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Original Article

Rutaecarpine Induces Apoptosis via a Mitochondrial Membrane-Mediated Pathway in the Human Liver Cancer Cells (HepG2)

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ABSTRACT

Background: The desire for novel drugs and bioactive ingredients to combat serious illnesses like cancer has grown dramatically. A great deal of research is being done to discover and create new medications. **Aim:** According to research, cirrhosis brought on by the Hepatitis C and B Viruses (HCV) is thought to be responsible for almost half of occurrences of Hepatocellular Carcinoma (HCC). These viruses alter the DNA of the liver cells they infect, turning healthy liver cells into cancerous ones. HCC is a clinical condition where the cancer typically commences in the liver. Human liver cancer HepG2 cells are treated with Rutaecarpine (RUT), a versatile medicinal plant derived from *Evodia rutaecarpa*. **Materials and Methods:** RUT was administered to HepG2 cell lines at different concentrations and the IC_{50} value was found. The cell line treated with RUT was then stained with the dual dye AO/EtBr to confirm apoptosis. In a similar manner, HepG2 control and RUT-treated cell lines underwent 24 hr of mitochondrial labeling and estimated to have loss in mitochondrial membrane potential which releases proteins that significantly contribute to apoptosis. **Results:** Rutaecarpine significantly reduces cell viability by 50% inhibiting cell growth, losing mitochondrial membrane potential and Increased expression of apoptotic indicators, such as caspase-3, caspase-8 and caspase-9, were evaluated in RUT treated liver cancer cells using ELISA. **Conclusion:** According to the study's findings, in HepG2, RUT induces apoptosis and via a mechanism mediated by the mitochondrial membrane.

Keywords: Rutaecarpine, Hepatocellular carcinoma, Apoptosis, Markers.

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INTRODUCTION

Liver cancer is expected to continue to be a major global health concern, impacting over a million people globally before 2025. More than 90% of instances of liver cancer are attributed to Hepatocellular Carcinoma (HCC), the most widespread kind of the disease. The primary risk factors for the development of HCC are infection with the hepatitis B and C viruses and non-alcoholic steatohepatitis linked to metabolic syndrome or diabetes mellitus is increasing in the West. Moreover, there are differences in the molecular pathophysiology of HCC associated with non-alcoholic steatohepatitis.[1] Hepatocellular carcinoma accounts for three-quarters of all liver cancer cases.[2]

The herb *Evodia rutaecarpa* is a traditional Chinese herb that contains the bioalkaloid Rutaecarpine (RUT), on tumor cells, it exhibits anti-proliferative properties.[3] According to the study, RUT inhibited the growth of several human malignancies. Moreover, it was discovered that RUT caused colorectal cancer cells to undergo apoptosis. Furthermore, RUT prevented colorectal cancer cells from growing and from metastasizing to the lungs *in vivo*.[4] Rutaecarpine injection suppresses the growth of prostate cancer cells in mice when loaded subcutaneously with TRAMP-C1 cells; the anti-cancer effects are associated with an *in vivo* Th1-polarized immune balance.[5]

Proteins that control mitochondrial morphology by altering mitochondrial fusion or fission play a role in the development of liver cancer resistance or vulnerability in animal models.[6] The bioenergetics, metabolic and signalling organelles known as mitochondria help cells recognize stress and react to the environment. Therefore, it is not unexpected that mitochondria have a crucial role in the development of cancer, since this process requires adaptation to alterations in the environment and in cells, as well as to cancer treatments. In addition to bioenergetics, a number of aspects of mitochondrial biology, such as metabolism, signaling, oxidative stress management, cell death susceptibility,

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fission and fusion dynamics, mitochondrial biogenesis and turnover, all play a role in transformation. Comprehending the mechanisms underlying mitochondrial activity throughout the carcinogenesis process will be essential for developing the next wave of cancer treatments.[7]

Mitochondria are key organelles responsible for several physiologic functions, including energy production, cellular metabolism, apoptosis and calcium and redox balance.[8] Consequently, the idea of "metabolic reprogramming" has been thoroughly researched. It has been shown throughout time that cancer cells adopt a "metabolic phenotype" that gives them an advantage in proliferating through increased aerobic glycolysis, significant alterations in specific TCA cycle enzymes and increased glucose uptake.[9] The macrostructures known as mitoribosomes are in charge of accelerating the synthesis of mitochondrial proteins. For the oxidative phosphorylation system's synthesis, mitochondria have their own ribosomes. As a result, control over mitoribosome biogenesis affects cell survival, proliferation and differentiation in close relation to mitochondrial respiration.[10]

HepG2 cells carry out a variety of specialized liver tasks. They are non-tumorigenic cells with high rates of proliferation and an epithelial-like appearance.[11] The liver cancer cell line HepG2 was found to be very susceptible to the cytotoxic effects of the ethanolic extract of *S. nigrum*.[12] HepG2 cells can be used to examine anticancer drug metabolism because phase I, II and III drug transport proteins are expressed equally in HB and HCC cells. Likewise, the cell line can be employed in research with CYP inducers because of the low basal activity of CYP proteins such as CYP1A2, CYP3A4 and CYP2B6.[13]

The primary goal of this research is to induce apoptosis in Human liver cancer cells (HepG2) via the mitochondrial membrane-mediated mechanism. Biochemical *in vitro* methods were used to examine Rutaecarpine's impact on apoptosis, cell proliferation, mitochondrial membrane potential and levels of apoptotic markers such as caspase 3, 8 and 9 in HepG2 cell lines, demonstrating its exceptional contribution to liver cancer treatment.

MATERIALS AND METHODS

Materials

Rutaecarpine and Rhodamine 123 were procured from Sigma-Aldrich (USA). Acridine orange and ethidium bromide procured from Thermo Fisher Scientific, USA. The investigations employed only analytical-grade chemicals and reagents.

Cell line and cell culture

The human cell line HepG2 was procured from ATCC, USA. It was grown in 25 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) with 1% penicillin/streptomycin antibiotics and 10% fetal bovine serum as a supplement. Cells were cultivated and incubated at room temperature in a humidified CO_2 incubator with 5% CO_2 and 95% air. Prior to subculture, the medium was replaced every 48-72 hr until confluence reached 80-90%.

Determination of Cell Proliferation by XTT Assay

HepG2 cells were seeded in triplicate in a 96-well plate with varied concentrations of RUT for 24 hr. The XTT assay was employed to measure cell proliferation. The media was withdrawn after 24 hr and the plates were examined using the XTT reagent. A microplate reader was used to measure the XTT-formazan derivative's absorption. Enzymes called dehydrogenase convert the yellow tetrazolium salt XTT into a formazan dye when the cell is alive. The amount of formazan generated varies with the number of active cells. At 450 nm, the absorbance was measured.[14]

Estimation of Apoptosis by Dual AO/EB Staining

RUT capability to induce apoptosis in human HepG2 cells was assessed using the AO/EtBr staining technique. After adding Rutaecarpine to the cells at IC_{50} dosage, the cells were left to incubate for 24 hr. Following incubation, they were stained using ethidium bromide and acridine orange in equal proportions. The stained cells were viewed under a fluorescence microscope.[15]

Determination of Mitochondrial Membrane Potential (MMP) with Rh-123

MMP assessments are critical for determining the commencement of the apoptotic process. To determine the change in MMP, rhodamine-123 (Rh-123), a cationic dye, is frequently utilized as an excellent fluorescence indicator.[16] Rh-123 dye was used to stain the mitochondria in order to detect changes in MMP. RUT was administered to the HepG2 cells at the IC_{50} value and incubated for 24 hr at 37°C with 5% CO_2 supplied.[17] Using a fluorescent microscope, the fluorescence of Rh-123 in the cells was determined.

Identification of apoptotic markers, like caspase 3, 8 and 9

The cell lysates of control and RUT treated cells were prepared. The concentrations of Caspase 3, 8 and 9 were determined in the samples using an ELISA kit. Statistical analysis was carried out with the representative data to evaluate the study.[15,18]

Statistical Analysis

The mean±SD of the findings is reported. To determine if there were any differences between the groups, a one-way ANOVA test was applied. The differences between the control and sample means were considered statistically significant if $p<0.05$.

RESULTS

Role of Rutaecarpine on Cell Proliferation

The study was conducted to identify the role of RUT on HepG2 cells at different concentrations from 3.13 to 200 µM. Experiments were performed in triplicates to assess if any variations are observed and to ascertain the test's specificity. The study's conclusions established IC_{50} value and the dose dependent inhibition of cell proliferation. The representative data revealed that RUT remarkably down-regulates cell viability at different concentrations resulting in 50% inhibition of the growth. In Figure 1, the percentage of cell viability decreases with increasing concentration. Further it is evident that the maximum effect was observed at 25 µM concentration of the drug and thus estimated to be the IC_{50} value.

Significance of Rutaecarpine on Apoptosis

The ability to induce apoptosis by RUT on HepG2 cancer cells were evaluated and exhibited through morphological alterations. AO/EtBr staining is used to make distinctions between healthy and apoptotic cells. Where, the red fluorescent dye ethidium bromide only selectively marks the broken nuclei of apoptotic cells, the green fluorescent dye acridine orange only reaches healthy cells. As seen in Figure 2, the control cells displayed green fluorescence, whereas the RUT-treated cells displayed orange fluorescence, signifying apoptotic cells. However, the appearance of red-stained cells indicates an early stage of apoptosis. Thus, the fluorescent microscopic image shows no discernible apoptosis in the control group, but apoptosis was found in the RUT-treated cells.

Effect of Rutaecarpine on Mitochondrial membrane potential of HepG2 cancer cells

Mitochondria are necessary for life, owing to their involvement in important metabolic processes such as ATP production. Mitochondrial membrane potential is becoming increasingly crucial in sustaining mitochondrial activity and preventing hepatocyte apoptosis. An indication of bioenergetic stress, the loss of mitochondrial membrane potential can trigger the release of proteins that cause apoptosis, which is one of the first steps in the cascade of apoptosis and leads to cell death. MMP loss is measured using rhodamine-123. The cells were administered a 25 µM dose of RUT treatment and compared to untreated control cells. Using a fluorescent microscope, the fluorescence intensities of cells treated with Rutaecarpine and that of control showing the liver cancer cell's MMP patterns were investigated. Figure 3 demonstrates that the control cells fluoresced bright green, the rutaecarpine-treated cells fluoresced dull green, indicating a drop in MMP.

Rutaecarpine inhibits (HepG2) cancer cells and induces apoptosis

Proteases called caspases trigger and carry out programmed cell death. Using the ELISA technique, caspase 9, 8 and 3 levels were assessed after treating HepG2 cells with 25 µM RUT in order to determine the role of the apoptotic gene in the process of cell death. We performed three independent consecutive tests. The increased expression of Caspase 3, 8 and 9 in RUT-treated cells as compared to control is seen in Figure 4. Data is reported as mean±standard deviation and analyzed using One-way ANOVA.

DISCUSSION

Hepatoxicity can be caused by viral infections, high levels of alcohol, medicines, environmental toxins and other conditions. Hepatic toxicity can result in cirrhosis, hepatic cancer, hepatitis and hepatic fibrosis. It is a prevalent pathological characteristic of many liver illnesses.[19] The induction of programmed cell death is a significant and crucial process in treatment choices for cancer cells.[15] Apoptosis, frequently referred to as Programmed Cell Death (PCD), is a necessary step for proper development, the prevention of cancer, aging generally and neurological conditions like Parkinson's, Alzheimer's and amyotrophic lateral sclerosis.

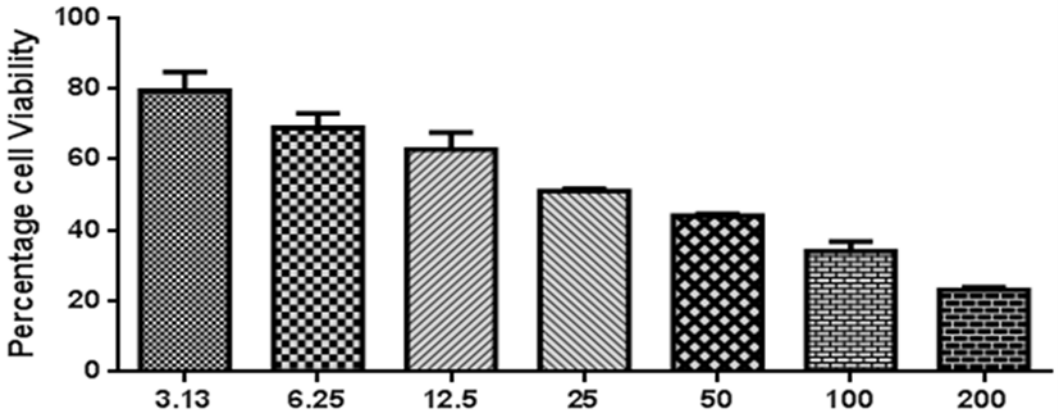

Figure 1: Assessment of HepG2 Cell viability at Various Rutaecarpine Doses (3.13-200 µM). Experiments were performed in triplicate to determine the IC50 value and representative data are shown here for the dose resulting in 50% inhibition of growth.

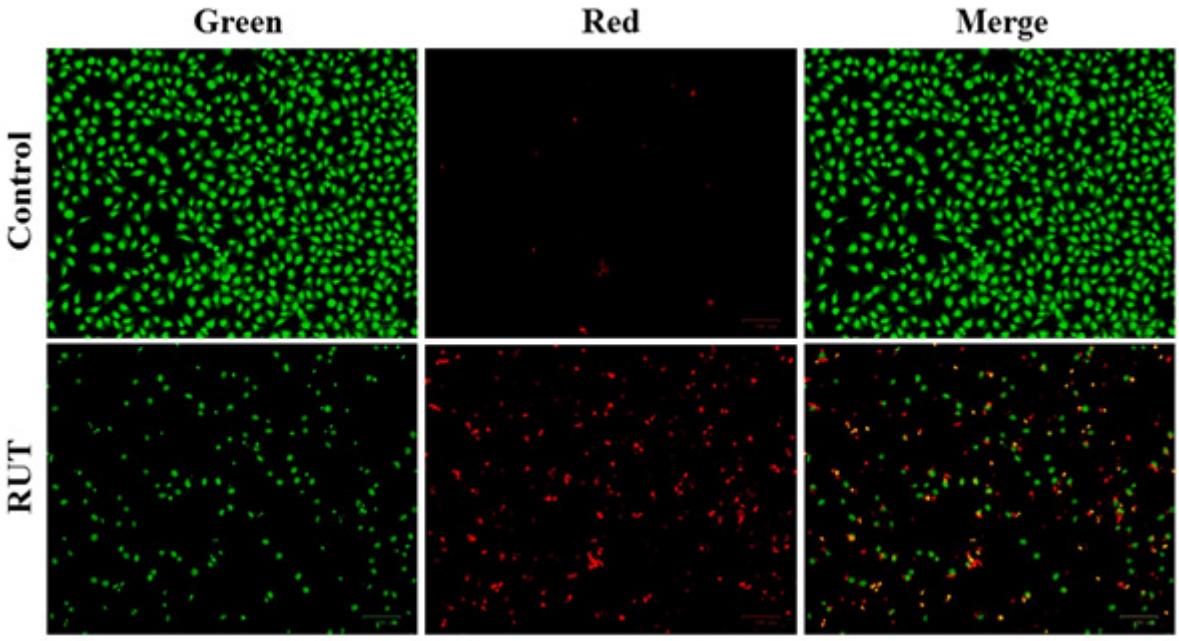

Figure 2: Influence of Rutaecarpine-Induced Apoptosis on HepG2 Cell Line via AO/EtBr Staining approach. The fluorescent microscopic images of HepG2 cells incubated for 24 hr with control exhibit bright green, while Rutaecarpine at IC_{50} displays dull green with orange and red stained cells. Values are expressed as mean±SD of three experiments.

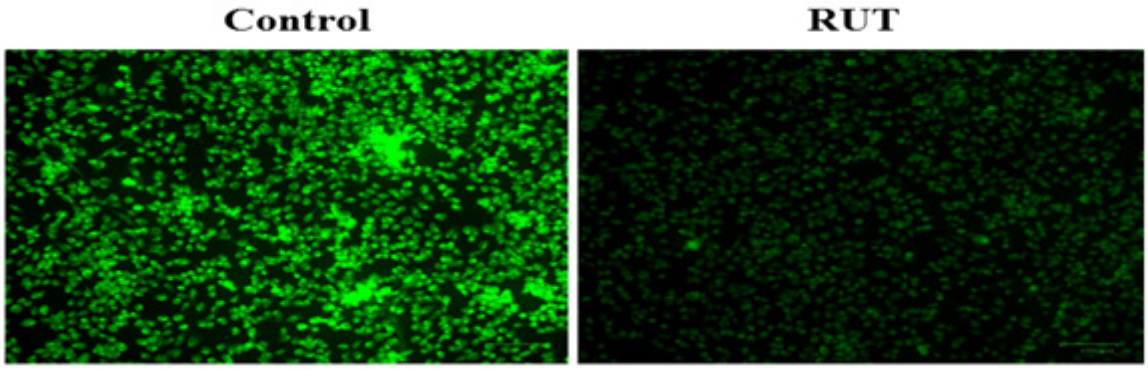

Figure 3: Mitochondrial Membrane Potential Analysis in Rutaecarpine treated HepG2 Cells incubated for 24 hr by mitochondrial Staining with Rh-123. The MMP patterns evaluated by a fluorescent microscope of control cells show vivid green, but Rutaecarpine treated HepG2 cells show dull green, indicating a decrease in MMP. This is a representative image of the experiment performed in triplicate with magnification at 20X.

There are two basic ways to cause apoptosis: either the death receptors (the extrinsic pathway) or the mitochondria (the intrinsic pathway). Although caspase-independent forms of apoptosis have been reported, both pathways converge to promote the activation of caspases, the final arbiters of cell death.[20] To learn more about the compound's effects, this research has examined the pathway mediated by the mitochondrial membrane.

With few available treatment options, a dismal prognosis and high rates of mortality and morbidity, HCC tends to be an aggressive cancer. Chemotherapy is among the most effective forms of treatment for HCC, but patients find it difficult to bear its harsh side effects. Consequently, the creation of innovative and potent medications that are both more effective and have fewer severe side effects is necessary.[21] In this investigation, we assessed Rutaecarpine's potential to treat hepatocarcinoma in HepG2 cells. RUT possesses a number of characteristics, including analgesic, anti-inflammatory, anti-tumor, anti-depressant, anti-obesity properties. Recent research has demonstrated that RUT clearly functions as an inhibitor in a variety of cancer pathways. For instance, RUT inhibits the cancer cell's proliferation in prostate cancer by controlling the immunological balance by T helper type 1 (Th1)-polarized. RUT inhibits the growth of ovarian cancer cells as well. Their research revealed that RUT halted the growth of HepG2 cells, brought about apoptosis, elevated the expression of caspase and hastened the cell-cycle arrest. Nonetheless, further research needs to be done on RUT's regulation mechanism.[22]

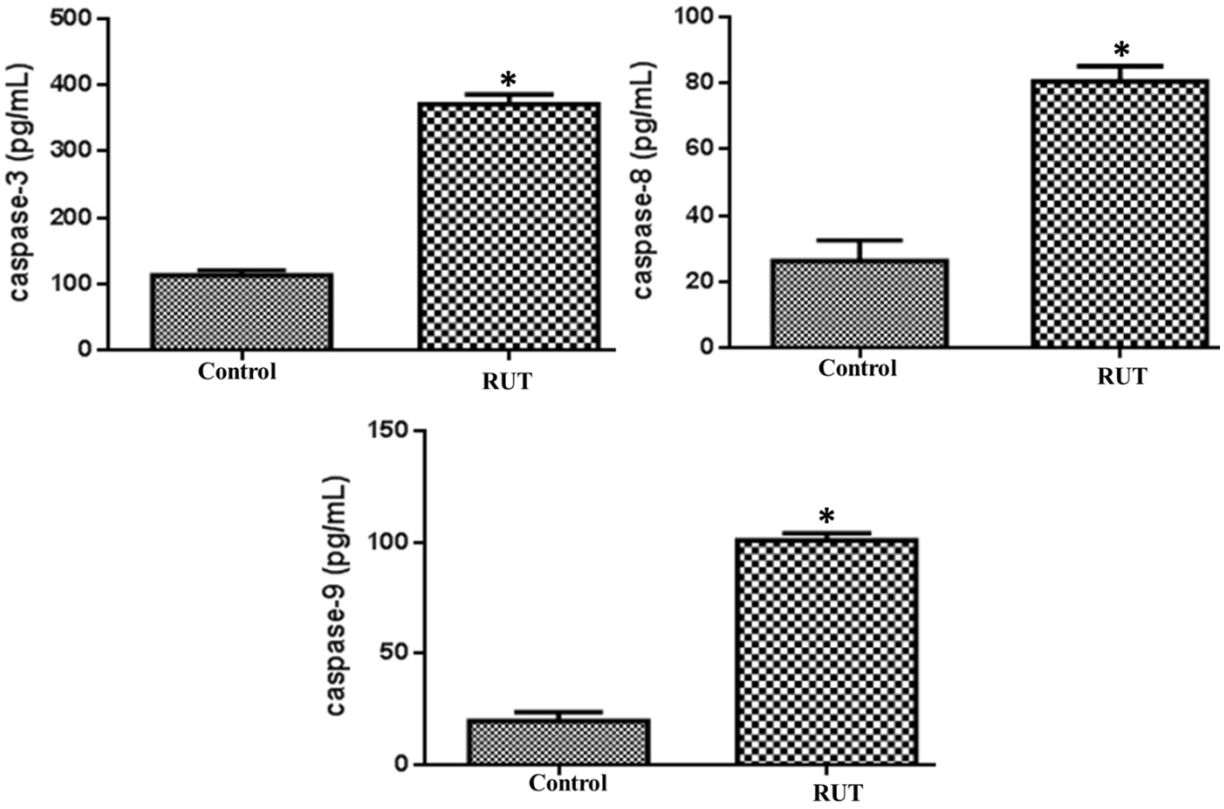

Figure 4: Expression of Caspase-3, 8 and 9 in 25 µM Rutaecarpine treated HepG2 cells analysed by ELISA. Triplicate tests were carried out. '*' indicates $p<0.05$, which is deemed statistically significant. The data was analyzed using One-way ANOVA and presented as mean±standard deviation.

RUT showed reduced cytotoxicity at the same dose as the clinical chemotherapeutic medication 5-fluorouracil, according to earlier research. RUT, on the other hand, dramatically slowed down the HCT116 and SW480 CRC cell lines' growth and multiplication *in vitro*. Additionally, the study showed a significant decrease in the CRC cells' ability to migrate and penetrate following treatment with RUT.[4] Nonetheless, our analysis included one research study that was responsible for half of the growth inhibition. When tested against HepG2 cancer cell lines, it has an IC_{50} value of 25 µM. The aggregate evidence from previous investigations supports the alkaloid's nature, which lowers the proliferation of liver cancer cells while also controlling relevant signaling pathways to promote apoptosis.[23]

Employing AO-EtBr staining, investigations and comprehend morphological alterations were brought about by RUT on the HepG2 cell line. When coupled to DNA, AO fluoresces green and it can enter both early apoptotic and normal cells with intact membranes. EB only entered cells with broken membranes, such as late apoptotic and dead cells and fluoresced orange-red when attached to concentrated DNA fragments or apoptotic entities. Moreover, slight DNA damage can be detected with combined AO/EB staining. Thus, evaluations were conducted on early-apoptotic, normal and late-apoptotic cells.[24] Chemotherapeutic medications are known to directly target DNA in order to kill cancer cells. The process is associated with many features such as membrane blebbing, chromatin condensation and nuclear disintegration. Apoptotic bodies are created when the cells go through these stages and macrophages subsequently consume them. Overproduction of ROS causes the aforementioned alterations in the cells, which ultimately result in apoptosis. This has been shown in the RUT-treated cells and the AO/EtBr staining, which shows the apoptotic characteristics previously indicated, confirms this.[15]

One of the most important metrics for evaluating cellular energy metabolism in both healthy and pathological circumstances is the Mitochondrial Membrane Potential (MMP).[25] Increased Rh123 fluorescence suggests that ATP may cause mitochondrial membrane depolarization. By comparing the ATP-induced mitochondrial membrane depolarization of cells under different treatments, it is possible to ascertain the scientific significance of the modification in mitochondria as well as the degree and stage of apoptosis.[16] Originally, it was thought that changes in MMP were obligatory, early steps in the apoptotic signaling pathway. Numerous research avenues indicate that, in certain regimes of apoptosis induction, alterations in mitochondrial structure and MMP occur prior to the nuclear characteristics of apoptosis.[26] According to our research; there has been a drop in MMP, which eventually serves as a signpost for the triggering of apoptosis.

A class of cysteine proteases known as caspases controls the apoptotic reaction.[27,28] Caspases-3, an executioner and caspases-8 and 9, initiators of mitochondrial-mediated apoptosis, are the main members of the caspase family.[15] The main apoptotic proteins, including caspase 3, caspase 8 and caspase 9, were measured for expression using the ELISA method. The results showed that the HepG2 cells treated with RUT had higher expression levels of these caspases, indicating the occurrence of apoptosis in the cells. According to earlier research, RUT has an anti-apoptotic role. Apoptosis was detected in a number of markers, which helped to clarify the method by which RUT causes apoptosis. The outcomes demonstrated that following RUT treatment in colon cancer cells, the expression levels of cleaved Caspase-3, 8 and 9 increased. This result is consistent with the mechanism that has been published, according to which RUT can up-regulate the expression of Bax and Caspase-3, downregulate the expression of Bcl-2 and activate Caspase-3, all of which help to increase the apoptosis of the gastric cancer cell SGC 7901. This finding implies that RUT functions differently in various cell types or in various illnesses. But additional research is required. Collectively, we show that apoptosis is the mechanism by which RUT inhibits tumor growth.[4] As a result of the current research, it can be concluded that RUT effectively inhibits the growth of HepG2 hepatocellular cancer cells. It also demonstrated how this effect is mediated by apoptosis induction and the loss of mitochondrial membrane potential in cancer cells. RUT may therefore be further research will be required in the future studies are warranted to understand the conducting *in vivo* studies and cellular mechanisms responsible for its HCC cancer protective effects.

CONCLUSION

Hepatocellular Carcinoma (HCC) is prevalent due to a dismal prognosis and lack of efficient therapies. The ongoing search for novel therapeutic approaches underscores the significance of discovering plant-based compounds with potential efficacy in the treatment of cancer. RUT, an indolopyridoquinazolinone alkaloid extracted from the unripe fruit of *Evodia rutaecarpa*, was the focus of the study investigating its anti-cancer traits. According to our findings, Rutaecarpine significantly reduces cell viability by 50% inhibiting cell growth, losing mitochondrial membrane potential and significantly triggering apoptosis through elevated concentrations of caspase 3, 8 and 9. These findings supported the theory that RUT, which induces apoptosis and mitochondrial-mediated mechanism, may be a promising anticancer agent for liver cancer. However, further research will be needed in the future to establish additional evidence of Rutaecarpine's effects on Liver Cancer, revealing Rutaecarpine as a potent anti-cancer medicine for effective management of liver cancer and a less harmful method of treatment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

HCC: Hepatocellular carcinoma; **RUT:** Rutaecarpine; **AO:** Acridine Orange; **EB:** Ethylene Bromide; **IC_{50}:** Half maximal inhibitory concentration; **PI:** Propidium Iodide; **ELISA:** Enzyme-linked immunosorbent assay; **Th1:** T helper type 1; **TCA:** Tricarboxylic Acid Cycle; **HB:** Hepatoblastoma; **CYP:** Cytochrome P450; **CYP1A2:** Cytochrome P450 1A2; **CYP3A4:** Cytochrome P450 3A4 ; **CYP2B6:** Cytochrome P450 2B6; **XTT:** 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetraz olium-5-Carboxanilide; **MMP:** Mitochondrial Membrane Potential; **Rh-123:** Rhodamine-123; **CO_2:** Carbon dioxide; **SD:** Standard Deviation; **ANOVA:** Analysis of Variance; **ATP:** Adenosine triphosphate; **PCD:** Programmed cell death; **CRC:** Colorectal Cancer; **DNA:** Deoxyribonucleic acid; **ROS:** Reactive oxygen species; **Bax:** Bcl-2 Associated X-protein; **Bcl-2:** B-cell lymphoma 2.

SUMMARY

Through a mitochondrial-mediated mechanism, Rutaecarpine was shown in this research to trigger apoptosis in HepG2 cells. In liver cancer cells, Rutaecarpine inhibited growth by 50% and decreased cell proliferation. Rutaecarpine decreased MMP, indicating an apoptotic response Additionally, ELISA showed that Rutaecarpine enhanced the activity of caspase-3, 8 and 9 in HepG2 cells, suggesting that a signaling pathway that is dependent on mitochondria was responsible for inducing death in liver cancer cells.

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