT Assay. These six compounds are Luteolin, Glutathione (GSH), Angelica sinensis polysaccharide (ASP) from Angelica sinensis, Ammonium glycyrrhizin (CAG) from Glycyrrhiza glabra, Antimycin A (AA), and Glutamine (Gln). The mechanism of action of these compounds involves preventing further damage to liver cells and repairing cells that have already been damaged. In summary, these compounds play a significant role in addressing hepatotoxicity caused by toxic substances and drugs.*

Keywords: *Hepatotoxicity, bioacyive compounds, MTT assay, Primary Cell Culture*

INTRODUCTION

Since the early 1990s, it has been known that human cells can be proliferated in vitro (Leland & Ginocchio, 2007). Cell culture was first developed in the early 1920s as a method to study animal cells in vitro. Cell culture involves a complex process concerning the isolation of cells from their natural environment (in vivo) or under controlled environmental conditions (in vitro). Cells from specific tissues or organs can be widely used in research and diagnosis, especially in virus infection. Cell culture is an essential tool in modern medicine and the diagnosis of infections in humans (Hudu et al., 2016).

Cell culture is a technique extensively employed in research focusing on human metabolism and physiology, areas often challenging to investigate directly in vivo. This method involves the extraction of cells from tissues, followed by their cultivation over periods ranging from days to weeks. Ethical and clinical considerations permitting, cells may be sourced from normal tissues (e.g., skin tissue), or alternatively from diseased tissues (e.g., liver tumor cells) obtained during surgical interventions as part of patient treatment regimens. Typically, cell culture begins with the preparation of cell suspensions derived from original tissues via enzymatic, mechanical, or chemical dissociation methods, or alternatively utilizes primary cultures or established cell lines. These procedures are conducted under sterile laboratory conditions within controlled environments, encompassing regulated temperature, gas composition, and pressure. Such adjustments aim to replicate the in vivo cellular environment, enabling cells to thrive

and proliferate in a controlled manner (Otero et al., 2012).

Primary cells closely resemble their tissue of origin, being directly isolated from tissues and cultured under optimized conditions. Due to their unaltered nature, they exhibit physiological characteristics closely resembling the in vivo state, making them valuable for studying various aspects of cell physiology and biochemistry, such as metabolism, aging, and signaling pathways, as well as for assessing the effects of pharmaceuticals and toxic agents. However, researchers encounter challenges related to variability arising from differences among donors and during subculture procedures, particularly when investigating cell signaling pathways. Hence, it is common practice for researchers to screen cells for responsiveness to standard stimuli before conducting signaling studies(Ramos et al., 2014). Epithelial cells, fibroblasts, keratinocytes, melanocytes, endothelial cells, muscle cells, hematopoietic cells, and mesenchymal stem cells are among the most commonly utilized types of primary cells in research.

In this article, research on the types of primary cell cultures used and compound sources has been summarized. The aim is to facilitate other researchers in conducting in-depth in vitro studies on various potentials of existing phytopharmaceuticals.

MATERIALS AND METHODS

This review is based on research articles discussing the effects of various plant extracts in addressing hepatotoxicity issues. The data sources for this review were obtained from international databases such as Google Scholar, PubMed, and Science Direct. The search used keywords within the PICO strategy, relevant to the research with inclusion parameters such as: "Hepatotoxicity", "Active Compound", "MTT Assay", and "Primary cell culture", or a combination of these keywords with Boolean operators. The following keywords utilized logical operators like "hepatotoxicity treatment" and "primary cell culture with MTT Assay". Articles were analyzed and selected according to inclusion and exclusion criteria.

Inclusion criteria were tailored to the goals of the review article, so the journal inclusion criteria are research data providing primary information on the therapeutic effects of plant extracts on liver cells, original articles, animal experimental research subjects, with publication years in the last 15 years, i.e., 2007-2022, full-text articles, and indexed in Scopus Q1 - Q4. Articles related to hepatotoxicity issues were used as exclusion criteria for article selection. Non-specific articles discussing plant extracts on rat hepatocytes, and research subjects are humans or microorganisms were excluded. Then, literature in the form of journal reviews, abstracts, case reports, and clinical trials were also excluded. Journals that met the keywords, title, and abstract criteria were examined in full text so that their content could be understood and aligned with the researched topic. Journal searches were used for evaluation by searching and observing inclusion and exclusion criteria. The analysis conducted in this article review was descriptive.

RESULT AND DISCUSSION

34 journals were identified for initial systematic review. These journals were documented from Elsevier using keywords like hepatotoxicity, Primary cell culture, plant extracts, natural compounds, and the MTT Assay method as a toxicity testing method. Other search pages such as PubMed, Science Direct, and Google Scholar were also utilized to facilitate the search. The first selection was made based on the title and study of hepatotoxicity, resulting in 34

inclusion journals and no exclusion journals. The second selection based on the remaining keywords resulted in 6 inclusion journals and 28 exclusion journals. The selection process is displayed in the form of a chart in Figure 1.

The publication years of the selected journals range from 2008 to 2022. From these journals, several types of primary hepatocyte cell cultures were identified, including mouse primary hepatocytes, rats primary hepatocytes, canine primary hepatocytes, and chicken primary hepatocytes. Some studies also combined these with cell lines to compare various cases. The use of plant extracts and natural substances was also very diverse, including Luteolin, glutathione, extracts from Angelica sinensis, Ginkgo biloba L, Silybum marianum, and other natural compounds. Detailed information such as publication year, authors, types of primary hepatocyte cell culture, plants, compounds, medium, antibiotics, and incubation periods applied in each journal are included in Table 1.

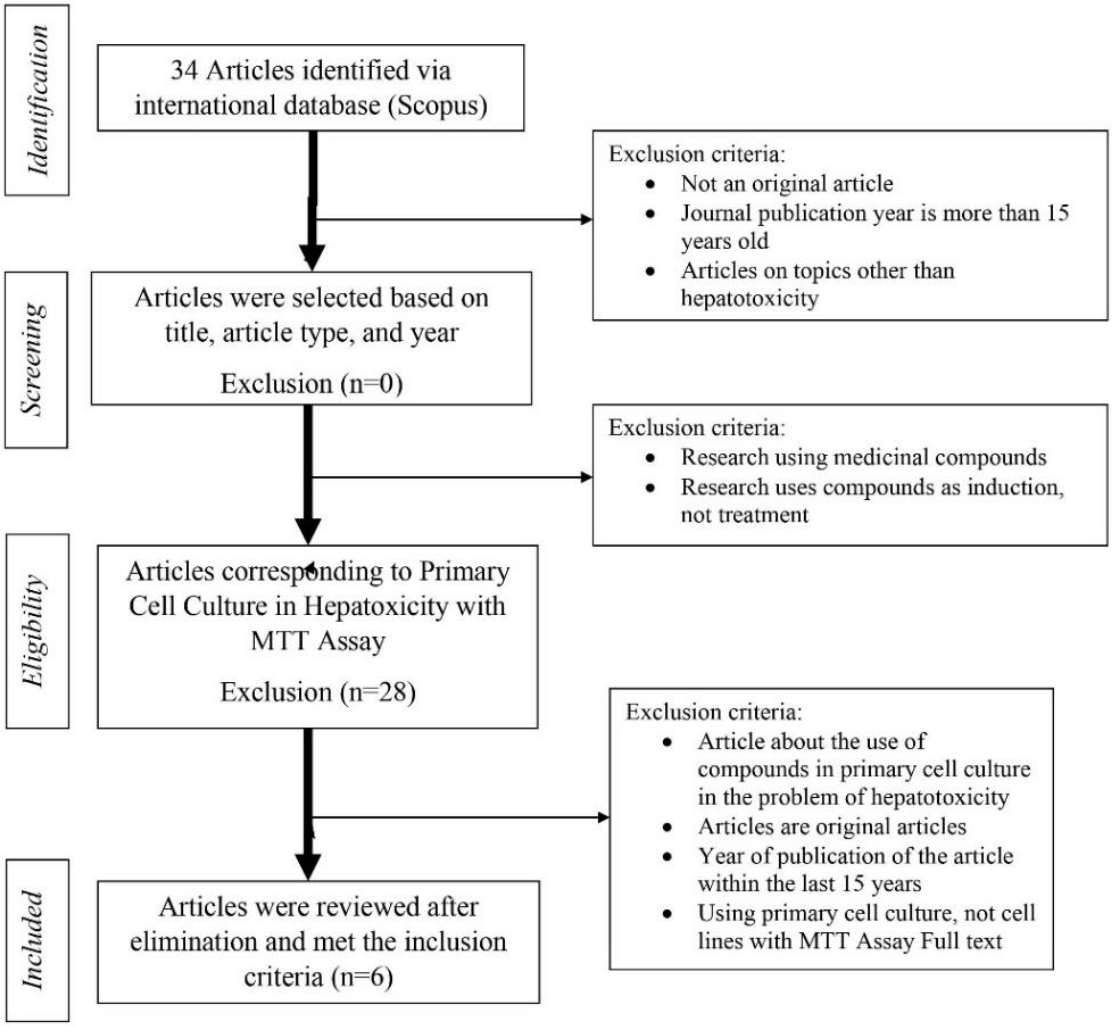

Figure 1 Prism diagram in article selection

Table 1. Summary of Inclusion Articles

No.	Author (Year)	Primary cell culture	Compound	Medium (Incubation time)	Antibiotics	serum	Results and conclusions or explanation
1 .	Lachin Mousavi, Elham Zadeh-Hashem , dan Mehdi Imani (2022)	primary culture of rat hepatocytes (RHs).	Luteolin, β-cyfluthrin	DMEM (Overnight and 4 hours)	penicillin G sodium/streptomycin sulfate	Fetal bovine serum (FBS)	Results: In the first experiment, exposure doses for 0, 24, and 48 hours of β-cyfluthrin 0, 10, 20, 40, and 80 µL were found at doses of 40 and 80 µL at 24hour exposure to reduce the viability of hepatocyte cells. And the exposure dose was decided at 40 µL to assess Luteolin's ability as an antioxidant for hepatotoxicity. The second experiment was exposure to 40 µL of β-cyfluthrin combined with luteolin at doses of 20, 40, and 60 µM at exposures for 0, 24, and 48 hours. The results showed that 40 µM could increase hepatocyte cell viability. Conclusion: β-cyfluthrin exhibits hepatotoxic effects at particular doses, and this study demonstrates that luteolin effectively enhances hepatocyte cell viability in mouse primary cell cultures exposed to β-cyfluthrin-induced hepatotoxicity.
2.	Natalie M. Kirk, Miranda D. Vieson , Kim A. Selting, and Jennifer M. Reinhart (2021)	Primary dog hepatocytes	glutathione (GSH) dan itraconazole (ITZ)	William's E medium (4 Hours and 24 hours)	penicillin/streptomycin	Fetal bovine serum (FBS)	Results: Hepatocyte cytotoxicity exhibited a notable increase over time ($P = 0.004$) and with escalating concentrations of ITZ ($P < 0.001$). Conversely, cytotoxicity demonstrated a significant decrease in correlation with elevated GSH concentrations ($P < 0.001$). Furthermore, a significant interaction was observed between time and ITZ concentration ($P = 0.014$). Conclusion: There is no drastic comparison regarding the effect, the higher the concentration and the longer the exposure time to itraconazole, the more it increases cytotoxicity, as well as glutathione (GSH) further reduces cytotoxicity.
3.	Chunfeng Lia , Shumin	Rats primary	angelica sinensis polysaccharid	Williams' E medium (ThermoF	-	-	**Results:** ASP rescues cell proliferation and viability in DB-treated hepatocytes, ASP triggers

No.	Author (Year)	Primary cell culture	Compound	Medium (Incubation time)	Antibiotics	serum	Results and conclusions or explanation
	Liub , Jian Zhengc , and Yingwei Xue (2021)	hepatocytes	e (ASP) dan Diosbulbin-B (DB)	isher Scientific, USA) (4 hours)			autophagy to protect hepatocytes from DB-induced cell death, and ASP promotes autophagic flux in DB-treated hepatocytes through activating the MEK/ERK pathway. **Conclusion:** ASP can be used as an adjuvant. DB agents in cancer treatment.
4.	ZUGONG YU, FENG WU, JING TIAN, XUEWEN GUO dan RAN AN (2018)	chicken primary hepatocytes	ammonium glycyrrhizin, L-arginine, silymarin and glucurolactone yang diinduksi ochratoxin (OTA)	DMEM ((4 Hours and 24 hours)	-	fetal bovine serum	Results: cell viability decreased compared to the control group when exposed to OTA for 24 hours. However, cell viability increased significantly when hepatocytes were treated with CAG, L-Arg, Sil and GA at concentrations of 0.1, 1 and 10 μg/ml in comparison with the OTA alone group. Conclusion: These results suggest that CAG, L-Arg, Sil, and GA exhibit hepatoprotective, antioxidant, and antiapoptotic effects in cultured hepatocytes exposed to OTA.
5.	Hasan Turkez, Fatime Geyikoglu, Mokhtar I. Yousef, Kubra Celik, Tulay O. Bakir (2012)	primary hepatocytes cultures	Lglutamine (Gln)	n Dulbecco's modified eagle medium (DMEM), 75 % (v/v) Eagle's minimum essential medium and 25 % (v/v) medium 199 (48 hours)	-	10 % (v/v) fetal calf serum containing streptomycin (100 lg/mL), penicilin (100 IU/mL), bovine insulin (5 lg/mL), bovine serum albumin (1 mg/mL)	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a dioxin compound known for its hepatotoxic effects. L-glutamine (Gln) possesses antioxidant properties that may mitigate hepatotoxicity induced by TCDD exposure. This study aims to investigate the efficacy of L-glutamine in reducing TCDD-induced hepatotoxicity in primary cultures of rat hepatocytes. Hepatocytes were exposed to TCDD and Gln for 48 hours, and cell viability was assessed using the [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide] (MTT) assay. Results from the MTT and lactate dehydrogenase (LDH) assays indicated that TCDD reduced cell viability, while L-glutamine had no such effect. TCDD exposure also led to elevated

No.	Author (Year)	Primary cell culture	Compound	Medium (Incubation time)	Antibiotics	serum	Results and conclusions or explanation
							levels of total oxidant status (TOS) and a significant decrease in total antioxidant capacity (TAC) and total glutathione (TGSH) levels in rat hepatocytes. Furthermore, TCDD administration resulted in a dose-dependent increase in hepatocyte micronuclei (MNHEPs) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) compared to control cultures. This study underscores the potential therapeutic significance of L-glutamine in attenuating TCDD-induced liver injury, offering novel insights into its hepatoprotective effects.
6.	Madhulika Tripathi, Brijesh Kumar Sing , Chetna Mishra, Sheikh Raisuddin , Poonam Kakkar (2009)	Rat hepatocyte	nimesulide	RPMI-1640 (24 hours)	fetal bovine serum	Penicilin , Streptomycin, Amphotericin B, sodium pyruvate and glutamine	Nimesulide can cause apoptosis in primary cultured rat hepatocytes through oxidative stress in the mitochondria of primary cultured rat hepatocytes. Fumaria parviflora extract can inhibit the toxicity caused by Nimesulide by modulating the apoptotic pathway

One of the causes of liver damage is medications. Drugs said to be hepatotoxic are those that can induce liver damage, often referred to as drug-induced liver injury (DILI). The mechanism of DILI is not entirely understood, but broadly involves two mechanisms: direct hepatotoxicity and adverse immune reactions. Direct hepatotoxicity occurs when the liver is directly damaged, and the other reaction involves the liver converting drugs into chemicals that can be harmful to the liver. The liver is a vital organ with diverse functions. It plays a crucial role not only in synthesis, metabolism, and storage but also in the detoxification of endogenous and exogenous compounds, converting them into less toxic substances for excretion. Hepatotoxicity implies liver damage due to chemicals.

Research on the use of active compounds to address the issue of hepatotoxicity aims to observe the ability of active compounds to cause changes or improvements in liver cells using in vitro studies, specifically primary cell culture. This systematic review identified 9 types of compounds applied through cell culture

mechanisms. Here is a review of the 9 types of compounds against articles that meet the inclusion criteria.

1. Luteolin

The current study demonstrates the protective efficacy of luteolin against the hepatotoxicity induced by β-cyfluthrin in rat hepatocytes, as evidenced by improved cell viability and reduced levels of malondialdehyde (MDA). Previous research has revealed that luteolin, a flavonoid class, possesses strong ROS scavenging effects (Oh et al., 2020). In this context, it has been shown that the glycosylation form of luteolin (cynaroside) reduces ROS formation and ultimately prevents the damaging effects of oxidative toxicity in cardiomyocytes. This study also revealed the hepatoprotective role of luteolin against toxic doses of β-cyfluthrin. the observed enhancement in cellular viability following luteolin treatment, particularly under conditions of oxidative challenge, correlates with a dose-dependent upregulation of the antiapoptotic protein Bcl-2 expression and a concurrent downregulation of the proapoptotic protein Bax expression (Sun et al., 2012).

2. Glutathione (GSH)

The observed benefits of prior administration with GSH offer further substantiation to the hypothesis implicating oxidative damage in the pathogenesis of hepatotoxicity induced by ITZ. Studies conducted in animal models have revealed significant alterations in hepatic redox status during ITZ treatment, characterized by increased levels of liver myeloperoxidase, nitric oxide, and malondialdehyde, accompanied by diminished activities of superoxide dismutase, glutathione peroxidase, and reduced glutathione levels (Sozen et al., 2015). One conceivable mechanism underlying the protective effect of GSH against cytotoxicity involves its ability to bind and neutralize toxic metabolites of ITZ. A similar mechanism has been proposed for KTZ-induced hepatotoxicity, wherein metabolites of KTZ undergo oxidation to generate reactive intermediates that form complexes with hepatic proteins. GSH effectively diminishes the formation of KTZ metabolite-protein adducts by interacting with reactive moieties on KTZ derivatives, thereby facilitating their elimination (Rodriguez & Buckholz, 2003).

3. Angelica sinensis Polysaccharide (ASP) from *Angelica sinensis*

The MEK/ERK pathway plays a role in regulating liver injury by influencing both cell apoptosis (Fu et al., 2020) and autophagy. Notably, ASP regulates the MEK/ERK pathway in osteoarthritis chondrocytes, as supported by our data indicating that DB suppresses the MEK/ERK pathway in chondrocytes, but its activity is restored when cells are co-treated with ASP, suggesting ASP activation of the MEK/ERK pathway in DB-treated hepatocytes. Furthermore, published evidence demonstrates ASP-induced cell autophagy in DB-treated hepatocytes through MEK/ERK pathway activation. Additionally, we have shown that inhibiting the MEK/ERK pathway reduces cell viability and enhances apoptosis in hepatocytes treated with ASP and DB together, indicating ASP restoration of cellular function in DB-treated hepatocytes via MEK/ERK pathway activation.

CAG, L-Arg, Sil, and GA enhance cell viability and suppress the elevation of ALT levels. Additionally, L-Arg, Sil, and GA reduce AST activity in the supernatant, while CAG does not affect AST activity. Both CAG and GA demonstrate dose-dependent increases in cell viability and decreases in ALT activity. L-Arg displays optimal

protective efficacy at lower doses, whereas Sil exhibits maximum functionality at a concentration of 1 µg/ml. These findings suggest that CAG, L-Arg, Sil, and GA can safeguard the survival of chicken hepatocytes. Furthermore, these compounds elevate SOD and GSH levels and reduce MDA levels, with Sil exerting the most pronounced effect among the four agents. Thus, the results imply that the four hepatoprotective agents investigated in this study may confer antioxidant properties on chicken hepatocytes (Gupta et al., 2015).

4. Antimisin A (AA)

Various concentrations of ROT (0-20 µM) and AA (0-8 µg/ml) were administered to hepatocytes. The results of the MTT assay indicated that only high concentrations of ROT and AA sustained cell viability. Consequently, a concentration of 5 µM for ROT and 1 µg/ml for AA was chosen for subsequent experiments due to their minimal impact on cell survival. Prior investigations have documented that AA, by impeding electron transport at complex III, safeguards mitochondria during ischemic events, pinpointing MRCC III as a critical site in the Electron Transport Chain (ETC) responsible for mitochondrial impairment during ischemia. Conversely, ROT, by obstructing electron transport, maintains respiration through cytochrome oxidase during ischemia. However, in our present study, neither ROT nor AA exhibited protective effects but rather exacerbated Cr (VI)-induced cytotoxicity.

5. Glutamin (Gln)

Investigating the Impact of Glutamine (Gln) Administration on TCDD-Induced Liver Injury. The research delved into the effects of Gln treatment on liver injury triggered by TCDD. Findings revealed that Gln administration not only prevented liver damage but also exerted a notable beneficial influence by averting MNHEPs and modulating antioxidant status. The study illustrated that TCDD led to significant hepatocyte injury, as evidenced by elevated LDH levels, whereas higher doses of Gln demonstrated a discernible capacity to attenuate the toxic effects of TCDD. Serum LDH levels, serving as a biomarker for liver injury, were elevated (Park et al., 2010). Physiological plasma concentrations of Gln typically range between 0.5 and 0.7 mM in humans (Oehler et al., 2002). The dose-dependent protective effects of Gln supplementation at concentrations of 0.5, 1, and 2 mM were previously documented in human lymphocytes cultured by Greig in 2001 (Greig et al., 2001).

The results of animal experiments indicated that supplementation with 1.25 mM of glutamine (Gln) could confer cellular protection (Khogali et al., 2002). In this investigation, Gln supplementation was found to reduce oxidative stress by increasing Total Antioxidant Capacity (TAC) and glutathione (GSH) levels in a dose-dependent manner. Notably, the highest dose of Gln (2 mM) exhibited superior efficacy compared to lower doses (0.5 and 1 mM), providing partial explanation for its protective effects. Gln serves as a substrate for glutathione synthesis, and its depletion correlates with elevated reactive oxygen species (ROS) levels. It has been suggested that amino acids like Gln play a role in scavenging free radicals and ROS. Thus, under conditions of heightened oxidative stress, dietary antioxidants, including Gln, become crucial for maintaining the balance between oxidants and antioxidants. The observed increase in TAC in liver tissue aligns with findings from previous murine models. Gln demonstrates significant antioxidant activity in cultured hepatocytes, consistent with its known ability to scavenge and

quench ROS in lipid bilayers (Qing et al., 2015). Consequently, Gln supplementation enhances immune response (Kumar & Anandan, 2007). Nevertheless, the precise mechanisms underlying Gln's protective effects against organ and tissue damage remain incompletely understood (Jia et al., 2006).

CONCLUSIONS

A total of 6 compounds were tested in vitro on primary cell culture for their influence and treatment against hepatotoxicity. These compounds are Luteolin, Glutathione (GSH), *Angelica sinensis* polysaccharide (ASP) from *Angelica sinensis*, Ammonium glycyrrhizin (CAG) from *Glycyrrhiza glabra*, Antimycin A (AA), and Glutamine (Gln). These bioactive compounds play a crucial role in the prevention and repair of liver cells damaged by toxic chemical compound.

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