Effect of Rhubarb (*Rheum rhabarbarum*) Extract against Hepatocarcinoma (HepG2) Cell Line and Hepatotoxicity Induced by Paracetamol in Rats

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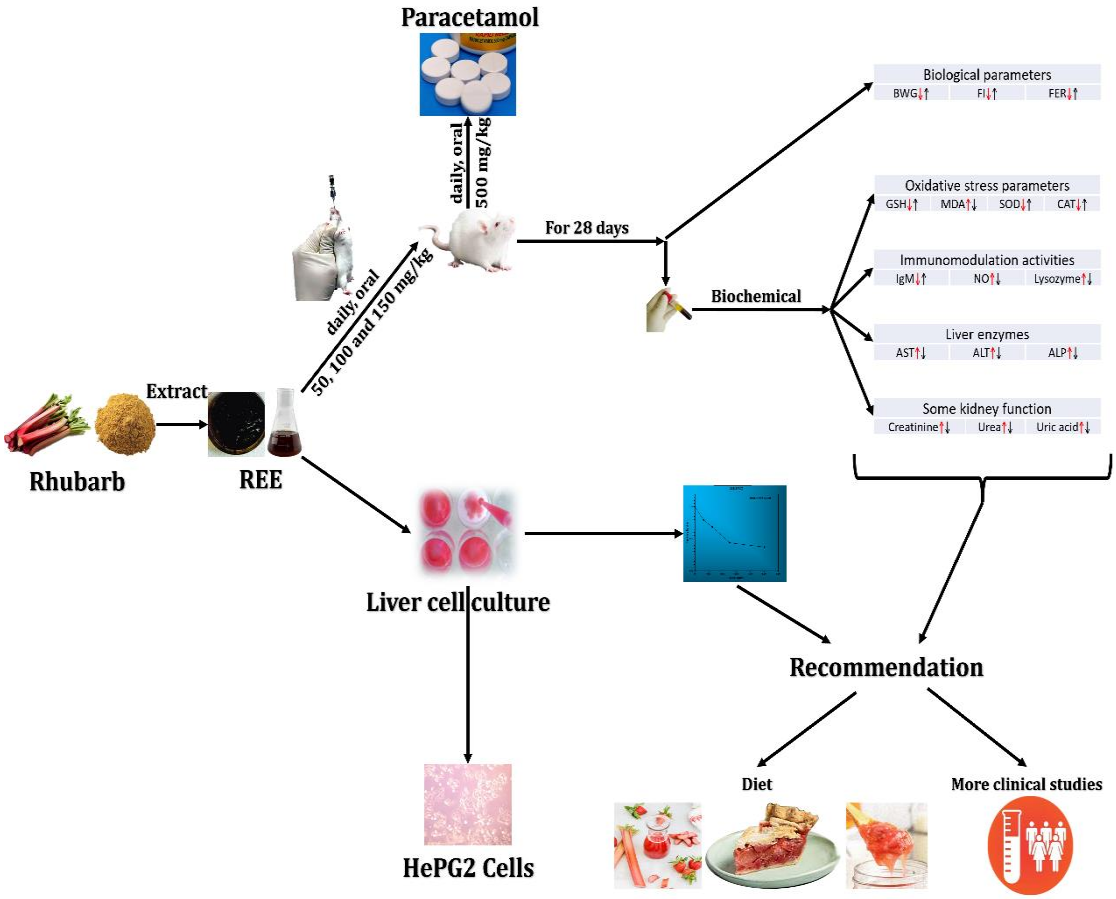
Abstract

The liver is the primary organ in the body for intense metabolism and excretion. Liver disease can be brought on by a variety of substances and medications that are frequently used in daily life. Therefore, this study evaluated the impact of rhubarb ethanolic extract (REE) against hepatotoxicity induced by paracetamol in male albino rats and its potential effect on the human hepatocarcinoma (HepG2) cell line in vitro. In this respect, thirty male adult albino rats weighing (160±8.65g) were separated into 5 groups (6 rats per group) and fed on a basal diet. Group 1(ve^-): kept as normal group. Group 2(ve^+): paracetamol hepatotoxicity rats as model group. Group 3, 4, and 5: paracetamol hepatotoxicity rats and treated with REE (50, 100, and 150 mg/kg b.wt) daily oral dose throughout the experiment period. For inducing paracetamol hepatotoxicity, the rats were given 1.5 mL of 500 mg/kg paracetamol orally by gavages. After completing 28 days, all animals were sacrificed; blood samples were collected and subjected to biochemical analysis. Paracetamol toxicity has been associated with various health hazards that result in a variety of body disorders, such as defects in biological parameters, oxidative stress, immune disorders, excessive biochemical disturbances of liver enzymes, and some renal functions. While REE (50, 100, and 150 mg/kg b.wt.) intervention improved biological parameters (body weight gain (BWG), feed intake (FI), and feed efficacy ratio (FER), it reduced the oxidative stress significantly ($p\leq0.05$) by increasing glutathione (GSH) level, superoxide dismutase (SOD) activity, catalase (CAT) activity, decreasing malondialdehyde (MDA) level, improving immunomodulation activities (IgM, NO, and lysozyme), and significantly ($p\leq0.05$) enhancing some kidney functions (urea, creatinine, and uric acid). REE reversed liver enzymes, especially at higher dose (150 mg/kg b.wt.), as evidenced by decreasing AST (38.48%), ALT (60.7%), and ALP (56.14%) compared to

the model group. It is interesting that the administration of REE at doses of 50, 100, and 150 mg/kg b.wt. showed a significant ($p \leq 0.05$) reduction in these toxic effects in a dose-dependent manner; REE at a dose of 150 mg/kg b.wt. showed the greatest results. While investigating the human hepatocarcinoma (HepG2) cell line, REE demonstrated apoptotic activity on human cancer liver cells, perhaps because of the intrinsic anticancer properties. The half maximal inhibitory concentration (IC50)-value in HepG2 for REE was 222 µg/mL. These findings suggested that REE has great potential for the development of healthy diets that treat paracetamol overdose and toxicity. As well as providing a direction to isolate possible anti-cancer compounds in REE for hepatocellular carcinoma. Pending more studies to discover the underlying mechanism, especially under clinical trials.

Keywords: Rhubarb, Acetaminophen, Liver injury, Anthraquinone compounds, HepG2 cells.

Graphic abstract



تأثير مستخلص الراوند المضاد لخلايا سرطان الكبد (HepG2) والسمية الكبدية الناجمة عن الباراسيتامول في الفئران

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الملخص :

الكبد هو العضو الأساسي في الجسم الذي يقوم بعملية التمثيل الغذائي والإخراج. ويمكن أن يحدث مرض الكبد نتيجة لمجموعة متنوعة من المواد والأدوية التي تستخدم بشكل متكرر في الحياة اليومية. لذلك، أجريت هذه الدراسة لتقييم تأثير مستخلص الراوند الإيثانولي ضد السمية الكبدية التي يسببها الباراسيتامول في ذكور الفئران البيضاء وتأثيرها المحتمل على خط خلايا سرطان الكبد البشري (HepG2)في المختبر. وفي هذا الصدد، تم تقسيم ثلاثين من ذكور الفئران البيضاء البالغة التي يبلغ وزنها (١٦٠ ± ٨,٦٥ جم) إلى ٥ مجموعات (٦ فئران لكل مجموعة) وتغذيتها على نظام غذائي أساسي. المجموعة ١ (السالبة): تم الاحتفاظ بها كمجموعة طبيعية. المجموعة ٢ (الموجبة): الفئران المصابة بالتسمم الكبدي لتناول الباراسيتامول كمجموعة نموذجية. المجموعة ٣ و٤ و٥: الفئران المصابة بالتسمم الكبدي لتناول الباراسيتامول وعولجت بجرعات يومية من مستخلص الراوند الإيثانولي (٥٠ و١٠٠ و١٥٠ مجم / كجم من وزن الجسم) عن طريق الفم طوال فترة التجربة. تم إعطاء فئران التسمم الكبدي الباراسيتامولي ١,٥ مل من ٥٠٠ مليجرام / كجم من الباراسيتامول عن طريق الفم من خلال أنبوب المعدة. بعد إكمال ٢٨ يومًا، تم ذبح جميع الحيوانات؛ وتم جمع عينات الدم وإخضاعها للتحليل. ارتبطت سمية الباراسيتامول بمخاطر صحية مختلفة تؤدي إلى مجموعة متنوعة من اضطرابات الجسم، مثل الخلل في المعايير البيولوجية، والإجهاد التأكسدي، واضطرابات المناعة، والاضطرابات الكيموحيوية المفرطة في إنزيمات الكبد، وبعض وظائف الكلى. في حين أدى التدخل بمستخلص الراوند الإيثانولي (٥٠ و١٠٠ و١٥٠ مجم / كجم من وزن الجسم) إلى تحسين المعايير البيولوجية (زيادة وزن الجسم (BWG) والمأخوذ الغذائي (FI) ومؤشر كفاءة التغذية(FER)، فقد قلل من الإجهاد التأكسدي بشكل ملحوظ ($p \leq 0.05$) عن طريق زيادة مستوى الجلوتاثيون (GSH) ونشاط أكسيد الفائق ديسميوتاز (SOD) ونشاط الكاتالاز (CAT) وتقليل مستوى مالونديالدهيد (MDA) وتحسين المناعة (IgM و NO وlysozyme) وتعزيز بعض وظائف الكلى بشكل ملحوظ ($p \leq 0.05$) (اليوريا والكرياتينين وحمض البوليك). عكس مستخلص الراوند الإيثانولي إنزيمات الكبد، وخاصة عند التركيز المرتفع من مستخلص الراوند الإيثانولي (١٥٠ مجم / كجم من وزن الجسم)، كما يتضح من انخفاضAST (٣٨,٤٨٪)، و ALT بنسبة (٦٠,٧٪)، و ALP(٥٦,١٤٪) مقارنة بالمجموعة الموجبة النموذجية. ومن المثير للاهتمام أن إعطاء مستخلص الراوند الإيثانولي (٥٠ و١٠٠ و١٥٠ مجم/كجم من وزن الجسم) أظهر القدرة على التخفيف المعنوي ($p\leq0.05$)من هذه التأثيرات السامة اعتمادًا على الجرعة المعطاه. وسجل مستخلص الراوند الإيثانولي (١٥٠ مجم/كجم من وزن الجسم) أفضل النتائج. كما أظهر مستخلص الراوند الإيثانولي نشاطًا مميتا للخلايا السرطانية الكبدية البشرية، ربما بسبب خصائصه المضادة للسرطان. كانت قيمة IC50 في HepG2لـ مستخلص الراوند الإيثانولي ٢٢٢ ميكروجرام/مل. وتشير هذه النتائج إلى أن مستخلص الراوند الإيثانولي لديه إمكانات كبيرة لتطوير أنظمة غذائية صحية تعالج الجرعة الزائدة من الباراسيتامول والسمية. بالإضافة إلى امكانية توفير اتجاه لعزل المركبات المضادة للسرطان المحتملة في مستخلص الراوند الإيثانولي لسرطان الخلايا الكبدية. وتتطلع الدراسة لمزيد من الدراسات لاكتشاف الآلية الأساسية، وخاصة في إطار التجارب السريرية

الكلمات المفتاحية: الراوند ، الأسيتامينوفين، إصابة الكبد، مركبات الأنثراكينون، خلايا سرطان الكبد HepG2

Introduction

The liver is an essential organ in humans; it regulates many physiological functions, particularly the biotransformation of xenobiotics (**Lindamood, 2020; Acharya *et al*., 2021)**. Consequently, liver damage from long-term exposure to toxic xenobiotics is likely to occur, and this damage can lead to cirrhosis, liver cancer, and sudden liver failure **(Barouki *et al*., 2023).**

Paracetamol, also known as acetaminophen, N-acetyl-p-aminophenol, or N-acetyl-p-aminophenol, is one of the most commonly used medications. It is available over the counter both as a single-entity formulation and in combination with other medications, as well as by prescription **(Michaut *et al*., 2014; Bibi *et al*., 2024)**. Due to the high incidence of paracetamol poisoning, the idea that paracetamol is a safe medicine has become extremely deceptive. The majority of paracetamol (80%–90%) that is taken at therapeutic levels is conjugated with glucuronic acid or sulfate and eliminated by the kidneys. Cytochrome P450 enzymes, like Cyp2E1 and Cyp1A2, operate upon a minor component to generate N-acetyl-p-benzoquinone imine (NAPQI), a reactive metabolite **(Mazaleuskaya *et al*., 2015)**. In paracetamol overdose (>4000 mg/day), only a portion of the excess NAPQI generated can be detoxified by conjugation with glutathione (GSH), as GSH has a limited capacity to detoxify it. The residual portion of NAPQI then attaches itself to liver proteins, causing an inflammatory response, oxidative stress, mitochondrial malfunction, apoptosis, hepatotoxicity, centrilobular necrosis, and liver failure **(El Faras and Elsawaf, 2017; Ahmed *et al*., 2023; Singh, 2023; Prescott, 2024).** Over 300,000 hospitalizations and up to 42% of all cases of abrupt liver failure are caused by paracetamol-induced hepatotoxicity each year **(Bibi *et al*., 2024)**. Paracetamol-induced liver damage in mice is a widely used experimental paradigm for evaluating drugs with possible hepatoprotective properties **(Bezzag *et al*., 2018)**. In addition, regular paracetamol use has been related to an increased risk of liver cancer **(Tian *et al*., 2024)**. Paracetamol increases apoptosis of the HepG2 cell line by inducing inflammation and oxidative stress **(Palabiyik *et al*., 2016; Behrends *et al*., 2019; Sharafudeen and Abraham, 2024).** HepG2 is a human cell line produced from hepatoblastoma that exhibits several different metabolic activities relevant to the liver as well as intriguing characteristics of differentiated hepatocytes **(Van Summeren *et al*., 2011)**. The human hepatoma cell line HepG2, which was created in 1979, has been used to study a variety of hepatotoxicity processes and is the most well-characterized and widely utilized cell line in terms of hepatotoxic endpoints **(Noor *et al*., 2009)**. Thus, HepG2 cell lines are used as a model in experiments to

investigate hepatotoxicity in vitro (**Sharafudeen and Abraham, 2024)**. Regretfully, the current choices for treating liver diseases are largely inefficient, frequently cause serious side effects, and are extremely costly, especially in developing nations **(Langmead and Rampton 2001**). Growing evidence points to medicinal plants as possible sources of innovative therapeutic molecules helpful against cancer and hepatotoxicity as more and more synthetic conventional drugs fail **(Zheng *et al*., 2022)**.

Rhubarb *(Rheum rhabarbarum)*, is a wild plant species belonging to the *Polygonaceae* family **(Al-Khatib *et al*., 2022)**. Rhubarb consumed fresh or cooked after peeling **(Şat *et al*., 2023),** has a lot of minerals, including zinc, phosphorus, potassium, sodium, calcium, magnesium, iron **(Özcan *et al*., 2007)**, and vitamin C **(Munzuroğlu *et al*., 2000)**. Moreover, it has a high phenolic content and antioxidant activity **(Öztürk *et al*., 2007; Doğan and Meral, 2016)**. **Alkaya *et al*. (2019)** reported that the polyphenols extracted from the stems and roots of rhubarb had high antioxidant activity. Additionally, rhubarb contains pharmacologically active compounds that have antitumor activity by inhibiting the growth of certain cancer cells and bioactive components like anthraquinone, which are recognized as the plant's key characteristics. Rhubarb also has laxative, anti-inflammatory, antibacterial, and antiviral properties **(Wang *et al*., 2014; Malik and Müller, 2016; Bhat, 2021)**. There are two types of rhubarb anthraquinones: mixed and free. Rhein, emodin, aloe-emodin, physcion, and chrysophanol are free anthraquinones. Anthrone derivatives include substances like sennoside A-D. Combined anthraquinones, usually glycosides, are formed when free anthraquinones are linked with glycosyl groups **(Cao *et al*., 2017)**. Another important component is tannins, which have antibacterial, hemostatic, anti-inflammatory, antioxidant, and anti-diarrheal properties **(Qin *et al*., 2011; Laddha and Kulkarni, 2019; Marcińczyk *et al*., 2022)**. Furthermore, stilbene compounds, including piceatannol, rhapontigenin, and their derivatives **(Wang, 2019)**, have been identified as having significant biological potential; they have been shown to have anti-inflammatory **(Dvorakova and Landa, 2017)**, anti-cancer **(De Filippis *et al*., 2017)** and anti-aging **(Dutta *et al*., 2023)**, properties. Analgesic and anti-inflammatory qualities are well-known for phenylbutanone, which includes lindleyin and isolindleyin **(Zhang *et al*., 2022)**. Pharmacologically, anthraquinone reverses the progression of acute liver injury **(Arosio *et al*., 2000; Neyrinck *et al*., 2017)**. Anthraquinone may reduce acute liver injury by controlling oxidative stress, inflammation, and fibrosis diseases, according to the metabolic pathway study **(Gong *et al*., 2023)**. Cooked rhubarb had a maximum non-lethal concentration that was almost nine times greater than raw rhubarb, suggesting a major detoxifying impact **(Wang *et al*., 2022)**. Processing considerably reduces the "bitter cold" effect of rhubarb on the stomach,

reducing gastric mucosa damage and gastrointestinal dysfunction in rats **(Zhang *et al*., 2019)**. Processing provides a scientific basis for "processing attenuating toxicity" by changing the distribution and effect of its constituents within the body in addition to changing the content. While several research works have examined the modifications in active chemicals brought about by different methods of processing rhubarb, variations in the temperature, time, and solvents used during processing cause variations in the results **(Wen *et al*., 2024)**.

Thus, the current research aims to investigate how rhubarb ethanolic extract (REE) protects male albino rats from the hepatotoxicity induced by paracetamol and whether it has any effect on the in vitro culture of the human hepatocarcinoma (HepG2).

Materials and Methods

Materials

Fresh rhubarb was obtained from National Research Center, Cairo, Egypt. The botanist from Menoufia University in Egypt's Department of Plant Protection and Production, Faculty of Agriculture, identified the plant materials as rhubarb (*Rheum rhabarbarum*).

Paracetamol (Panadol®): 500 mg tablets purchased from El-Nasr Pharmaceutical Chemicals Company in Egypt, grinded and dissolved in distilled water before administration. Corn oil, wheat bran, and corn starch were acquired from a local market in Shebin El-Kom, Menoufia, Egypt. Egypt's Cairo Corporation for Chemical Trade supplied the ethanol 70%, casein, sucrose, vitamins, minerals, choline bitartrate, and L-cysteine.

Rats

A total of thirty adult male albino rats (*Sprague Dawley* strain) weighing 160 ± 8.65 g were procured from the Medical Analysis Department of the Research Institute of Ophthalmology in Cairo, Egypt.

Ethical Approval

This study was granted ethical approval (**Reg. No., MUFHE /F/NFS/23/24)** from Menoufia University's Institutional Animal Care and Use Committee (IACUC).

Methods

Preparation of the rhubarb ethanolic extract(REE)

The rhubarb was repeatedly cleaned using distilled water and after that, all the different parts were separated and dried in the shade away from the sun. An electric grinder (Moulinex, France) was used to grind the dried rhubarb into a powder. The plant extract was made by combining the powdered rhubarb with 70% ethanol (1:10 by weight) and adding it to individual Erlenmeyer flasks. The flasks were sealed and stored out of direct sunlight for 72 hours before being shaken for 120 minutes to combine the contents. After passing the liquid through filter paper, the

contents of the flasks were heated to 40 °C in an oven to allow the alcohol and water to evaporate. Using a spatula, the dry extract was gathered and utilized to make the extract solution **(Lasibi and Moshtaghi, 2023)**. REE dissolved in sterile water and was given orally by gavages at a daily dose of 50, 100, and 150 mg/kg b.wt.

Diet

The components of the basal diet were produce were prepared in accordance with **Reeves *et al*. (1993)**.

Experimental design

This research was applied at the biology lab of the Menoufia University of Egypt's Faculty of Home Economics. Rats were kept in well-aerated cages under hygienic circumstances, fed on a basal diet, and had free access to water **(Reeves *et al*., 1993)**. Following acclimation, thirty rats were separated into 5 groups, 6 rats per group. Group 1(ve^-) fed only the basal diet and kept as normal group. Group 2 (ve^+) paracetamol hepatotoxicity rats fed a basal diet and used as model group. Group 3, 4, and 5: paracetamol hepatotoxicity rats fed a basal diet and treated with REE (50, 100, and 150 mg/kg b.wt) daily oral dose throughout the experiment, which lasted for 28 days. Feed intake was calculated daily, and rats were weighed weekly.

Induction of paracetamol hepatotoxicity

The paracetamol hepatotoxicity rats were given 1.5 mL of 500 mg/kg paracetamol orally by gavages **(Pandey *et al*., 2008)** throughout the experiment period

Biological evaluation

According to **Chapman *et al*. (1959)**, biological assessments including feed efficiency ratio (FER), body weight gain (BWG), and feed intake (FI) were determined and calculated during the experiment.

Blood samples

Rats were sedated with ether, and blood samples were taken from the abdominal aorta following a 12-hour fast after the 28-day experiment. As stated by **Stroev and Makarova (1989)**, blood samples were placed into sterile, dry centrifuge tubes, allowed to clot at room temperature, and then centrifuged for ten minutes at 3000 rpm to separate the serum. The serum was thoroughly aspirated, put into sterile capillary tubes, and kept frozen at -20°C until it was analyzed.

Biochemical analysis

Serum GSH, MDA, SOD, and CAT levels were determined according to **Vuolo *et al*. (2022); Esterbauer and Cheeseman (1990); Misra and Fridovich (1972)**; **Aebi (1984)**. Following the methods of **Falkenburg (2015); Wo *et al.* (2013); and Thomas *et al.* (1981),** respectively, IgM, NO, and lysozym**e** were measured. The **Reitman and**

Frankel (1957) approach was used to determine serum ALP, AST, and ALT quantitatively. Serum samples were used to calculate the concentrations of creatinine, urea, and uric acid in line with **Bartels *et al*. (1972); Patton and Crouch (1977); and Fossati *et al*. (1980)**, respectively.

Statistical analysis

The data is shown as mean ± standard deviation (SD). A computerized software costat program was used to statistically evaluate the data using one-way ANOVA. According to **Snedecor and Cochran (1967),** differences between treatments at $p<0.05$ were considered statistically significant.

Determination of potential cytotoxicity of REE on human hepatocarcinoma (HepG2) cell line

The American Type Culture Collection (ATCC, Minnesota, U.S.A.) obtained the human hepatocarcinoma (HepG2) cell line utilized in this investigation.

Serial subculturing was used to preserve the tumor cell lines at the National Cancer Institute in Cairo, Egypt.

REE samples were obtained by dissolving a 1:1 stock solution and kept at -20°C in dimethyl sulfoxide (DMSO). A range of concentrations measured in µg/ml were used for the REE.

Using a sulphorhodamine-B (SRB) assay, the cytotoxicity was evaluated in accordance with the methodology described by **Skehan *et al*. (1990)**. The sulforhodamine B colorimetric assay is employed in the screening of cytotoxicity. 1112-1116. Nat. Protoc. 2006:1. Aminoxanthrene dye containing two sulphonic groups, vivid pink in color, is called SRB. It is a protein stain that, under slightly acidic environments, attaches to the amino groups of intracellular proteins to produce a sensitive indicator of the amount of protein present in cells.

Buffers and reagents

1. Glacial acetic acid: 1% was utilized to dissolve the SRB dye that was not bound.
2. Sulphorhodamine-B (SRB): utilized as a protein dye, a 0.4% concentration was dissolved in 1% acetic acid.
3. Trichloroacetic acid (TCA): a 10% solution was utilized for protein precipitation, and a 50% stock solution was made.
4. To solubilize the SRB dye, 10 mM (pH 10.5) tris base was utilized. 121.1 g of tris base was dissolved in 1000 ml of distilled water to prepare it, and 2 M HCl was added to correct the pH.

Methods of (HepG2) cell line:

1. After seeding cells in 200 µl of fresh media in 96-well microtiter plates at a starting concentration of 4x103 cells/well, the cells were given 24 hours to adhere to the plates.
2. REE was added at various concentrations (0, 62.5, 125, 250, and 500) µg/ml.
3. Three wells were used for each concentration of REE. For 48 hours, the plates were incubated.
4. For one hour at 4 °C, the cells were fixed with 10 µl of cold trichloroacetic acid at a final concentration of 10%.
5. The plates were stained with 50 µl of 0.2% SRB dissolved in 1% acetic acid for 30 minutes in the dark at room temperature. They were then cleaned with distilled water using an automatic washer (Tecan, Germany).
6. After air drying, the plates were cleaned with 1% acetic acid.
7. 200 µl/well of 10M tris base (pH 10.5) was used to solubilize the dye, and each well's optical density (O.D.) was determined spectrophotometrically at 570 nm.

Calculation

The following formula was used to determine the percentage of cell survival:

The surviving fraction is calculated as O.D. (treated cells) / O.D. (control cells).

Furthermore, Prism version 5 was used to compute the half maximal inhibitory concentration (IC50) values, or the medication concentrations needed to achieve a 50% inhibition of cell growth.

Results and Discussion

Effect of rhubarb ethanolic extract (REE) on biological parameters (BWG, FI, and FER) in normal and paracetamol-intoxicated groups

Effects of REE on biological parameters (BWG, FI, and FER) in normal and paracetamol-intoxicated groups were shown in Table 1. These findings showed that rats induced with paracetamol toxicity had lower BWG, FI, and FER (91.27, 67.3, and 72.73%) compared to the normal group. The outcome was consistent with the findings of **Patra *et al.* (2018)**, who found that due to reduced feed and water intake, gastrointestinal toxicity, and paracetamol toxicity, the percentage increase in body growth was significantly lower than intoxicated groups. Additionally, paracetamol overdose reduces rats' body weight significantly when compared to normal rats **(Rumack, 2004)**. This loss in body weight may be the result of decreased appetite **(Hegazy *et al*., 2021)**. On the other hand, **Payasi *et al*. (2010)** observed that there was no discernible difference in the mean body weight when compared to the control group after administering a low dose of 66.6 mg/kg paracetamol infusion for 28 days. This may be due to the use of lower doses of paracetamol than in our

current study. However, intervention with REE (50, 100, and 150 mg/kg b.wt.) led to a significant increase ($p \leq 0.05$) on the BWG, FI, and FER by the rates of 586.36, 422.73, and 336.36%, 110.54, 84.56, and 78.15%, and 221.43, 180.95, and 142.86% when compared to the model group, respectively. Similar investigations revealed that supplementing with rhubarb extract increased the abundance of *Akkermansia muciniphila* and was linked to improvements in metabolism **(Neyrinck *et al*., 2017)**. Previous research has demonstrated that *Akkermansia muciniphila* can treat high fat diet (HFD)-induced obesity and diabetes by influencing metabolism and enhancing the function of the intestinal barrier **(Depommier *et al*., 2019)**. To prevent metabolic problems, rhubarb supplementation is sufficient **(Régnier *et al*., 2020)**. Our findings demonstrated that the group receiving a low dose of REE (50 mg/kg b.wt) experienced the greatest impact. The laxative qualities of rhubarb, as documented by **Zhang *et al.* (2022)**, may help to explain this. Greater laxative action is associated with higher doses. The investigation conducted by **Huang *et al*. (2023)** revealed that although rhubarb has a purgative side effect, there were no notable adverse effects associated with rhubarb-based therapy. Therefore, when used in clinical settings, it has a high safety profile.

Table 1: **Effect of rhubarb ethanolic extract (REE) on biological parameters (BWG, FI, and FER) in normal and paracetamol-intoxicated groups**

Groups		BWG (g/day)		FI (g/day)		FER	
		Mean±SD	% of change	Mean±SD	% of change	Mean±SD	% of change
Normal group		2.52±0.11^{a}	-----	16.24±0.11^{a}	------	0.154±0.006^{a}	------
Paracetamol intoxicated groups	Model group	0.22±0.04^{e}	-91.27	5.31±0.13^{d}	-67.3	0.042±0.006^{e}	-72.73
	REE (50 mg/kg)	1.51±0.09^{b}	586.36	11.18±0.14^{b}	110.54	0.135±0.006^{b}	221.43
	REE (100 mg/kg)	1.15±0.07^{c}	422.73	9.8±0.42^{c}	84.56	0.118±0.003^{c}	180.95
	REE (150 mg/kg)	0.96±0.07^{d}	336.36	9.46±0.07^{c}	78.15	0.102±0.007^{d}	142.86
LSD		0.142	-----	0.392	----	0.01	-----

Each value is expressed as mean ± SD. Means under the same column with different superscript letters are significantly different ($p \leq 0.05$). BWG, body weight gain; FI, feed intake; and FER, feed efficiency ratio.

Effect of rhubarb ethanolic extract (REE) on oxidative stress parameters (GSH, MDA, SOD, and CAT) in normal and paracetamol-intoxicated groups

The effects of rhubarb ethanolic extract (REE) on GSH, MDA, SOD, and CAT) in normal and paracetamol-intoxicated groups are displayed in Table 2. In comparison to the normal group, the paracetamol-intoxicated group exhibits considerably ($p \leq 0.05$) decreased GSH, SOD, and CAT levels (80.94, 65.64, and 84.63%, respectively), whereas MDA level (876.64%) increased. These findings are consistent with the findings of **Islam *et al*., (2021); and Abdallah *et al*., (2023)** who showed that high MDA, decreased catalase, and decreased serum total antioxidant capacity all indicated that a toxic dosage of paracetamol significantly induced tissue damage through an oxidative stress mechanism. When paracetamol is hazardous to the body, it damages tissue by releasing oxidants and free radicals that disrupt the lipid bilayer of the cell membrane and produce MDA. Regarding GSH, our findings are corroborated by **Kuriakose and Kurup's (2010)** findings, which indicate that animals given 500 mg/kg.b.wt of paracetamol experienced substantial glutathione depletion and an increase in the production of toxic reactive metabolites, mitochondrial dysfunction, and oxidative stress. This was due to the saturation of the conjugation pathway leading to glutathione. Significantly more oxidative stress was caused by the production of ROS, free radicals, and a drop in GSH levels **(Nagaraj *et al*., 2011)**.

The research reports that rats given paracetamol experience severe oxidative stress in their livers, which results in increased MDA and decreased GSH, SOD, and CAT **(Edo *et al*., 2023; Okiljević *et al*., 2024; Shams *et al*., 2024)**. When compared to the model group, REE administration at doses of 50, 100, and 150 mg/kg b.wt. significantly ($p \leq 0.05$) decreased MDA and increased GSH, SOD, and CAT levels. The expression of the oxidative stress parameters mentioned above was modulated in a dose-dependent manner. With significantly ($p \leq 0.05$) lower levels of MDA (86.48%) and significantly ($p \leq 0.05$) higher levels of GSH (377.06%), SOD (188.84%), and CAT (540.96%), the groups receiving high dosages (REE 150 mg/kg b.wt) shown the greatest improvement when compared to the model group. According to related studies, rhubarb can improve oxidative damage, lower liver MDA levels, scavenge free radicals, increase total antioxidant capacity, stabilize cell membranes, decrease lipid peroxidation, and benefit hepatocytes **(Wang *et al*., 2015)**. The active components of rhubarb, known as anthraquinones, have been shown in rats to significantly lower MDA and ROS levels and improve SOD activity **(Zhong *et al*., 2012; Lai *et al*., 2015)**.

Table 2: Effect of rhubarb ethanolic extract (REE) on oxidative stress parameters (GSH, MDA, SOD, and CAT) in normal and paracetamol-intoxicated groups

Groups		GSH (U/l)		MDA (nmol/ml)		SOD (U/l)		CAT (U/l)	
		Mean±SD	% of change	Mean±SD	% of change	Mean±SD	% of change	Mean±SD	% of change
Normal group		5.72±0.11^{a}	-----	0.65±0.04^{d}	-----	114.73±2.47^{a}	------	10.79±0.76^{a}	------
Paracetamol intoxicated groups	Model group	1.09±0.06^{e}	-80.94	6.36±0.42^{a}	878.46	39.41±0.63^{d}	-65.64	1.66±0.04^{d}	-84.43
	REE (50 mg/kg)	3.73±0.09^{d}	242.2	2.45±0.18^{b}	-61.48	90.28±1.01^{c}	129.08	4.36±0.05^{c}	162.65
	REE (100 mg/kg)	4.54±0.09^{c}	316.51	1.79±0.13^{c}	-71.86	98.23±1.52^{b}	149.25	6.71±0.41^{b}	304.21
	REE (150 mg/kg)	5.2±0.22^{b}	377.06	0.86±0.18^{d}	-86.48	113.83±2.43^{a}	188.84	10.64±0.34^{a}	540.96
LSD		0.21	-----	0.416	-----	3.229	----	0.758	-----

Each value represents the mean value of three replicates ± SD. Means under the same column with different superscript letters exhibited significance at $P \leq 0.05$. REE, rhubarb ethanolic extract; GSH, Glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; and CAT, catalase.

Effect of rhubarb ethanolic extract (REE) on immunomodulation activities (IgM, NO, and lysozyme) in normal and paracetamol-intoxicated groups

Table 3 shows the impact of rhubarb ethanolic extract (REE) on IgM, NO, and lysozyme in both normal and paracetamol-intoxicated groups. There were significant reductions in IgM (66.99%), whereas NO **(**245.56%) and lysozyme (289.07%) significantly increased compared to the model group**. Gong *et al*. (2010); Morsy *et al*. (2013); and Shams *et al*. (2024)** reported that paracetamol administration to normal rats resulted in a significant increase in NO and lysozyme levels due to epithelial cells being exposed to oxidative stress**.** Regarding IgM, paracetamol administration led to spleen damage, inhibited lymphoid follicular and sinus histiocytosis in the spleen, and decreased the production of antibodies (IgG and IgM) **(Talaat *et al*., 2023; Shams *et al*., 2024)**.

Treated groups with different doses of REE (50, 100, and 150 mg/kg b.wt.) significantly restored these immunomodulation activities (IgM, NO, and lysozyme) in a dose-dependent manner and to be approximately near the normal limits in most of the cases. In line with these results, **Kounsar *et al*. (2011)** speculated that the ethyl acetate extract of rhubarb has an immuno-enhancing effect. Rhubarb immunomodulation mechanisms are focused on its monomer components such as emodin, rhein, and aloe-emodin **(Cao *et al*., 2017)**. Also, these results may be traced back to anthraquinone aromatic organic natural pigments found in rhubarb for better improvement of innate and adaptive immune parameters such as

IgM, lysozyme activity, and NO production; this is when using anthraquinone-enriched 5 mg/kg diet **(Harikrishnan *et al*., 2019)**.

Table 3: **Effect of rhubarb ethanolic extract (REE) on immunomodulation activities (IgM, NO, and Lysozyme) in normal and paracetamol-intoxicated groups**

Groups		IgM (ng/ml)		NO (ng/ml)		Lysozyme(ng/ml)	
		Mean±SD	% of change	Mean±SD	% of change	Mean±SD	% of change
Normal group		653.38±6.86[a]	-----	21.42±1.32[e]	------	3.66±0.46[e]	------
Paracetamol intoxicated groups	Model group	215.68±5.3[e]	-66.99	74.02±2.36[a]	245.56	14.24±0.64[a]	289.07
	REE (50 mg/kg)	332.22±5.85[d]	54.03	44.69±0.51[b]	-39.62	5.47±0.37[b]	-66.59
	REE (100 mg/kg)	298.31±3.06[c]	84.67	31.47±1.02[c]	-57.48	4.45±0.23[c]	-68.75
	REE (150 mg/kg)	450.35±7.44[b]	108.81	24.93±2.01[d]	-66.32	4.53±0.47[c]	-68.19
LSD		10.735	-----	2.89	----	0.822	-----

Each value represents the mean value of three replicates ± SD. Means under the same column with different superscript letters exhibited significance at $P \leq 0.05$. REE, rhubarb ethanolic extract; IgM, immunoglobulin M and NO, nitric oxide.

Effect of rhubarb ethanolic extract (REE) on liver enzymes (AST, ALT, and ALP) in normal and paracetamol-intoxicated groups

Effects of rhubarb ethanolic extract (REE) on liver enzymes (AST, ALT, and ALP) in normal and paracetamol-intoxicated groups were shown in Table 4. Albino rats induced with paracetamol (500 mg/kg) alone developed significant hepatocellular damage, as evidenced by an increase in liver tissue biomarkers AST (133.13%), ALT (157.39%), and ALP (92.34%) when compared to the normal group. High levels of ALT and AST are caused by reactive species (NAPQI) that are created when a paracetamol overdose damages hepatic cells through lipid peroxidation and cellular permeability. ALP levels rise as a result of hepatotoxicity, which induces biliary congestion and makes it difficult for the body to excrete ALP **(Islam *et al*., 2021; Koç *et al*., 2023)**. These results were also confirmed by **Ayenew and Wasihun (2023); and Bibi *et al*. (2024)**, who pointed out that the values of AST, ALT, and ALP were all substantially raised by paracetamol. However, intervention with REE (50, 100, and 150 mg/kg BW) led to a significantly ($p \leq 0.05$) decrease on the AST, ALT, and ALP which was nearly equivalent to normal rats by the rates 41.48, 50.57, and 56.14%; 42.72, 48.08, and 60.7%; and 35.1, 38.01, and 38.48% compared to the model control group, respectively. The REE demonstrated a dose-dependent action, as indicated by the levels of decline in AST, ALT, and ALP. In solidarity with these results, prior research has demonstrated that rhubarb can effectively lower serum ALT and AST levels. This is primarily because of free anthraquinone compounds, which enhance the

effectiveness of suppressing hepatic oxidation and oxidative stress by lowering lipid peroxidation damage to cell membranes, scavenging oxygen free radicals, suppressing intracellular ROS, and boosting hepatocyte activity **(Zhong *et al.*, 2012; Lai *et al.*, 2015).** Moreover, rhubarb significantly inhibits the progression of liver cirrhosis and fibrosis. The process is associated with hepatic stellate cell activity reduction **(Lin *et al.*, 2009; Wang *et al.*, 2018).** On the other hand, **Xing *et al.* (2012); and Zhang *et al.* (2016)** demonstrated that rhubarb would cause hepatotoxicity in rats at high dosages. This can be explained by the usage of extremely high quantities of rhubarb, which causes harm to the cells in the liver.

Table 4: Effect of rhubarb ethanolic extract (REE) on liver enzymes (AST, ALT, and ALP) in normal and paracetamol-intoxicated groups

Groups		AST (U/l)		ALT (U/l)		ALP (U/l)	
		Mean±SD	% of change	Mean±SD	% of change	Mean±SD	% of change
Normal group		85.2±1.8^{d}	-----	48.56±1.92^{d}	------	201.36±0.78^{d}	------
Paracetamol intoxicated groups	Model group	198.63±2.51^{a}	133.13	124.99±2.64^{a}	157.39	387.3±2.86^{a}	92.34
	REE (50 mg/kg)	116.23±1.75^{b}	-41.48	71.6±1.55^{b}	-42.72	251.37±1.58^{b}	-35.1
	REE (100 mg/kg)	98.19±1.11^{c}	-50.57	64.89±1.16^{c}	-48.08	240.08±2.12^{c}	-38.01
	REE (150 mg/kg)	87.11±1.5^{d}	-56.14	49.12±0.99^{d}	-60.7	238.25±2.54^{c}	-38.48
LSD		3.263	-----	3.193	----	3.84	-----

Each value represents the mean value of three replicates ± SD. Means under the same column with different superscript letters exhibited significance at $P \leq 0.05$. REE, rhubarb ethanolic extract; AST, aspartate aminotransferase; ALT, alanine aminotransferase, and ALP, alkaline phosphatase.

Effect of rhubarb ethanolic extract (REE) on some kidney function (creatinine, urea, and uric acid) in normal and paracetamol-intoxicated groups

Table 5 presents the impact of rhubarb ethanolic extract (REE) on some kidney function parameters, including uric acid, urea, and creatinine, in both normal and paracetamol-intoxicated groups. The data showed that the rats given paracetamol had considerably ($p \leq 0.05$) higher levels of creatinine (118.75%), urea (159.07%), and uric acid (87.5%) compared to the normal group. These findings were consistent with those of **Ijaz *et al.*, (2016)**, who found that administering paracetamol raised serum creatinine and urea levels significantly ($P<0.05$) when compared to the control

group's levels of each. It has been reported that paracetamol toxicity reduced glutathione, which in turn led to lipid peroxidation and its intracellular accumulation. Its reactive metabolite, NAPQI, then formed a covalent bond with renal tissues and caused cell deterioration and death. Moreover, paracetamol caused biochemical and histological alterations in the rat kidneys, which were linked to an increase in oxidative damage, apoptosis, and elevated creatinine, urea, and uric acid (**Hegazy *et al*., 2021; Edo *et al*., 2023; Hasan *et al*., 2024)**.

However, intervention with REE (50, 100, and 150 mg/kg b.wt) led to significantly ($p \leq 0.05$)decreases on the creatinine, urea, and uric acid by 47.89, 39.05, and 51.43%; 41.02, 52.19, and 51.72%; and 24.04, 36.49, and 37.72% compared to the model group, respectively. The rate of decrease in creatinine, urea, and uric acid among the hepatotoxic rats was exhibited in a dose-dependent manner. Also, there was no significant difference between treatment with REE (100 mg/kg BW) and REE (150 mg/kg BW) urea and uric acid levels. These results are in line with those of other authors who found that rhubarb slows proteolysis, lowers intestinal absorption of amino acids, and decreases the liver's production of urea **(Zhang *et al*., 2018; Huang *et al*., 2023)**. REE encourages the excretion of urea and creatinine from the urine and feces and increases the frequency of bowel motions. Its antioxidant qualities and humoral immune system regulation help to ameliorate the kidneys' hypoxic status. Furthermore, by limiting cell division, lowering extracellular matrix (ECM) deposition, and preventing the synthesis of tumor necrosis factor (TNF), rhubarb enhances the metabolism of amino acids, nitrogen, and lipids **(Moon *et al*., 2006)**.

Positive therapeutic effects of rhubarb on patients with chronic renal failure were shown by a systematic review and meta-analysis. In addition to increasing the creatinine clearance rate (CCR) and improving the overall effective rate, rhubarb also decreased serum creatinine (SCr) , urea, and uric acid **(Huang *et al*., 2023)**. Male mice are more severely affected when rhein is administered at high doses over an extended period, as demonstrated by **Hu *et al*. (2019)**.

Table 5: Effect of rhubarb ethanolic extract (REE) on some kidney function (creatinine, urea, and uric acid) in normal and paracetamol-intoxicated groups

Groups		Creatinine (mg /dl)		Urea (mg /dl)		Uric acid (mg /dl)	
		Mean±SD	% of change	Mean±SD	% of change	Mean± SD	% of change
Normal group		0.48±0.02^{d}	-----	18.86±0.38^{d}	------	3.04±0.19^{d}	------
Paracetamol intoxicated groups	Model group	1.05±0.05^{a}	118.75	48.86±0.65^{a}	159.07	5.7±0.39^{e}	87.5
	REE (50 mg/kg)	0.71±0.03^{b}	-47.89	28.82±0.64^{b}	-41.02	4.33±0.27^{b}	-24.04
	REE (100 mg/kg)	0.64±0.04^{c}	-39.05	23.36±0.45^{c}	-52.19	3.62±0.11^{c}	-36.49
	REE (150 mg/kg)	0.51±0.02^{d}	-51.43	23.59±0.41^{c}	-51.72	3.55±0.11^{c}	-37.72
LSD		0.056	-----	0.931	----	0.441	-----

Each value represents the mean value of three replicates ± SD. Means under the same column with different superscript letters exhibited significance at $p \leq 0.05$. REE, rhubarb ethanolic extract.

Effect of rhubarb ethanolic extract (REE) on human hepatocarcinoma (HepG2) cell line

Table 6 and Figure 1 illustrate the impact of rhubarb ethanolic extract (REE) on the human hepatocarcinoma (HepG2) cell line. According to the table data, REE may have had an intrinsic anticancer effect since it caused human cancer liver cells to undergo apoptosis. Apoptosis is a homeostatic process that keeps the cell population in tissues stable and happens naturally during development and aging. The IC_{50} value in HepG2 for REE was 222 µg/mL. These findings are in line with those of several researchers who found that compounds found in rhubarb, including emodin, rhein, stilbene, and aloe-emodin, can influence biological processes related to cancer through a variety of signaling pathways. These compounds also provide advantages over conventional cytotoxic drugs by lowering tumor drug resistance because of their multi-targeting capabilities. Emodin at a dose of 10 µM has been demonstrated to enhance the vulnerability of tumor cells to radiation and chemotherapy by impeding P-glycoprotein (P-gp) function and starting the mitochondrial apoptotic pathway in vitro **(Liu *et al*., 2012; Li *et al*., 2016; Cao *et al*., 2017)**.

Rhubarb plays a critical role in several stages of tumor progression by targeting distinct pathways. It efficiently suppresses tumor invasion and migration **(Chen *et al*., 2010; Zhou *et al*., 2017)**, inhibits tumor cell growth **(Liu *et al*., 2018)**, and prevents the creation of tumor neovascularization. Hepatocellular carcinoma is inhibited in its growth and multiplication by the anthraquinone chemicals found in rhubarb. According to a study conducted in vitro, emodin inhibited the growth of orthotopic tumors of

human hepatocellular carcinoma in male mice by regulating the activation of signal transducers and activators of transcription 3 in tumor tissues. This inhibition was observed in hepatoma and was dose- and time-dependent **(Subramaniam *et al*., 2013).** Additionally, by producing ROS, damaging DNA, and changing the ATP level, chrysophanol may cause hepatoma cells to necrotize **(Lu *et al*., 2010)**.

According to reports, rhein may prevent hepatoblastoma G2 (HepG2) cells from growing and proliferating by upregulating the production of p53 and p21/WAF1 proteins and obstructing the G1 phase of the cell cycle **(Kuo *et al*., 2004).** Emodin prevented HepG2 cells from growing, which disrupted ATP synthesis and significantly reduced mitochondrial membrane potential. This, in turn, caused the mitochondria's permeability transport pores to open, allowing calcium ions to exit and activating the caspase protein family, which in turn caused apoptosis **(Hsu *et al*., 2010)**. Aloe-emodin, a further bioactive component of rhubarb with anti-cancer properties, inhibited HepG2 and Hep3B cells' ability to proliferate by blocking the p21-dependent and p53-induced apoptotic pathways **(Yang *et al*., 2019)**. By up-regulating miR-370 and controlling the AMPK/Sp1/DNMT1 signaling pathway, Physcion caused HCC cells to undergo apoptosis **(Pan *et al*., 2016)**.

Table 6: Effect of rhubarb ethanolic extract (REE) on human hepatocarcinoma (HepG2) cell line

REE concentration(ug/ml)	HepG2 (ug/ml unit)
0.000	**1.000**
62.500	**0.796**
125.000	**0.686**
250.000	**0.440**
500.000	**0.371**

REE, rhubarb ethanolic extract. HepG2, human hepatocarcinoma.

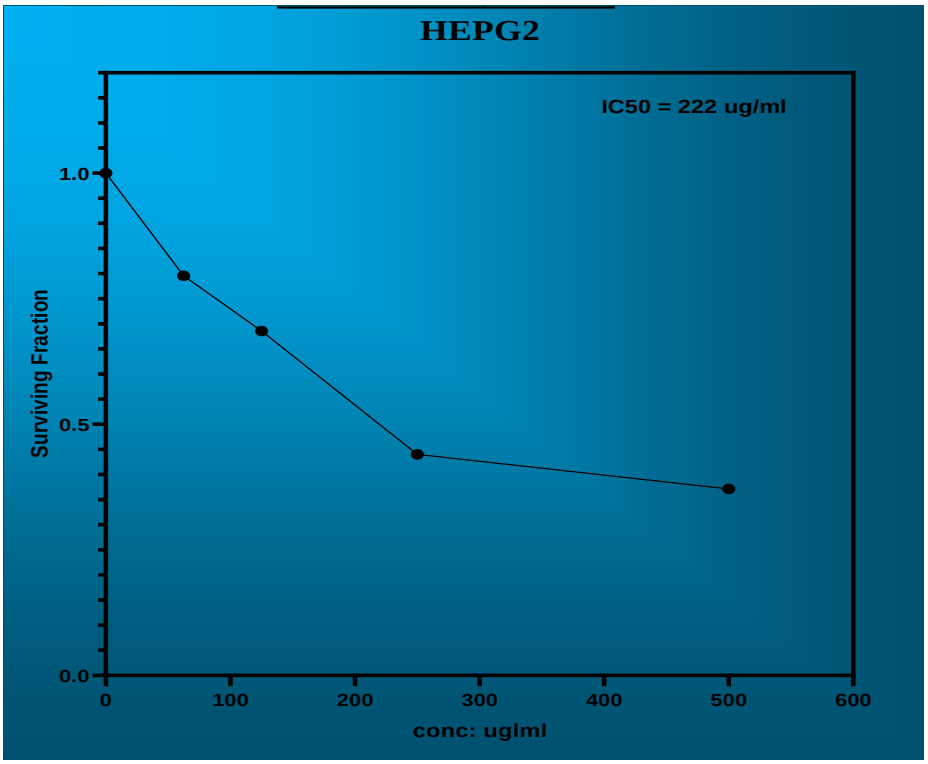

Figure 1: Effect of rhubarb ethanolic extract (REE) on human hepatocarcinoma (HepG2) cell line

4. Conclusion

Paracetamol toxicity produces an oxidative stress condition that leads to significant liver damage. REE had attenuated paracetamol-induced hepatotoxicity and exhibited ameliorative effects against oxidative stress, as well as restoring antioxidant status and biochemical alterations (some immunomodulation parameters, liver enzymes, and some renal functions) in paracetamol-intoxicated rats toward normal levels. In addition, the results indicated that REE had apoptotic action on human cancer liver cells, possibly as a result of the inherent anticancer, this is when studied on the human hepatocarcinoma (HepG2) cell line. Based on these results, it appears that REE offers a lot of promise for creating nutritious meals to combat toxicity and overuse of paracetamol. As well as providing a direction to isolate possible anti-cancer compounds in REE for hepatocellular carcinoma. In addition, reduce the dose of paracetamol. Pending more studies to discover the underlying mechanism, especially under clinical trials.

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