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FULL PAPER

Application of *Phyllanthus emblica* extract in manufacturing pharmaceutical composition for repairing liver damage

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Abstract

The objective of this study was to prepare a medicinal formulation with the extract of *Phyllanthus emblica* to repair liver damage. The hepatocyte cell line Hep-G2 was used in the experiment. The experimental sample was prepared by adding 20,000 hepatocytes to each well of a 24-well Seahorse XF24 analyzer special cell culture plate and cultured for 24 hrs. Subsequently, the culture medium from each well was removed. The hepatocytes in the wells were processed according to the conditions of the experimental, control, or comparator group. The oxygen consumption of the hepatocytes in the experimental, control, and comparator groups in the wells of the cell culture plate was measured using the Seahorse XF24 analyzer bioenergy meter. Treating cells with hydrogen peroxide can simulate the intracellular oxidation of free radicals, permitting examination of the mitochondrial activity of cells under oxidative stress. The mitochondrial activity in the hepatocytes was maintained. The mitochondria produced a sufficient amount of adenosine triphosphate (ATP), allowing the hepatocytes to maintain their normal metabolic functions. Owing to the improved synthesis efficiency and capability of triphosphate required for cell damage repair, damaged hepatocytes were able to obtain adequate energy for repair. Thus, liver repair was accelerated, and it returned to its normal condition.

1. Introduction

The liver is one of the major metabolic organs in the body. The liver's work involves metabolizing sugars, proteins with lipids, and decomposing, converting and removing toxins from the body. However, free radicals are also produced as a by-product in the process of liver work, especially in the decomposition, conversion and elimination of toxins in the body. Furthermore, for the liver that is damaged by the disease, the amount of free radicals produced when the liver is working is also increased in the case where the function of the liver is affected by the disease (Malhi and Gores, 2008; Lobo *et al.*, 2010; Guicciardi *et al*., 2013). For hepatocytes, the free radicals have a strong oxidative force, making the contact with free radicals of liver cells and hepatocytes within the organ is greatly increased the chance of damage caused by oxidation. In particular, mitochondria are oxidized and phosphorylated into hepatocytes and synthesize adenosine triphosphate (ATP) (Degli Esposti *et al.*, 2012; Bergman and Ben-Shachar, 2016). When the mitochondria are damaged by oxidative damage caused by large amounts of free radicals, the liver cells are unable to obtain sufficient energy from the self-active mitochondria. In this way, the low activity of mitochondria will work on the liver cells and have a serious negative impact, and thus the liver function has a negative impact (Degli Esposti *et al.*, 2012; Mullebner *et al.*, 2015). This study provided the use of Emblica extract for the preparation of a pharmaceutical composition for repairing liver injury, thereby enhancing the activity of granulocytes in hepatocytes of the liver, thereby maintaining the normal function of liver cells and repairing liver damage (Chularojmontri *et al.*, 2013; Zhao *et al.*, 2016).

2. Materials and methods

2.1 Emblica extracts treatment method

In this study, the extraction of Emblica extract, using carbon dioxide in the supercritical fluid extraction of

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Emblica fruit, or solvent extraction using methanol, ethanol, acetone, ethyl acetate, weight percentage 0.1 to 5% of sodium chloride aqueous solution, aqueous solution of potassium chloride, aqueous solution of calcium chloride, an aqueous solution of magnesium chloride or 0.1 to 5% by weight sodium chloride in ethanol solution, potassium chloride ethanol solution, calcium chloride ethanol solution, magnesium chloride ethanol solution Sub-fruit and to obtain raffinate. Then, the extract was purified by filtration to obtain the Emblica extract used in this study. The Emblica extract was subjected to a drying procedure using spray dry or vacuum drying to obtain an easily preserved Emblica extract powder.

The antioxidant effect of Emblica comes from low molecular weight hydrolysable tannins: Emblicanin A, Emblicanin B, Pedunculagin, and Punigluconin. As shown in Figure 1, the *Phyllanthus emblica* fruits structure of chemical components (Dasaroju and Gottumukkala, 2014).

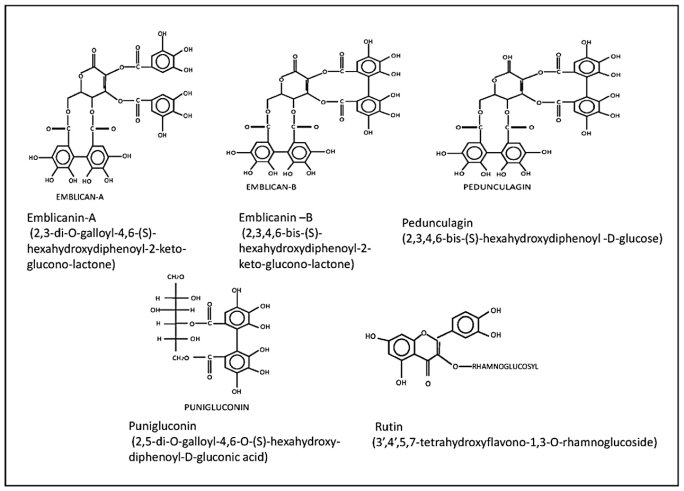

Figure 1. *Phyllanthus emblica* fruits: structure of chemical components

Emblica extract containing 35% to 55% by weight of Emblicanin-A with Emblicanin-B mixture, 4% to 15% by weight of Punigluconin, 10% to 20% by weight of Pedunculagin, 5% to 15% by weight Rutin with 10% to 30% by weight of Gallo-ellagitannoids.

2.2 Infrared absorption spectrum and HPLC do view Emblica extract

As shown in Figure 2, the infrared absorption spectrum of Emblica extract was 3403.6±5 cm - 1,2931.6±5 cm - 1, 1385.0±5 cm - 1,1318.6±5 cm - 1,1623.5±5 cm - 1,1451.3±5 cm - 1 1352.1±5 cm - 1,1218.4±5 cm - 1,1148.6±5 cm - 1,1035.7±5 cm - 1,3403.6±5 cm - 1 with characteristic absorption peak.

As shown in Figure 3, the HPLC chromatogram of the *Phyllanthus emblica* extract shows characteristic peaks at 1.620, 2.148, 3.265, and 4.370 mins.

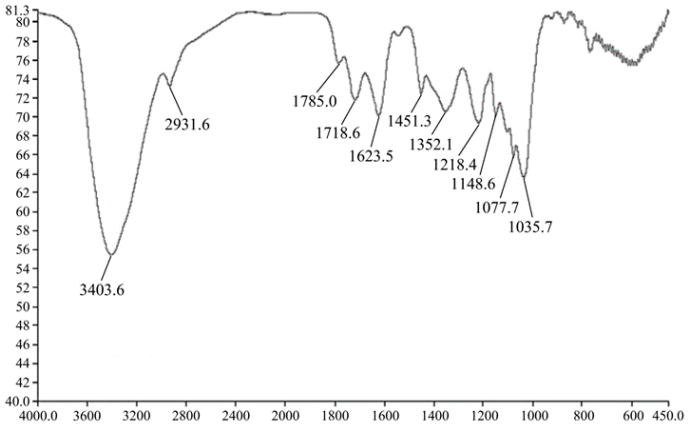

Figure 2. The infrared absorption spectrum of the *Phyllanthus emblica* extract used in Examples 1 to 3 of the present invention.

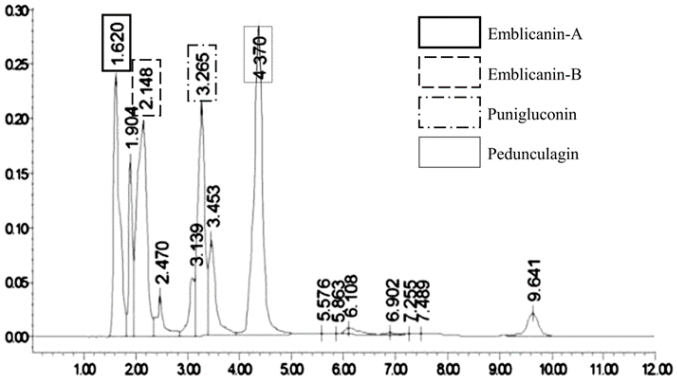

Figure 3. HPLC chromatogram of the *Phyllanthus emblica* extract shows characteristic peaks at 1.620, 2.148, 3.265, and 4.370 mins.

2.3 Experimental cell treatment

ADSC cultures from donors were used in the study. Informed consent, according to (IRB) from the human subcutaneous adipose tissue separation of ADSC. The cells used were hepatocytes (Hep-G2 cells) (Das *et al.*, 2012). The experimental sample preparation was carried out for 24 hrs after the implantation of 20,000 cells in each well of a 24-well orifice. Then, the culture medium in the wells was removed, and the hepatocytes in the wells were treated according to the conditions of the respective examples, the control examples and the comparative examples.

2.4 Preparation of experimental cell samples

During the experiment, when preparing experimental samples (Examples 1-3 in Table 1), an aqueous solution containing a predetermined concentration of *Phyllanthus emblica* extract was first added to the wells containing the hepatocytes and incubated for 24 hrs. Next, the aqueous solution was removed. A 1.5-mM aqueous solution of H_2O_2 was added, and the cells were incubated in this solution for 60 mins. Thereafter, the aqueous solution of H_2O_2 was removed, and the hepatocytes were washed with phosphate-buffered saline (PBS),

Table 1. Experimental parameters and experimental measurement results of Examples 1-3, Comparative Examples and Control Examples

	Phyllanthus emblica extract concentration	H_2O_2 aqueous solution concentration	Synthesis triphosphate line glycoside oxygen consumption	Overcoming the oxygen consumption of hydrogen ion leakage	The maximum oxygen consumption of mitochondria	The efficiency of mitochondrial ATP matchmaking	Mitochondrial pre-storage oxygen consumption capacity
	(μg/mL)	(Mm)	(Pmol/min/μg protein)	(Pmol/min/μg protein)	(Pmol/min/μg protein)	%	%
Example 1	200	1.5	6.3	1.9	16.5	77	205
Example 2	250	1.5	6.35	1.8	17	78	210
Example 3	500	1.5	6.6	1.95	18	79	215
Comparative Example	-	1.5	4.2	4.85	8	48	85
Control Example	-	-	7.1	1.6	16	82	180

completing the preparation of experimental cell samples (Examples 1-3 in Table 1).

2.5 Preparation of the test sample of a comparative example

When the control sample is prepared, the liver cells left in the wells in which the hepatocytes are removed are the experimental samples of the control examples. When the experimental sample of the comparative example was prepared, an aqueous solution of H_2O_2 having a concentration of 1.5 mM was first added to the pores to immerse the hepatocytes for 60 mins. Next, the aqueous H_2O_2 solution was removed and the cells were washed with phosphate-buffered saline (PBS), the preparation of the experimental samples of the comparative example was then completed.

2.6 The control concentration of the aqueous solution of Emblica extract

In Example 1, the concentration of the aqueous solution of Emblica extract was 200 (μg/mL). In Example 2, the concentration of the aqueous solution of Emblica extract was 250 (μg/mL). In Example 3, the concentration of the aqueous solution of Emblica extract was 500 (μg/mL).

2.7 Seahorse bioscience XF24 analyzer

The Seahorse XF24 analyzer was used to measure the oxygen consumption of the hepatocytes of Examples 1-3, the control example, and the comparative example (Table 1) in the wells. Treating the hepatocytes with H_2O_2 simulates free radical production due to intracellular oxidation, aiding the study of the mitochondrial activity of cells under oxidative stress. Thus, the hepatocytes of Examples 1-3 and the comparative example in Table 1 were treated with H_2O_2. Next, the oxygen consumption of the hepatocytes was measured using the Seahorse analyzer to determine the effect of the *Phyllanthus emblica* extract on mitochondrial activity in hepatocytes.

2.8 Seahorse bioscience XF24 analyzer measurement principle and process

First, the basal oxygen consumption of the cells in the well was determined. Next, an ATP synthase inhibitor was added to inhibit ATP synthesis by the mitochondria. At this time, the decrease in oxygen consumption is due to oxygen consumption by the mitochondria for the synthesis of ATP by oxidative phosphorylation, which is the basal respiration of the mitochondria. An example of an ATP synthase inhibitor is oligomycin. Next, an uncoupler was added at an appropriate concentration to allow the mitochondria to function in a limited, idle state without disrupting the electron transport chain in the mitochondrial inner membrane to evaluate the maximal respiration of the mitochondria. Finally, an electron transport chain inhibitor was added to completely stop oxygen consumption in the mitochondria to confirm the measured background value, which indicates non-mitochondrial respiration. An example of an electron transport chain inhibitor is a combination of rotenone and antimycin A.

2.9 Calculation of oxygen consumption of mitochondria

Basal mitochondrial respiration is equal to the basal respiration of the cell minus non-mitochondrial respiration. The basal respiration of mitochondria minus the amount of oxygen consumed from ATP synthesis is equal to the oxygen consumption needed to overcome proton leakage. The maximal respiration of the mitochondria minus the basal respiration of the mitochondria is equal to the spare respiratory capacity of the mitochondria. The coupling efficiency of the mitochondria is equal to the oxygen consumed from ATP synthesis divided by the basal respiration of the mitochondria.

3. Results and discussion

3.1 Comparative example and control example of oleic acid-induced hepatocyte free radical production

The experimental parameters and measurement results of Examples 1-3, the comparative example, and the control example (Table 1) are shown in Figures 4-8. The experimental measurement results of picomoles of oxygen consumed per minute per microgram of protein (pmol/min/μg protein) shown in Table 1 are standardized by cell mass.

3.2 The relationship between mitochondrial oxygen consumption and liver function

Figure 4 shows the schematic of oxygen consumption from ATP synthesis in Examples 1-3, the comparative example, and the control example in Table 1. Figure 5 shows the schematic of oxygen consumption from overcoming proton leakage in Examples 1-3, the comparative example, and the control example in Table 1. Figure 6 shows the schematic of maximal respiration in Examples 1-3, the comparative example, and the control example in Table 1. Figure 7 shows the schematic of coupling efficiency in Examples 1-3, the comparative example, and the control example in Table 1. Figure 8 shows the schematic of spare respiratory capacity in Examples 1-3, the comparative example, and the control example in Table 1. Figure 9 shows the schematic of the amount of free radical production induced in hepatocytes by oleic acid in Examples 1-3, the comparative example, and the control example in Table 1.

3.3 Comparing the amount of leakage of the inner mitochondrial membrane of hydrogen ions

As shown in Figure 4, the oxygen consumption from ATP synthesis in Examples 1-3 in Table 1 was higher than that of the comparative example. As shown in Figure 6, the maximal respiration of the mitochondria in Examples 1-3 in Table 1 was higher than that of the comparative example (Im *et al*., 2015).

As shown in Figure 7, the coupling efficiency of the mitochondria in Examples 1-3 in Table 1 was higher than that of the comparative example. As shown in Figure 8, the spare respiratory capacity of the mitochondria in Examples 1-3 in Table 1 was higher than that of the comparative example. Thus, Figures 4, 6,

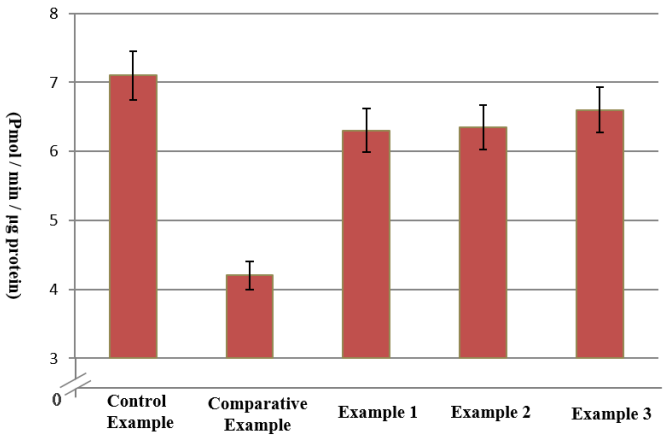

Figure 4. Shows the schematic of oxygen consumption from ATP synthesis in Examples 1-3, the comparative example, and the control example in Table 1.

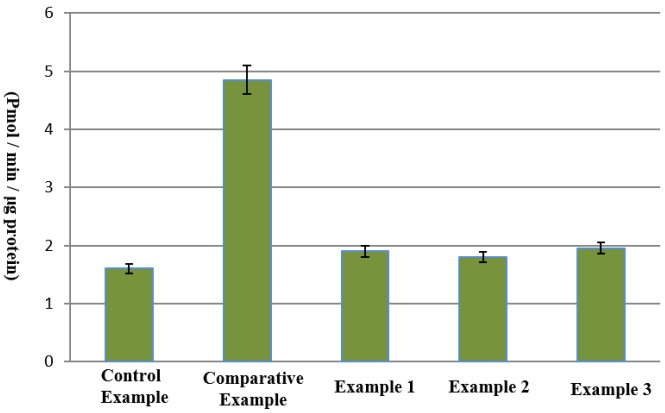

Figure 5. Shows the schematic of oxygen consumption from overcoming proton leakage in Examples 1-3, the comparative example, and the control example in Table 1.

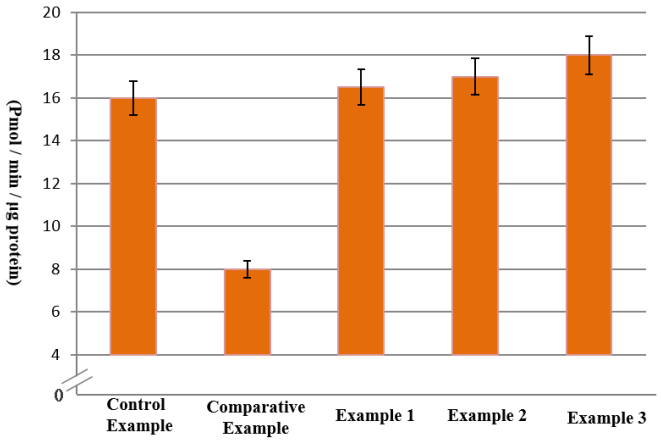

Figure 6. Shows the schematic of maximal respiration in Examples 1-3, the comparative example, and the control example in Table 1.

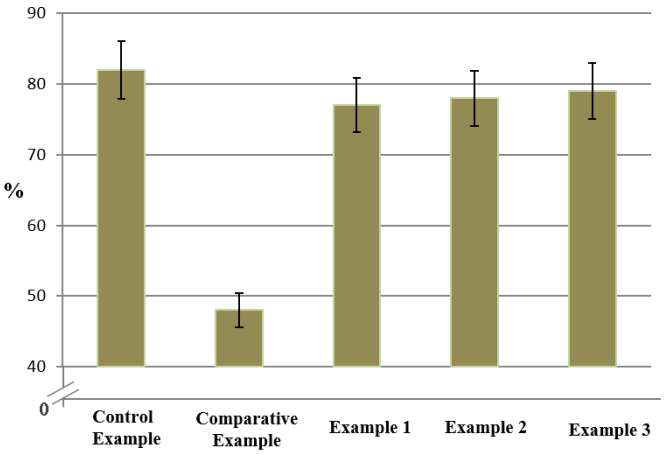

Figure 7. Shows the schematic of coupling efficiency in Examples 1-3, the comparative example, and the control example in Table 1.

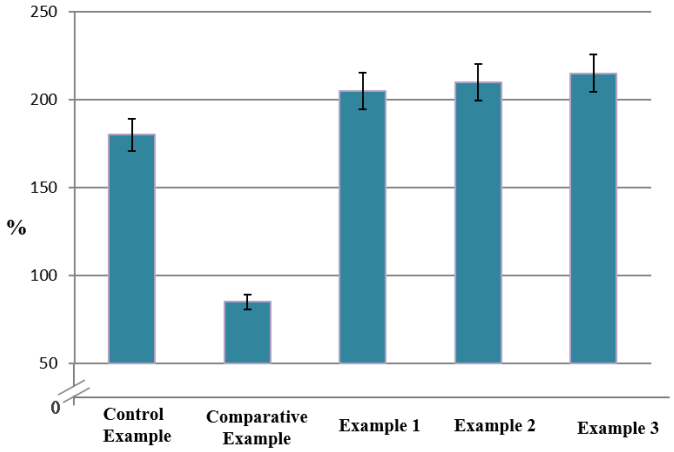

Figure 8. Shows the schematic of spare respiratory capacity in Examples 1-3, the comparative example, and the control example in Table 1.

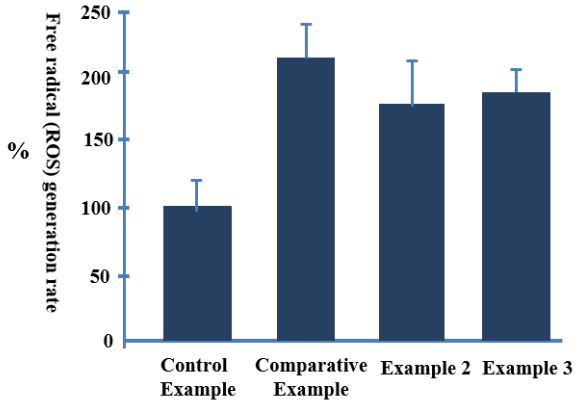

Figure 9. Shows the schematic of the amount of free radical production induced in hepatocytes by oleic acid in Examples 2 -3, the comparative example, and the control example in Table 1.

and 8 show that mitochondrial activity in the hepatocytes of Examples 1-3 in Table 1 improves after treatment with *Phyllanthus emblica* extract. Furthermore, the increased spare respiratory capacity of the mitochondria also represents an improved ability of the mitochondria and hepatocytes to respond to various cell stressors (Mullebner *et al*., 2015).

3.4 Enhance the ATP synthesis of mitochondria in hepatocytes exposed to free radicals to protect the liver

Hepatocytes treated with 200-500 µg/mL aqueous solutions of *Phyllanthus emblica* extract were protected from the destruction of the inner mitochondrial membrane by free radicals. Consequently, mitochondrial disintegration was delayed, thereby delaying hepatocyte apoptosis (Das *et al*., 2012).

Mitochondria perform oxidative phosphorylation and synthesize ATP. Accordingly, mitochondrial activity is maintained in hepatocytes, and the mitochondria in hepatocytes produce sufficient amounts of ATP for the hepatocytes to use, allowing the hepatocytes to maintain normal metabolism. By improving the efficiency and ability of ATP synthesis required for cell repair, the damaged hepatocytes obtain sufficient energy for repair, thereby accelerating liver repair to normal levels.

4. Discussion

During the normal liver function, especially during degradation, conversion, and elimination of toxins from the body, free radicals are also produced as by-products. However, in disease-induced liver injury, the amount of free radicals produced by the liver also increases when liver function is affected by the disease. Since free radicals have strong oxidizing power, the chances of oxidative damage to hepatocytes and their organelles that are exposed to free radicals are markedly increased (Widodo and Sismindari, 2020). In particular, the mitochondria are organelles responsible for oxidative phosphorylation and the synthesis of ATP in hepatocytes. When mitochondria experience oxidative damage caused by a large amount of free radicals, hepatocytes cannot obtain sufficient energy from mitochondria with decreased activity. Consequently, low mitochondrial activity has a markedly negative impact on hepatocyte function, leading to negative impacts on liver function as a whole. The *Phyllanthus emblica* extract used in the present study enhances the ability of mitochondria to perform oxidative phosphorylation and synthesize ATP in hepatocytes that have been exposed to free radicals. This leads to the maintenance of mitochondrial activity in the hepatocytes, and the mitochondria in hepatocytes can produce sufficient amounts of ATP for use by the hepatocytes, allowing them to maintain normal metabolism (Khopde *et al*., 2001).

Phyllanthus emblica is known for its high vitamin C (ascorbic acid) and polyphenol contents. To evaluate its antioxidant activity, Khopde *et al*. (2001) tested the ability of aqueous *Phyllanthus emblica* extracts to inhibit γ-radiation-induced lipid peroxidation (LPO) and superoxidase dismutase (SOD)-induced damage in rat liver mitochondria. For the LPO experiment, an aqueous solution of *Phyllanthus emblica* extract was used, and irradiation was performed at different time intervals. The extent of LPO was measured in terms of the levels of thiobarbituric acid reactive substances. The *Phyllanthus emblica* extract was found to serve as a suitable antioxidant against γ-radiation-induced LPO. The extract has also been found to inhibit damage caused by the antioxidant enzyme SOD (Storz and Imlayt, 1999).

Redox state represents an important context for many liver diseases, and it is involved in inflammatory, metabolic, and proliferative liver disease processes. Reactive oxygen species (ROS) are primarily produced

by cytochrome P450 enzymes in the endoplasmic reticulum of hepatocyte mitochondria. Under suitable conditions, cells employ specific molecular strategies for controlling oxidative stress and maintaining the balance between oxidant and antioxidant levels. Oxidative stress represents an imbalance between the levels of oxidants and antioxidants. Proteins, lipids, and DNA are cellular structures in hepatocytes that are affected by ROS and reactive nitrogen species. This process results in abnormal liver structure and function. Therefore, oxidative stress should be studied for the following reasons, it can help elucidate the mechanisms underlying the pathogenesis of various liver diseases, monitor oxidation marker levels in hepatocytes to help determine the extent of liver damage and ultimately allow the response to drug treatment to be observed (Cichoż-Lach and Michalak, 2014).

5. Conclusion

Chronic liver disease and cirrhosis are among the top ten causes of death in Taiwan. The mechanism underlying the development of liver disease has been extensively studied but remains poorly understood. The present study shows that a pharmacological compound containing *Phyllanthus emblica* extract can promote oxidative phosphorylation in the mitochondria of hepatocytes in contact with free radicals. By improving the efficiency and ability of ATP synthesis required for cell repair, damaged hepatocytes can be sufficiently repaired, thereby accelerating liver repair to normal levels.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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