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Research Article

Unveiling the Anti-cancer Efficiency of Chebulagic Acid-Mediated Apoptotic Mechanisms in HepG2 Cell Line

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Abstract

Background and Objective: Hepatocellular carcinoma (HCC) is a predominant type of Cancer in the liver than other cancers. World Health Organization predicted that the maximum mortality rate was caused by HCC (6th leading cause of death) and also by 2030, 1 million affected patients will die due to HCC. The molecular mechanism and treatment with plant-derived remedies/drugs giving better efficacy/resistance against HCC causing genes and its induced apoptosis. In the present (*in vitro*) study was designed to check the antitumor activity of the Chebulagic acid on HepG2 cells. **Materials and Methods:** *Terminalia chebular* derived Chebulagic acid anticancer activity on HepG2 cells was assessed by MTT assay, followed by DCFH-DA, Rhodamine123, acridine orange, Acridine orange/Ethidium bromide and DAPI fluorescent staining was done to check the apoptosis which was induced by Chebulagic acid on HepG2 cells. **Results:** The MTT assay revealed that Chebulagic acid treatment significantly inhibited cell proliferation and controlling ROS. Simultaneously, it shows a better effect on cells were pre-treatment with Chebulagic acid with dose and time-dependent based. Increased apoptotic cells (%) were found in mitochondrial membrane-associated and it's potential by Chebulagic acid on HepG2 cells. **Conclusion:** Overall findings of the study proposed that Chebulagic acid able to induce apoptosis HCC in human HepG2 cells by its apoptotic mechanisms.

Key words: Chebulagic acid, HepG2 cells, apoptosis, ROS

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The patients who had prolonged liver diseases and they become non-defensive against liver inflammation and its associated long-term disorders. However, such inflammation caused cirrhosis and favour for series of genetic and epigenetic culmination, which leads to a mutation in hepatic cells. Hence, there will be molecular amendments in dysplastic cells with rapid proliferation and survival encounters that turn into puffed called 'Hepatocellular carcinoma[1]. As per the recorded mortality rate worldwide, this Hepatocellular carcinoma (HCC) places in a 3rd position than other malignancies and nearly 600,000 people were get exposed are reported[2]. In HCC, in all types of malignancies, apoptosis is a significant issue and damages controlled proliferation and becomes dangerous to our health. In the case of normal conditions, apoptosis is mutual machinery called programmed cell death. The unwanted or expired cells were killed, then regeneration of those cells will occur[3]. Although apoptosis helps to inhibit indigenous inflammatory responses and its associated tissue damage was controlled by conserved plasma membrane integrity[4]. Besides, apoptosis prevents the anti-neoplasm movement by inhibiting attraction against modulation. Numerous reports have confirmed that apoptosis introduces the cells and serves as a lead role in treating Cancer. Also, cascade and caspases enzymes equally cover intrinsic and extrinsic pathways[5,6].

After apoptosis, oxidative stress of free radicals' product of reactive oxygen species also plays a significant role in the developing HCC of chronic liver disorders. In humans, the hepatic is an essential metabolic organ and serves as a reservoir of inner mitochondria. It helps to controls the production of ROS; in the meantime, balanced Antioxidants: ROS controls and maintains the homeostasis condition of the pathophysiology of liver cells[7]. Numerous studies reveal that enhanced ROS secretion creates an imbalance in the hepatic system and leads to chronic liver diseases of hepatitis B and C; However, some studies reported that this pathological condition could be a reason for developing HCC by enhanced oxidative stress created by hepatitis C[8,9]. Some degree of HCC was stimulated by oxidative stress, which was possibly driven via HBV-related dysfunction. Also, oxidative stress increases the secretion of ROS and acts against pathological redox mechanisms. Although there will be a relationship between mitochondrial dysfunction and it may lead to hepatic inflammation subsequently causes hepatocarcinogenesis[10]. Over two decades, researchers are trying to discover effective and advanced treatments like chemotherapy methods against this HCC. However, some of the anti HCC drugs caused significant contrary effects and that makes restrict to therapeutic uses in the health care center[11,12]. Meanwhile, this unconditional treatment makes us urge to discover or identify novel therapeutic compounds from naturally available sources with fewer side effects that will help to act against HCC[13].

Chebulagic acid is an isolated compound of the *Terminalia chebula* Retzius family plant that originated in Asia and Southeast Asian countries, noticeably in China, Nepal and India[14]. The dried ripen fruit of this plant is used as a pharmaceutical agent for a longer time by many countries worldwide. Apart from ripening fruit, the other parts of Terminalia plant families are used for numerous pharmaceuticals and therapeutics in treating various diseases against Cancer, cardiovascular disease, hypertension. Along with used as anti-inflammatory, antioxidant, antimicrobial and anti-viral functions[15-17]. Few studies reveal that this plant is used to treat sperm DNA damage and used as a cryopreservative agent too[18]. Also, it has been stated that Chebulagic acid suppressed HCC growth was done by convinced apoptosis and its associated autophagy mechanism[19]. Though, the role of Chebulagic acid on apoptosis in controlling HCC and its related pathophysiology accomplishment is still unknown. Hence, the present study was attempted to find the anti-HCC effect of Chebulagic acid-induced Apoptosis on HepG2 cell lines.

MATERIALS AND METHODS

Chemicals and reagents: All the plastic material was procured from Falcon labware (Becton Dickinson and Company, USA). Roswell Park Memorial Institute medium (RPMI-1640), Minimum essential medium (MEM), Phosphate buffered saline, BSA, trypsin, EDTA, antibiotics (streptomycin and penicillin) were purchased from Gibco-BRL (Grand Island, USA). The chemicals used in this study, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Butylated hydroxytoluene (BHT), were purchased from Sigma-Aldrich and Merck. For staining analysis, 2', 7'-dichlorine fluorescein diacetate (DCFH-DA) fluorescence probe, MTT cell proliferation assay kits, Propidium Iodide (PI), Hoechst 33342 staining reagent, Rhodamine 123 mitochondrial membrane potential kit, dimethyl sulfoxide (DMSO) was purchased from Nanjing Keygen Development Co. Ltd., China.

ABTS and DPPH radical scavenging activity of chebulagic acid: The antioxidants and free radical scavenging ability of Chebulagic acid were determined by ABTS and DPPH assay.

For ABTS assay, the procedure followed the method of Arnao *et al.*[20]. The ABTS scavenging capacity of the Chebulagic acid was compared with that of BHT and percentage inhibition calculated as:

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where, $\text{Abs}_{\text{control}}$ is the absorbance of ABTS radical in methanol, $\text{Abs}_{\text{sample}}$ is the absorbance of ABTS radical solution mixed with sample extract/standard. All determinations were performed in triplicate (n = 3). Similarly, 1, 1-Diphenyl-2-picryl-hydroxyl (DPPH) assay was used to confirm its radical scavenging activity with slight modifications from earlier reports[21]. DPPH as blank and butylated hydroxytoluene (BHT) is used as a standard positive control. The following formula determined the ability of Chebulagic acid in the DPPH scavenging function:

$$\text{DPPH scavenging (\%)} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \text{ x } 100$$

Selection of cell culture and its preparation: The human embryonic cell line of HEK293T and hepatoma cell line of HepG2 cell line was purchased from ADCC, USA. HEK293T cells were grown in Roswell Park Memorial Institute medium (RPMI). HepG2 cell was gown in Minimum essential medium (MEM) added with nutrition enriched 10% (v/v) heat- controlled FBS (fetal bovine serum) along adequate amount of antibiotics (100 U mL^{-1} of penicillin and 100 µg m^{-1} of streptomycin) were all used to improve the HepG2 cell lines and under preserved at 37°C in a CO_2 contained humidified incubator for humidity and nourishment of the live condition and further assessments.

Growth inhibition assay: With a density of 1×10^4 ratio, the HEK293T and HepG2 cells were taken and planted into 96-well culture dishes and allowed to reach full confluency[22]. Then, the cells were treated with two-fold concentrations of Chebulagic acid (6.25-100 µg mL^{-1}) for 24 hrs incubation. After the incubation period, 20 µL of MTT reagent (5 mg mL^{-1}) was added to the culture plate and kept at a 5% CO_2-limited humidified incubator for 37°C for 4 hrs. After 4 hrs, the culture dish was taken away and the crystals of formazan were dissolved in 150 µL of dimethyl sulfoxide. Immediately the plates were kept under the UV-microplate reader and measured at 570 nm. With the help of Sigma Plot 6.0 software was used to calculate the IC25, IC50 and IC75 concentration Chebulagic acid-treated HepG2 cell lines and the viability in percentage was measured by the following Eq:

$$\text{Cell viability (\%)} = 1 - \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}}$$

Screening of enzymatic antioxidants and lipid peroxidation: The oxidative stress-related markers of thiobarbituric acid-reactive species (TBARs) and antioxidants enzyme status were screened in HepG2cells. Briefly, the cells were treated with IC25, IC50 and IC75 concentrations of Chebulagic acid for 24 hrs incubation. After that Trypsin/EDTA solution was used to detach adherent cells, centrifuged at 3000 rpm for 10 min to collect the pellet. Then the pellet was resuspended in 500 µL of deionized water. It was used for biochemical analysis. Lipid peroxide level was monitored by using TBARS assay, according to the procedure Buege and Aust[23] with some modifications which standardized in our laboratory. At long last, the reading was recorded at 560 nm with the assistance of an ELISA reader. Also, enzymatic antioxidants activity of Superoxide dismutase (SOD) assay was performed and followed by standard protocol Marklund and Marklund[24]. The main principle of this method is that the SOD enzyme decays the superoxide ion into hydrogen peroxide in pyrogallol, which serves in an auto-oxidation reaction. One unit of SOD activity defined as an enzyme is required to cause 50% inhibition of pyrogallol autoxidation. SOD activity was expressed (U mL^{-1}). Catalase (CAT) assay was performed with the standardized protocol of Aebi (1984). The reduction in the removal of 1 hydrogen peroxide ion H_2O_2/unit time was measured as catalase activity, expressed (U mL^{-1})[25]. The method of Lawrence and Burk[26] was adopted to measure the Glutathione peroxidase (GPx) content. The reaction mixture consists of PBS, EDTA, Glutathione, Glutathione reductase, NADPH and NaN_3. The amount of GPx was measured in nanomoles per milligram of protein.

Mitochondrial membrane potential by rhodamine staining: Lipophilic natured cationic dye of Rhodamine-123 staining was used to measure the Mitochondrial membrane potential (∇ΨM) of Chebulagic acid-induced HepG2 cell lines with slight modification for our laboratory conditions. The ratio of 1×10^6 cells/well of HepG2 cells was taken into 6-well plates and frozen with Chebulagic acid with the different dosages for 24 hrs. After overnight frozen, the cells were treated with Rhodamine-123 dye and incubated for 30 min. The mitochondrial membrane potential of ∇ΨM was qualitatively

measured with the help of the cell imaging technique. Subsequently, the same cells were treated with trypsin EDTA digestion and measured the fluorescence intensity at 485/530 nm.

Estimation of intracellular ROS by DCFH-DA: DCHF-DA, a non-fluorescent, was used to calculate the intracellular ROS induced by Chebulagic acid-treated HepG2 cell lines. Its non-fluorescent nature can be easily diffused into cell membranes, reflected in intracellular ROS concentrations. If the cells are not affected, low intracellular ROS levels are observed; if the observed level is high, the cells are affected by Chebulagic acid treatment and promote more elevated ROS in intracellular membranes. Hence, the Fluorescence absorption was directly proportional to the higher generation of ROS. The primary step of this estimation, 1×10^6 cells/well of HepG2 cells, was taken into 6-well plates and treated with IC25, IC50 and IC75 concentration of Chebulagic acid and kept 24 hrs. After overnight incubation, the cells were added with 100 µL of DCFH-DA and kept at room temperature for 10 min. Subsequently, the plate was captured by a fluorescence microscope at 485-495 nm.

Determination apoptosis by AO/EtBr staining: Acridine orange and ethidium bromide (AO/EtBr) stains were used to identify the apoptotic changes in HepG2 cell lines. In brief, the harvested HepG2 cell lines were transferred into a 6-well plate for 24 hrs. The cells were soaked into a methanol: glacial acetic acid (3:1 ratio) mixture for half-hour and kept at 4°C. After, the cell was treated twice with PBS wash buffer, then finally stained with AO/EtBr with a 1:1 ratio and it was kept at room temperature for dryness for half-hour. The stained cell lines are again washed with PBS, wash buffer and observed under a fluorescence microscope.

Determination of nuclear damage by acridine orange and DAPI staining: AO and DAPI staining assessed apoptotic morphological changes in HepG2 cell lines. The Chebulagic acid in IC25, IC50 and IC75 of different concentrations against control proceeded. Also, Acridine Orange (AO) is easily accessible. It allows the cells to get fuse with nuclei and become green in colour if the cell is not influenced was considered normal nuclei. If the Nuclei show reduced or fragmented nuclear chromatin outcome, apoptotic and membrane integrity losses were noticed. Though the cells were seeded into a 6-well plate in the ratio of 3×10^4/well, the cells were treated with above mentioned Chebulagic acid concentrations and allowed for incubation for 24 hrs at humidified CO_2 chamber. After incubation, the cells were stained for 5 min with AO (100 mg mL^{-1}). Besides, Chebulagic acid-treated HepG-2 cells were identified by DAPI staining was determined by the presence of increased fragmented nuclei and membrane blebbing indicating yellow arrow marks of photographic images.

Statistical analysis: The results of biochemical estimations (TBARS, SOD, CAT, GPx), intracellular ROS species and *in vitro* assay were subjected to statistical analysis using SPSS 22.0 software. The data were presented as a mean±standard deviation (SD). The statistical significance of the correlation between the groups was tested using Duncan's Multiple Range Test (DMRT) and a $p<0.05$ was considered statistically significant.

RESULTS

***In vitro* antioxidants activity of chebulagic acid:** ABTS and DPPH analysis was used to know the antioxidant property of natural plant materials. Hence, we have proceeded with these estimations and it was depicted in Fig. 1a-b. The radical scavenging activity of Chebulagic acid was tested with different concentrations against the standard antioxidant of BHT of control. Also, the statistical values are represented here in Mean±SD. For ABTS, at a concentration of 100 µg mL^{-1}, the inhibition of Chebulagic acid and BHT was 71.68±1.56 and 92.54±2.95%, respectively. Similarly, at the same concentration, the DPPH inhibition of Chebulagic acid and BHT was 53.56±3.47 and 89.76±5.65%, respectively. The statistically significant changes that were noticed were *$p<0.05$ and **$p<0.01$ between the standard and Chebulagic acid-treated group. The ABTS and DPPH scavenging ability of Chebulagic acid were concentration-dependent.

Chebulagic acid effect on HepG2 cell cytotoxicity: The biocompatibility nature of the Chebulagic acid was tested in HEK293T cell lines and the results were shown in Fig. 2a. In a 5% CO_2 atmosphere, the HEK293T cell lines were incubated with various concentrations of 1.56-100 µg mL^{-1} for 24 hrs. MTT assays revealed that there are no inhibitory effects of HEK293T cell growth. It was confirmed the biocompatible nature of the Chebulagic acid. Figure 2b showed the cytotoxicity of Chebulagic acid effect on Human carcinoma cell lines of HepG2 was validated with MTT investigation. Diverse concentrations of (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µg mL^{-1}) Chebulagic acid were exposed to HepG2 cells for over a day. With the obtained data, the % of inhibition of

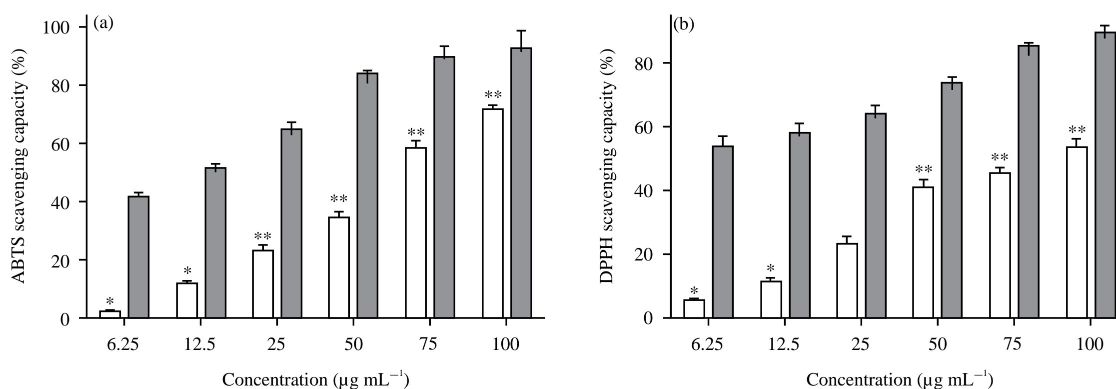

Fig. 1(a-b): (a) ABTS and (b) DPPH radical scavenging activity of chebulagic acid. Varying concentration of Chebulagic acid (6.25-100 µg mL^{-1}) was tested against the standard antioxidant of BHT

Value was expressed as Mean±SD. for three independent experiments. The statistical significance as *p<0.05 and **p<0.01 between the standard and Chebulagic acid-treated group

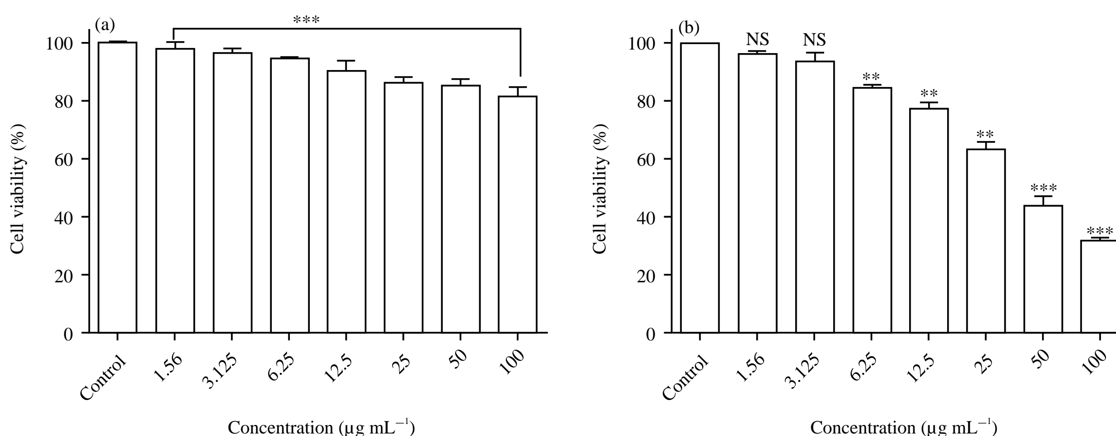

Fig. 2(a-b): Biocompatibility and cytotoxicity nature of chebulagic acid. (a) Varying concentrations of chebulagic acid (6.25-100 µg mL^{-1}) were tested against exponentially growing HEK293T cells for biocompatibility studies and (b) Cytotoxicity studies were screened by human hepatoma cells (HepG2) cells for 24 hrs incubation using MTT assay

HepG2 cells treated with chebulagic acid have a defendable anticancer activity with IC25, IC50 and IC75 values of 15.24±1.81 µg mL^{-1}, 42.86±0.94 g mL^{-1} and 92.53±2.06 µg mL^{-1}, respectively. NS: Non-significant at p = 0.01, significant differences from the control are indicated: **p<0.01, ***p<0.001

HepG2 cell proliferation was plotted against control. Significantly, the maximal cell viability percentage was noticed in 6.25-100 µg mL^{-1} concentrations. So, we have chosen the following doses of Chebulagic acid for further experimental studies the following dose of IC25, IC50 and IC75 values of 15.24±1.81 µg mL^{-1}, 42.86±0.94 g mL^{-1} and 94.53±2.06 µg mL^{-1}, respectively. In addition, we have noticed that except 1.56, 3.125 concentrations the rest of the selected doses was significant with % of cell viability in **p<0.01; ***p<0.001 vs. control. We did not observe any significant changes in or no significant with cell viability in 1.56 and 3.125 doses. The apoptogenic properties of Chebulagic acid (IC25, IC50 and IC75) were further examined by looking at morphological changes in HepG2 cells (Fig. 3). Cell shrinkage, nuclear condensation, membrane blebbing, chromatin cleavage and the development of pyknotic bodies of condensed chromatin were all seen in Chebulagic acid-treated cells. These characteristics were recognized as common hallmark changes in cell death and are commonly used to identify apoptosis.

Chebulagic acid influence on lipid peroxidation has driven antioxidants status in HepG2 cells: Oxidative stress is essential pathophysiology used to know the membrane stability and its damage by lipid peroxidative manner. In the present study, we have noticed that high elevation of lipid

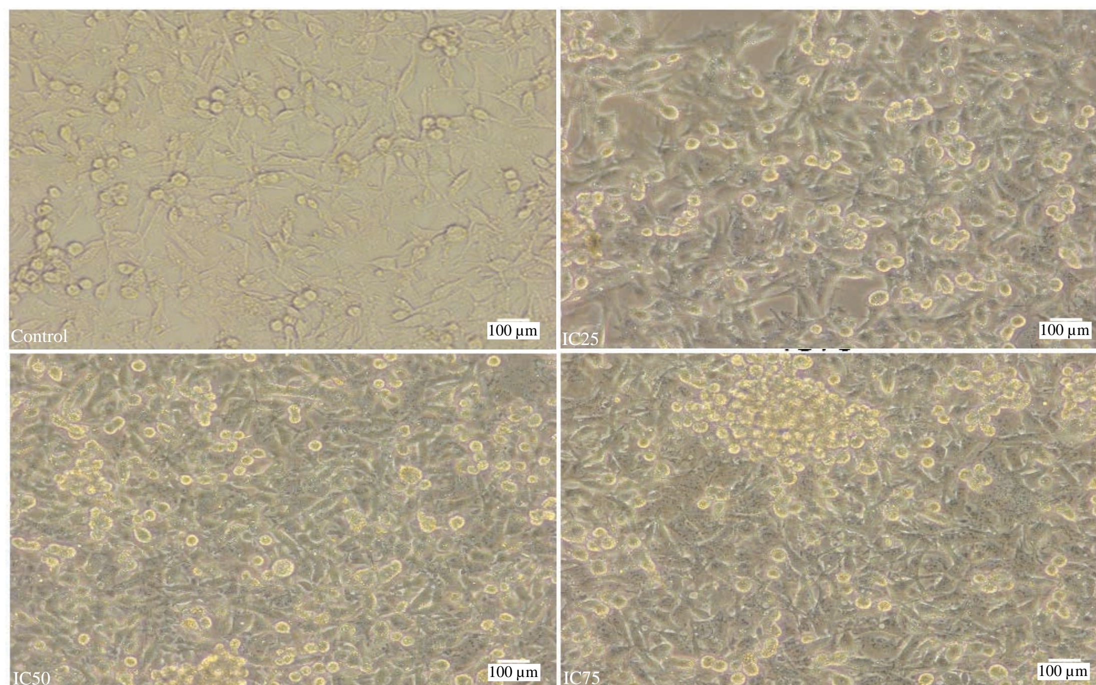

Fig. 3: Morphological changes of chebulagic acid-treated HepG2 treated cells
Cells were treated with IC25, IC50 and IC75 concentrations of chebulagic acid and imaged by an inverted phase-contrast microscope. Chebulagic acid treatment showing a condensed nucleus, membrane blebbing, cell shrinkage, bubbling apoptotic bodies and echinoid spikes

peroxidative markers such as TBARS, subsequently reduced antioxidants (SOD, CAT and GPx) elevation was observed in Chebulagic acid-induced HepG2 cells as well as compared with control. It clears that lipid peroxidation was directly proportional in reducing antioxidants levels in HepG2 cells based on a dose-dependent selection and was mentioned in Fig. 4(a-d) with its significance.

Chebulagic acid-treated mitochondrial membrane potential (MMP) in HepG2 cells: There are three stages in apoptosis. The early/primary stage of apoptosis was stimulated by altering mitochondrial membrane potential and it was evaluated by Rhodamine- 123, a lipophilic cationic dye. With IC25, IC50 and IC75 dose of Chebulagic acid-treated HepG2 cells imply that if the reduced or maintained mitochondrial membrane potential was observed, at the same time, the high amount of green fluorescence intensity was noticed in control than treated ones were shown in Fig. 5.

From these results, it was revealed that an increase in the concentration of Chebulagic acid leads to a malformed or diminished mitochondrial matrix of $\nabla\Psi M$ was observed in IC75 cells in an inverse proportioned manner than control subjects.

Chebulagic acid influenced an intracellular generation of ROS in HepG2 cells: HepG2 cells treated by Chebulagic acid and its intracellular ROS generation were identified by DCHF-DA staining, a non-fluorescent dye. Figure 6 depicted that less ROS generation was noticed by less green fluorescence intensity in untreated positive and negative control groups. On the other side, exciting, intense green fluorescence was observed in IC25, IC50 and IC75 of Chebulagic acid-treated HepG2 cells implies that if the increase in concentration helps generate more intracellular ROS. Furthermore, it came to know that increase in concentration leads to more ROS production in intracellular cells.

Effect of chebulagic acid on triggered apoptotic and its triggered morphological changes in HepG2 cells: The general mechanism of a red dye of EtBr and its emittance was observed as its shortened immature nuclei of apoptosis; meanwhile, the green emittance of AO was penetrated and observed in normal/healthy cells. The microscopic appearance of Apoptosis in HepG2 cells was stained with AO/EtBr and it's expressed in red and yellow fragmented nuclei cells of Chebulagic acid influenced apoptotic changes in HepG2 cells was shown in Fig. 7.

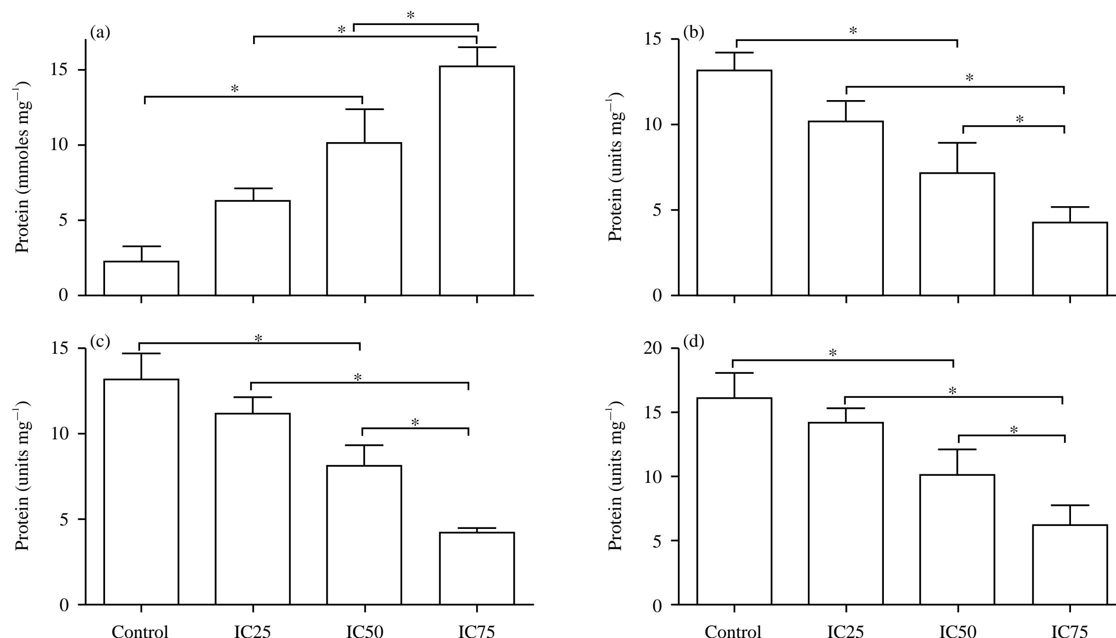

Fig. 4(a-d): Chebulagic acid-associated biochemical changes were screened by lipid peroxidation and antioxidants status in Hep-2 cells. (a) Bar diagram represents the TBARS, (b) Changes in the activities of SOD, (c) CAT and (d) GPx in IC25, IC50 and IC75 concentration of Chebulagic acid in HepG2 treated cells

*Enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in one minute. **mmol of hydrogen peroxide expended per minute. ***mg of glutathione expended per minute. Values are given as means SD. of six experiments in each group. Values not sharing symbols differ significantly at p<0.05 vs. control (DMRT)

The results obtained in the present study and the green fluorescence was high in untreated control groups than Chebulagic acid-treated (IC25, IC50 and IC75) HepG2 cells. Besides, we have noticed that orange or light yellow of apoptotic was developed by EtBr red staining. It was indicated that later apoptosis stages of DNA fragmentation were appeared by damaged/condensed Nuclei and membrane blubbing of HepG2 cells and it was decided by dose-dependent manner. Although, the strict selection of Chebulagic acid controls the apoptosis and avoids the nuclei condensation. Acridine orange (AO) is an alternative fluorescent dye frequently used to visualize the changes of apoptotic cells. IC25, IC50 and IC75 concentration of Chebulagic acid revealed an increase in the accumulation of acidic vesicular organelles compared with untreated control. However, this accumulation was expressively inhibited by Chebulagic acid (Fig. 8).

Nuclear damage of HepG2 cells by chebulagic acid: Chebulagic acid Treated HepG2 cells were treated with DAPI staining and checked its nuclear damage was shown in Fig. 9. An untreated control group was observed with standard and dense nuclei were emitted blue colour; inversely, HepG2 cells treated with various Chebulagic acid concentrations of IC25, IC50 and IC75 show increased fragmented nuclei and membrane blebbing indicate yellow arrow marks were also depicted in Fig. 9. Additionally, the exact damages in nuclear cells were visualized by DAPI staining in both control and Chebulagic acid-treated HepG2 cells.

DISCUSSION

In the present study, we explored the cytotoxic and anticancer efficacy of Chebulagic acid against HEK283T (non-cancerous cells) and HepG2 cells (Lung carcinoma). The findings of the study revealed that chebulagic acid does not produce any toxic or adverse effects on HEK283T cells, which endorsed the biocompatible nature. Nonetheless, the cell survival rate was dose-dependently decreased in chebulagic acid-treated HepG2 cells. Besides, the biochemical and fluorescent staining studies confirmed that chebulagic acid showed considerable anticancer activity. Over the past two decades, most researchers are paying attention to antioxidants from natural phytochemicals used for chemotherapeutic and act as a pharmaceutical agent than synthetically delivered drugs. At the same time, several

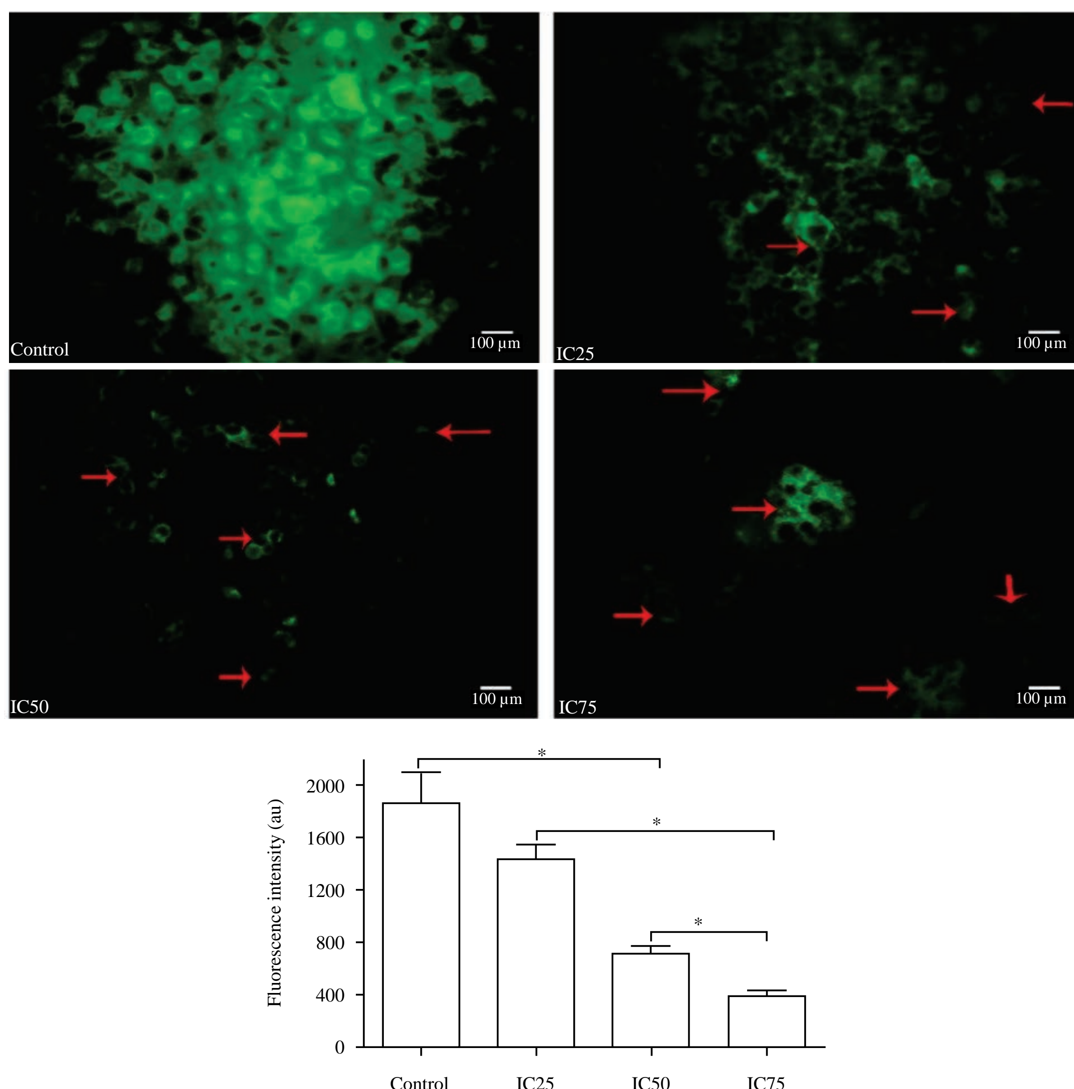

Fig. 5: Loss of mitochondrial membrane potential in chebulagic acid treated HepG2 cells using rhodamine 123 fluorescence dye staining

IC25, IC50 and IC75 concentration of chebulagic acid showing the decreased fluorescence intensity indicated that collapsed mitochondria matrix. Values are expressed as Mean±SD (*p<0.01)

epidemiological studies revealed that frequent intake of phytonutrients and that were acquired from natural sources are good in preventing the chances of mutagens and its related chronic diseases. One study from the journal of functional food suggested that natural plant extract-derived antioxidants act as a scavenger and produce resistant action against mutation-causing substance[27]. Although, as per pharmaceutical statistics, around 60% of the antimutant or anticancer drugs are discovered from naturally available plant/tree and related substances. Some studies were reported that isolated phenolic compounds of tea polyphenol, quercetin, *Terminalia arjuna* bark, Resveratrol, Tea polyphenol mediated zinc oxide, curcumin, gingerols and shogaol shows antioxidants properties along effective against various types of Cancer[28-31].

Further, so many cancer cell lines are exhibits and examined with the above-mentioned natural antioxidants and reported as an anticancer drug. The present study was done with a chebulic acid formulated benzopyran tannin compound called Chebulagic acid, a natural antioxidant from the dried *Terminalia chebula* fruit family[32]. The preliminary results of the present study were whether the Chebulagic acid effect reduces the proliferation of cancer cells by Inducing Apoptosis in Hep-2 cell lines. The obtained results are

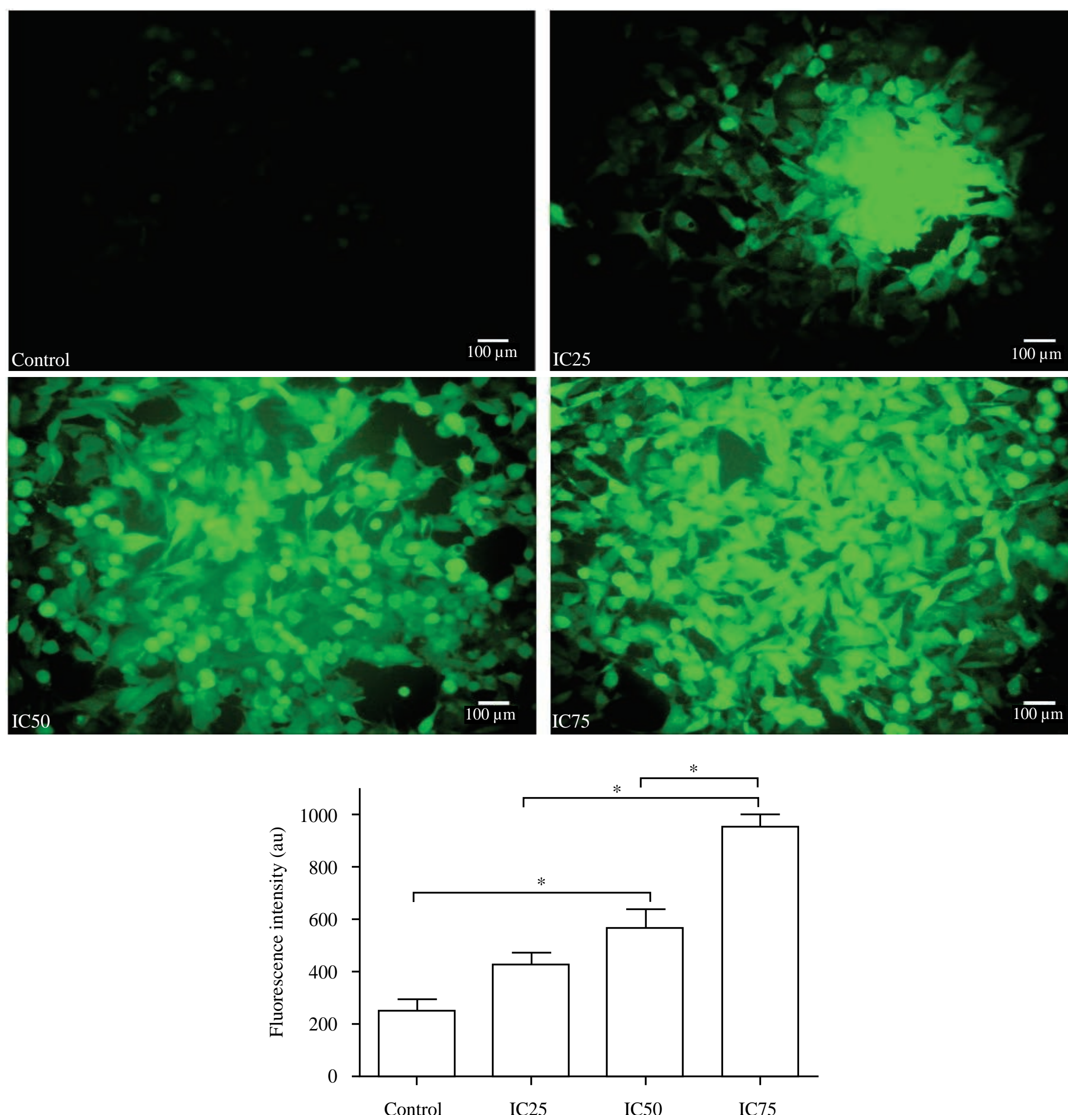

Fig. 6: Intracellular ROS status of chebulagic acid-treated HepG2 cells using DCFH-DA fluorescence dye staining

IC25, IC50 and IC75 concentration of chebulagic acid show the increasing fluorescent showing at 24 hrs incubation due to ROS induction. Values are expressed as Mean±SD (*p<0.01)

markedly indicated that Chebulagic acid efficiently acts on reducing the proliferation of cells which means of selected dose-dependent. But, when we increase the Chebulagic acid concentration is more than 75 µg mL^{-1} on HepG2 cells, it shows minimal at the same time noticeable viability changes were observed in the present prospective study. It understands that Chebulagic acid can serve as a potent inhibitor against carcinogens. Some earlier reports also agreed with tannin Chebulagic antioxidant was a potent mediator against cancers of breast, lung and hepatic colon as well as used to treat urinary tract diseases, dermatitis, diabetes mellitus, cardiovascular syndromes and hepatic disorders[33-36]. Followed by the present study and its results were reliable and indicate that significant inhibition function against cell proliferation of HCC.

Oxidative stress or free radicals are unbalanced extra uncoupled ions generated when there will be a higher production of ROS or discharge maximum cause effect on cell membranes[37]. Uncontrolled delivery of ROS by free radicals causes membrane damages called lipid peroxidation, a significant factor in triggering oxidative stress. If the endogenous antioxidants secretion is reduced, it will be directly proportional to produce abundant lipid peroxidation due to free radicals[38]. Also, maybe the primary stage of early

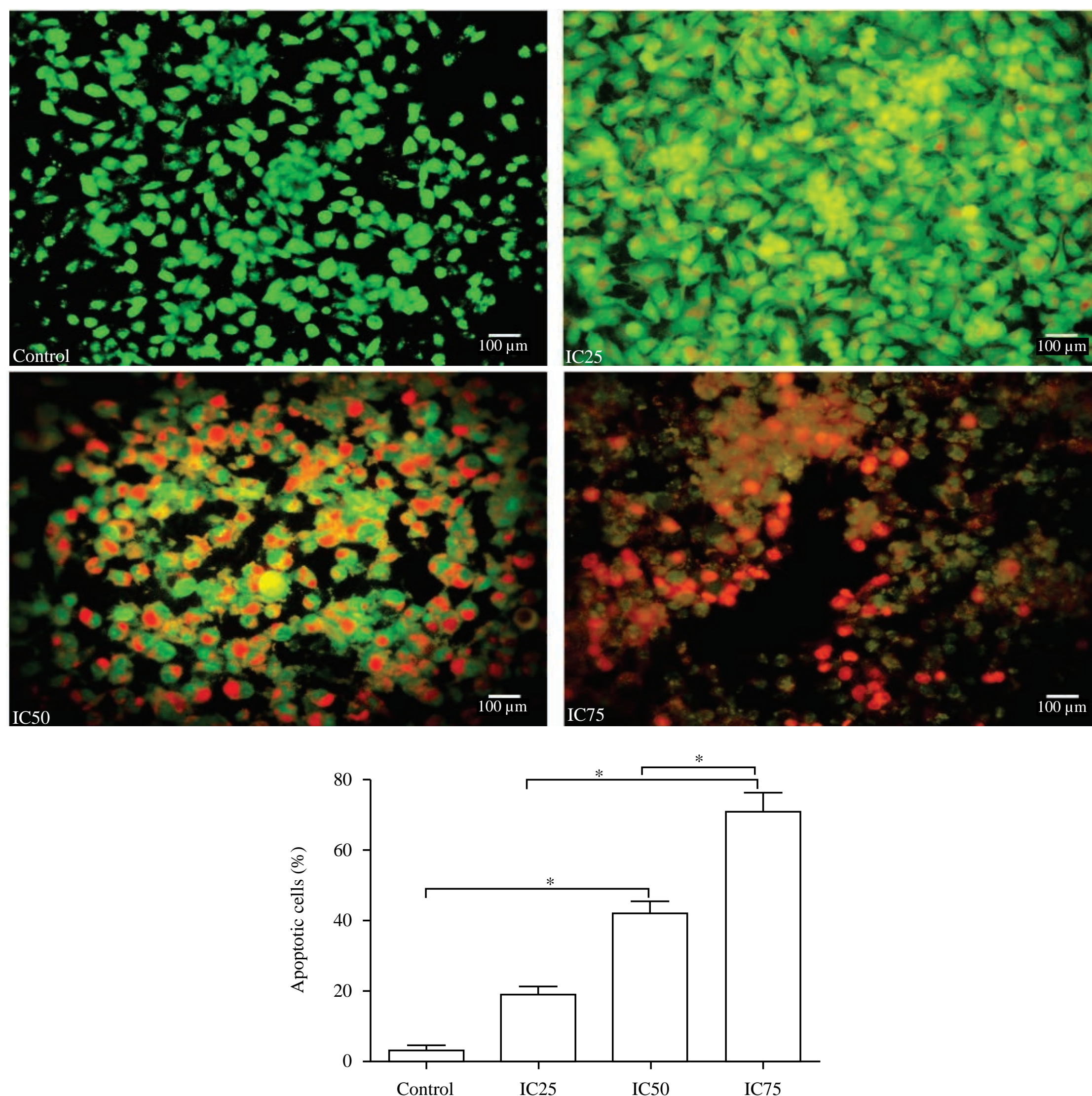

Fig. 7: Apoptotic changed in chebulagic acid-treated HepG2 cells using AO/EtBr staining

IC25, IC50 and IC75 concentrations of chebulagic acid showed orange colour cells indicate the early-stage apoptosis. 24 hrs incubation of chebulagic acid induces apoptotic and necrotic features of fragmented nuclei, shrunken cell and bulged necrotic cells values are expressed as Mean±SD (*p<0.01)

apoptosis will induce Cancer. This was agreed with the present study that we have noticed that if the low dose of Chebulagic acid is taken against to treat cancerous HepG2 cells, there will be reduced antioxidants levels, where supports to secrete more ROS more. Due to the increased intracellular generation of ROS, the cell membrane is damaged and mitochondrial membrane integrity is lost in HCC patients[39]. Hence, the HepG2 on Chebulagic acid on ROS: antioxidant conservation of Homeostasis balance solely depended on concentration. Followed by our observed results was inversely proportional with ROS based on Chebulagic acid concentrations.

Further, the tissue was exposed to Cancer, the condition in tissue and its membrane was a presence with increased uncoupled redox-transition metal ions was triggering the free hydroxyl radicals. Most of the plants in the Terminalia family and its phenolic formulated drugs are major exploits for their anticancer treatment, especially as tumor-producing proteome inhibitors[40]. Researchers reported antioxidants like vitamin C, vitamin E and Taurine, in combination with catalase as an antioxidant booster against ROS, mediated cell and its DNA damage Free radicals of ROS generation was against for all the type of antioxidants[41].

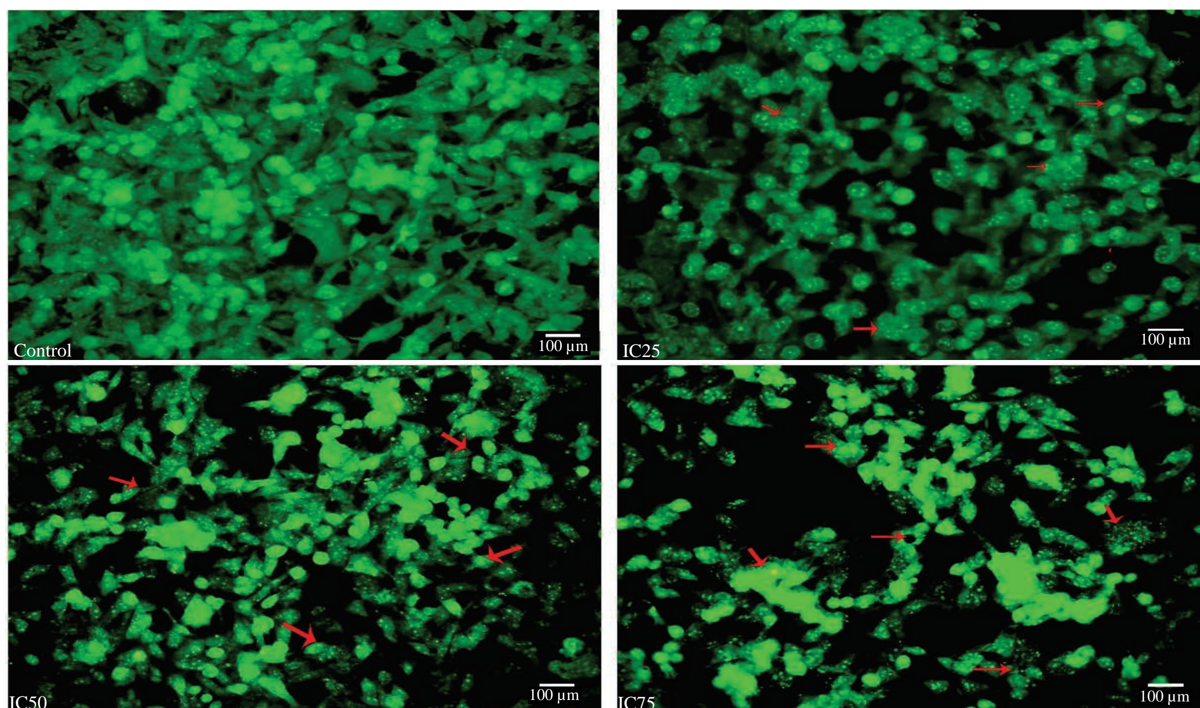

Fig. 8: Apoptotic morphological changes (acridine orange staining) were observed in control and chebulagic acid-treated cells
IC25, IC50 and IC75 concentration of chebulagic acid-treated HepG-2 cells shows increased fragmented nuclei and membrane blebbing indicate yellow arrow marks

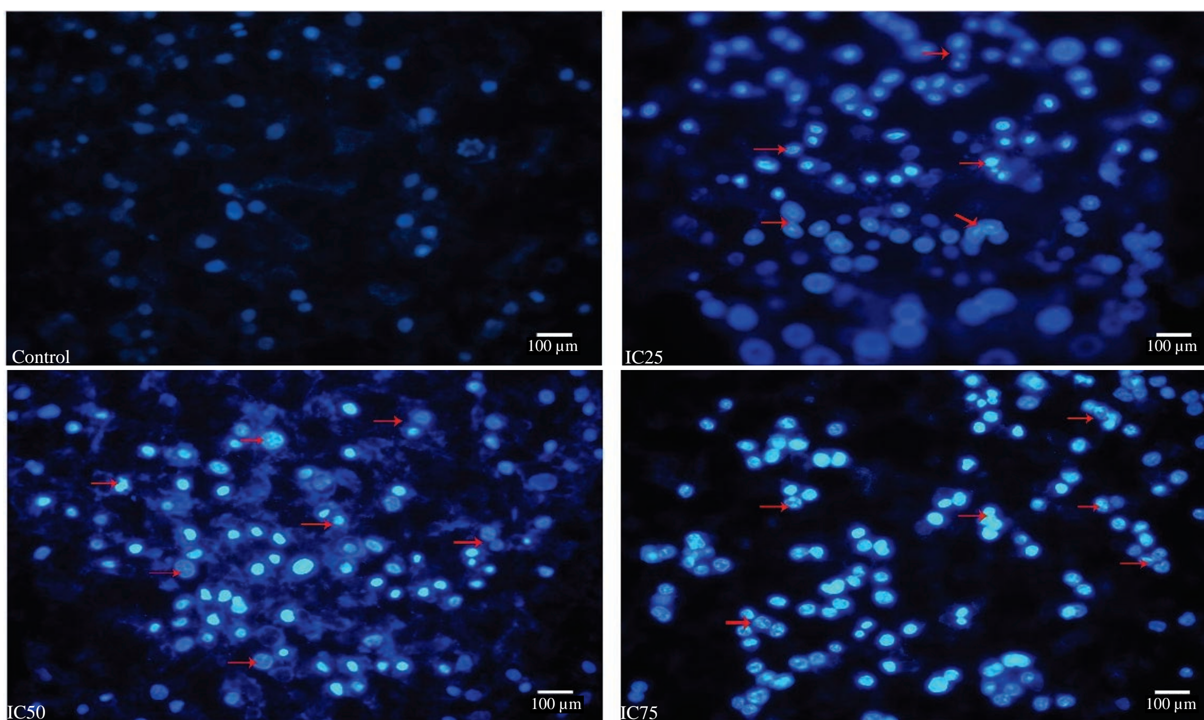

Fig. 9: Apoptotic morphological changes (DAPI staining) were observed in control and Chebulagic acid-treated cells
Different IC25, IC50 and IC75 concentration of chebulagic acid-treated HepG-2 cells shows increased fragmented nuclei and membrane blebbing indicate yellow arrow marks

To protect from oxidative stress produced tissue damage, the strong rigidity of membrane potential built by antioxidant system: ROS of balanced homeostasis in all the biological organisms is significant. Therefore, many hepatic therapeutic agents are used to stimulating antioxidant capacities instead of inhibiting or stop the intracellular ROS generation[42]. Here, an *in vitro* study was obtained with Chebulagic acid-triggered antioxidants and related intracellular ROS generation was inhibited by chebulic acid treatment.

In some cases, instead of maintaining ROS: Antioxidant direct action on mutated genome sequence in DNA was giving better results when coming to cancer treatment. So, apoptosis of programmed cell death is categorized by numerous statements and declared by an affected cell membrane, plasma membrane, nuclear chromatin shortening and DNA fragmentation lead to loss of DNA integrity which was identified by more apoptotic elevation[43]. Although, ROS of free radicals also favors the loss of membrane and DNA fragmentation which leads to a high apoptotic elevation that changes the biological and pathophysiology of DNA. Also, oxidative DNA damage by ROS triggers the alteration in nucleotides bases and its effects cause single-strand break and double-strand break[44]. Numerous studies reveal that natural phytoconstituents that treat cancer cells and its outcome apoptosis and its triggered intracellular ROS generation favors become oxidative DNA damage[45,46].

Meanwhile, the same result was also obtained in some previous studies, carcinoma cell line studies of HCC and A549 of lung cancer[47,48]. Remarkable notes that phytoconstituents from Ginger and its associated phenolic combinations enhance cell death by apoptosis through free radicals generated uncoupled ROS in pancreatic cancer cells[49]. Hence, apoptosis-induced cancer cell death was extensive therapy in case of late apoptosis. Apoptosis is an essential pathophysiological mechanism in the intracellular medium and favours eradicating the effective detrimental cells, which were mainly controlled by Bcl-2 family proteins. Suppose the uncontrolled downregulation of Bcl-2/bax leads to the absence of apoptosis in tumour cells[50]. Earlier reports explain that the tannin antioxidant property of Chebulagic acid was responsible for inhibiting the cytotoxicity in cancer cells[51]. Therefore, Chebulagic acid-enhanced ROS assembly leads to stimulates mitochondrial reliant Apoptosis in HepG2 cells. Also, this study was agreed with Kumar *et al.*[52] which revealed that Chebulagic acid-induced apoptosis through intracellular ROS generation activates the caspase (apoptotic marker) in human retinoblastoma cells. In regardless, the anticancer property of chebulic acid formulated tannin chebulagic acid was more antioxidant and the function against HCC was dose-dependent manner.

CONCLUSION

Whatever the reason for liver injury, if the inflammation stage was in the limit and the liver cells can be recovered. In case of liver injury was chronic, liver tissue recovery was difficult to control. Hence the present study, the HCC activity of Chebulagic acid on HepG2 cells *in vitro* was attempted in the present study. In a nutshell, we recommend that HepG2 cells produce excessive cell proliferation was inhibited by Chebulagic acid-induced ROS restricted mitochondrial interference in primary apoptosis stage was denoted by increased intracellular ROS generation in matrix cells which leads to deprived $\nabla\Psi M$, oxidative DNA damage and nuclear fragmentation was observed.

SIGNIFICANCE STATEMENT

The obtained results strongly recommend that Chebulagic acid can be a better and novel antitumor agent than synthetic and its associated adverse effects in chemotherapy. However, a suitable animal model might be used to investigate the cancer-preventive and immune-modulating effect Chebulagic acid will be explored in near future.

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