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Study On Anti-Cancer Activity Of Fruit Extract Of Terminalia Chebula Retz. By Exosome-Mediated Drug Delivery System In Hepg2 Cell Line

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ABSTRACT

Liver cancer is a serious clinical illness that has a poor prognosis, and elevated aggressiveness. Medicinal herbs have been used for millennia to treat a variety of liver-related issues. *Terminalia chebula Retz.* is widely utilized by traditional practitioners for a range of health benefits. In our study ethyl acetate extract of ripe fruits of *Terminalia chebula Retz.* (EaTCE) was tested for anti-cancer activity in HepG2 cell line. The plant extract was also loaded in exosome in an attempt to increase the bioavailability of the plant extract. However, EaTCE packed in exosomes (Ex-EaTCE) exhibited even superior results due to greater intracellular distribution of EaTCE. To evaluate the nature of phytochemicals present in the EaTCE the total phenolic, flavonoid and tannin contents were also determined. The MTT assay and apoptotic study were conducted for the assessment of in-vitro cytotoxicity. The percentage cell viability of EaTCE and Ex-EaTCE was found to be reversely concentration dependent. The IC_{50}values as determined by the MTT assay were found to be 34.3µg and 30.9 µg in of EaTCE and Ex-EaTCE respectively in HepG2 cell line

Keywords: Hepatoceluar carcinoma; *Terminalia chebula Retz.;* Exosome; Apoptosis; Cytotoxicity

Introduction

We have been gifted with an array of miraculous plants by Ayurveda that have the power to improve health, cure illnesses, and extend life. The herb *Terminalia chebula Retz*. has been used from the ancient times of Charaka. *Terminalia chebula* is one of the most often exploited plants in India and its subcontinent (Naik et al., 2004). This miraculous plant's anti-inflammatory and digestive qualities make it a component of many herbal medicines, including Triphala. It has also been revealed recently that *Terminalia chebula Retz.* has anticancer and antioxidant qualities. With a wide range of biological and pharmacological uses, including antibacterial, antifungal, antiviral, antimutagenic and anti-anaphylactic properties, *Terminalia chebula Retz*. is known as "the king of medicines" in Tibet. Other uses include hypolipidemic/hypocholesterolemic, gastrointestinal, anti-ulcerogenic, hepatoprotective, cardioprotective, radioprotective, antidiabetic and retinoprotective, antispasmodic, wound healing, purgative, immunomodulatory, and chemopreventive properties (Chattopadhyay & Bhattacharyya, 2007). The phytochemicals that are mainly responsible for the biological activity of this plant are tannins. Hydrolyzable tannins, makes up to approximately 32% of the total chemical makeup of *Terminalia chebula Retz*. There are about 14 different hydrolyzable tannin components present in this plant. Chebulic acid, chebulegic acid, ellagic acid, chebulinic acid, chebulanin, gallic acid, terchebulin, punicalagin, 1,6,-di-O-galloyl-D-glucose, casuarinin, corilagin, 1,2,3,4,5,6penta-Ogalloyl-ß-D-glucose, and 3,4,6-tri-O-galloyl-D-glucose are among them (Wadkar & Pinjari, 2023). This plant also contain certain other phyto-nutrients like protein, vitamin-c and minerals (Muhammad, 2012).

Cancer persists as one of the conditions that many individuals worldwide face a threat to their health. One of the most prevalent deadly cancer is liver cancer, and patients are frequently discovered at a stage that limits the effectiveness of treatment (Couri & Pillai, 2019). Hepatocellular carcinoma (HCC) is among the top five cancers worldwide and the third most significant cause of cancer-related death, with 840,000 newly reported

cases and at least 780,000 deaths annually (Anwanwan et al., 2020; Bray et al., 2018). There is still no established mechanism for HCC development. HCC has a sneaky commence, with over 85% of patients being diagnosed at intermediate to advanced stages. This makes therapy more challenging, and the prognosis is frequently dismal (Jiang et al., 2019). Thus, the search for a novel, non-invasive, sensitive, and specific biomarker is urgent.

Extracellular vesicles (EVs) are a novel form of structure that has gained interest recently for their potential significance in cancer. EVs are also referred as micro or nanovesicles, derived from the cell membrane. All prokaryotic and eukaryotic cells possess the ability to produce these double-membrane vesicles in a manner that has been evolutionarily conserved. Their size range is from 30 to 150 nm, with an average particle size of 100 nm. The way they work depends on where they came from. For instance, exosomes from tumour cells are primarily involved in invasion and migration and allow for cell-to-cell communication (Paskeh et al., 2022). Exosomes can be found in a variety of bodily fluids because they are composed of various proteins generated by tumour cells, which release more exosomes into bodily fluids than do normal cells. Since exosomes are found in a variety of bodily fluids, they may be used as novel biomarkers for disease identification and diagnosis (Keerthikumar et al., 2016). Patients frequently miss the best time to receive therapy because hepatocellular carcinoma frequently does not exhibit any particular symptoms in its early stages. Exosomes can be utilized to diagnose and prognosticate cancer since they can transport different proteases or other enzymes through the bloodstream to their intended targets (Li et al., 2019). Exosomes are a very promising technique for disease detection as a tumour marker for identifying different cancers as well as potent drug delivery agent, as evidenced by the growing number of research. Gene carriers and macromolecular, particle, and magnetic agent carrier systems are examples of contemporary drug delivery techniques. On the other hand, different approaches to targeted drug delivery have been developed in response to problems with non-specific cytotoxicity, biocompatibility, and delivery efficiency in individually tailored carrier systems. The following are some benefits of using exosomes as drug delivery vehicles. They are non-toxic and have low immunogenicity. They are derived from autologous cells and have a long circulation half-life, high permeability, small particle size, and the ability to traverse biological barriers (Chen et al., 2016). Exosomes are natural nanoparticles with exceptional biocompatibility, and their use is not restricted to the detection of hepatocellular carcinoma. In fact, they may prove to be a valuable tool in addressing treatment-related issues. There is a suggestion that exosomes are well-suited as drug delivery vehicles due to their biological functions and optimal natural structure that allows for the selective assembly and separation of their contents (Li et al., 2016). It has also been discovered that exosomes, which are naturally occurring signaling carriers, can successfully prevent cellular drug tolerance when anti-cancer medications are wrapped in them. This can increase the effectiveness of the treatment and kill tumour cells (Ma et al., 2016). For instance, Kim et al. discovered that paclitaxel treatment mediated via trans-exosomes demonstrated significant promise in the treatment of tumours. Tian et al. showed how specifically engineered exosomes might control the administration of adriamycin to cancerous cells (Tian et al., 2014). In this work, the total phenolic content, total tannin content and total flavonoid content of ethyl acetate *Terminalia chebula Retz*. fruit extract (EaTCE) has been evaluated utilising an array of assays. In our investigation, we have also used exosomes as a drug delivery vehicle in an effort to improve the plant extract's bioavailability. In the HepG2 cell line, the effect of EaTCE and exosome mediated EaTCE (Ex-EaTCE) are studied therapeutically by performing the MTT Assay and the apoptosis study.

Materials and methods

Collection and extraction

The *Terminalia chebula Retz*. sample of mature fruit were collected in September and November from various places within the Cachar district, situated in the southernmost region of the Indian state of Assam. The original recipient of the sample was submitted to the Department of Life Science and Bioinformatics, Herbarium, Assam University Silchar, Assam, India. The accession number is 8556 issued by the herbarium. After being cleaned, the gathered fruits were left to air dry for about a week. After that, the dried fruits werepowdered using a mixer. In order to prepare the extract, 100g of powdered ripe fruits of *Terminalia chebula Retz*. and ethyl acetate was used. The cold maceration method was followed while performing the extraction. Afterwards *Terminalia chebula Retz*. fruit extract (EaTCE) was lyophilized and stored for further use.

Phytochemical analysis of EaTCE

The plant extract was subjected to numerous phytochemical tests to confirm the presence of carbohydrates, alkaloids, tannins, phenolics, glycosides, flavonoids, saponins and steroids. Basic standardized protocol with certain minor modification was employed to ascertain the presence of phytocompounds (Morgan, 1990).

Determination of total phenolic content (TPC)

The determination of TPC of EaTCE was performed by Folin-Ciocalteu technique as mentioned in with some modifications. Gallic acid was used as reference and the absorbance was taken at 750 nm (Singleton V. L. et

al., 2016). For quantification of the curve linear regression was employed. The TPC was expressed in terms of mg of Gallic acid equivalents per g of dry weight (DW) (Kamtekar et al., 2014).

Determination of total flavonoid content (TFC)

The TFC of EaTCE was estimated by using the aluminium chloride colorimetric assay (Kamtekar et al., 2014). Quercetin was used as standard. First, 4ml of DW and 300µl sodium nitrite solution (5%) was added to 1ml of sample. After 5 minutes of incubation, 300µl aluminum chloride (10%) and 2 ml sodium hydroxide (1M) solution was added. By adding DW, the final volume was made up to 10 ml. The absorbance was observed at 510 nm. The TFC was calculated in terms of mg of quercetin equivalent/g dry weight.

Determination of total tannin content (TTC)

The total hydrolysable tannin content (TTC) present in EaTCE was estimated by means of Folin-Ciocalteu colorimetric method (Katoch, 2011). Tannic acid was used as standard for calibration curve. Sample was mixed with 0.5 ml of Folin-Ciocalteu reagent and 0.1 ml Na 2 CO 3 solution (7%). The resultant solution was then mixed thoroughly and kept for half an hour. The absorbance was recorded at 700 nm. The hydrolysable TTC of EaTCE was calculated as mg tannic acid equivalents (TAE) per g of dry extract (mg/g).

Isolation and drug loading in exosome

The juice of lemons was centrifuged at 500× g × 10 min. The supernatant was then filtered through 100 µm filter and subjected to repeated centrifugation at 2000× g for 20 min in order to remove cell debris, and at 15,000× g for 30 min in order to remove the fraction that was richer in micro vesicles. The supernatant was then subjected to an ultracentrifugation process for 90mins at 110,000× g to extract the nanovesicles. For further analysis, the pellet was again suspended in the PBS buffer (1X).Then, after 8 hours of incubation; EaTCE was loaded into exosomes via sonication. The exosome and EaTCE mixture were treated with ultrasonic treatment using a homogenizer probe. In an effort to preserve the integrity of the exosomal membrane, the exosomes were again incubated with EaTCE for 8 hours after undergoing ultrasonic treatment. EaTCE enclosed within exosomal membrane was called Ex-EaTCE in our study.

In-vitro analysis

The cell line HepG2, with hepatocellular carcinoma was used for the in-vitro investigations. The National Centre for Cell Science (NCCS), located in Pune, India, had provided the cell line. The cell line was first grown in MEM medium with 1% penicillin-streptomycin and 10% fetal bovine serum, and was then incubated at 37°C with 5% CO_2. In this test, the initial seeding density per well was approximately 10,000 cells. Next, trypsin-EDTA was used to subculture HepG2 cell line at 90% confluence for a 24hour incubation period at 37 ± 1°C to create a monolayer. Next, fresh medium was added to the culture medium for further cultivation of cells.

MTT Assay

10,000 cells per well were seeded from the HepG2 cell lines in quadruplicates for this analysis, and the cells were then incubated at 37 ◦C for the whole night to allow cell attachment. Afterwards, EaTCE and Ex-EaTCE was given in triplicates, at concentrations ranging from 10µg/mL to 100µg/mL to the cell line. The cells were then incubated with both the drugs for 18–24 hours at 37 ± 1°C. Following that, 1 mg/ml of MTT reagent was added to each well, and the mixture was allowed to incubate for 4 hours. Finally, formed formazan salts were dissolved in DMSO by adding it to the wells, and a spectrophotometer was used to record the absorbance at 570 nm. Then, from the obtained readings, the cell viability graph was plotted with concentration in the X-axis and cell viability in Y-axis. Make-Radical; model-RTC-7, a phase contrast microscope at 20X resolution was used to capture the images. The % cell viability was calculated using the formulae mentioned below.

Cell viability (%) = (Treated / Control) * 100

Apoptosis study

Using the acridine orange-ethidium bromide fluorescent dye method, apoptosis was investigated. To ensure total cell dissociation, the cells were then seen under an inverted phase contrast microscope (Model: RTC-7; Make: Radical; model-RTC-7). The cells were seeded at a density of about 105 and then incubated at 37 °C for 24 hours. Finally, triplicates of EaTCE and Ex-EaTCE at various concentrations (25µg/mL, 50µg/mL, 75µg/mL, and 100µg/mL) were dissolved in 0.1% dimethyl sulfoxide (DMSO) was introduced to the HepG2 cell line. After the incubation period, the media was removed and the cells were properly washed with PBS. The cells were then inspected under the same microscope once again after adding 1µg/ml of the Acridine orange-Ethidium Bromide dye system to each well and images were captured.

Statistical Analysis

All the experiments were performed in triplicates and the result were represented as mean ±standard error of mean (SEM). The statistical evaluation was done by using GraphPad Prism 10 (Version 10.2.3). One way analysis of variance (ANOVA) was employed to ascertain the significance (99.9% confidence, $p<0.001$) difference between two mean values.

Results & Discussion

Phytochemical analysis of EaTCE

The percentage yield was calculated using the formula mentioned below.

$$\text{Percentage Yield (\%)} = \frac{\text{Weight of Supernatant (gms)}}{\text{Weight of Plant material (gms)}} \text{ X } 100$$

The percentage yield obtained from 100 gm of dry *Terminalia chebula Retz.* ripe fruit powder in ethyl acetate solvent was found to be 34.44 %. The results of the phytochemical analysis are presented in **Table 1**. Ferric chloride (1%) was reacted with EaTCE and formed blue colour precipitate. This result indicated the presence of tannins in the extract. The saponins present in EaTCE gave positive results for the Frothing test. Flavonoids were reacted with NaoH to form yellow colour that confirms the presence of flavonoids. Quinine was reacted with alcoholic kOH solution and indicated a colour change from red to blue. The presence of alkaloids was also confirmed by means of Dragendroff's test, Mayer's test and Wagner's test. The formation of red colour when the extract reacted with Molisch reagent confirmed the presence of carbohydrates. All the results of the analysis were shown in **Fig. 1.**

Table 1: Phytochemical analysis of hydro-alcoholic *Terminalia chebula Retz.* fruit extract (TCE)

Test name	**Wagner's Test**	**NaOH Test**	**Lead acetate Test**	**Dragendroff's Test**	**Mayer's Test**	**Molisch's Test**	**Concentrated H_2SO_4 Test**	**Ferric Chloride Test**	**Saponin Test**	**Soluble starch Test**	**Fehling's Test for free sugar**	**Tannin's Test**	**Fehling's Reducing Test for combined Sugar**
Colour of precipitate	**Reddish brown**	**Yellow orange**	**White**	**Orange**	**Cream**	**Red\ Dull violet**	**Orange**	**Deep Blue**	**Foam**	**Yellow**	**Red**	**Blue Black Green**	**Reddish Brown**
Presence in Hydro-alcoholic TCE	+	+	+	+	+	+	-	+	+	+	-	+	+

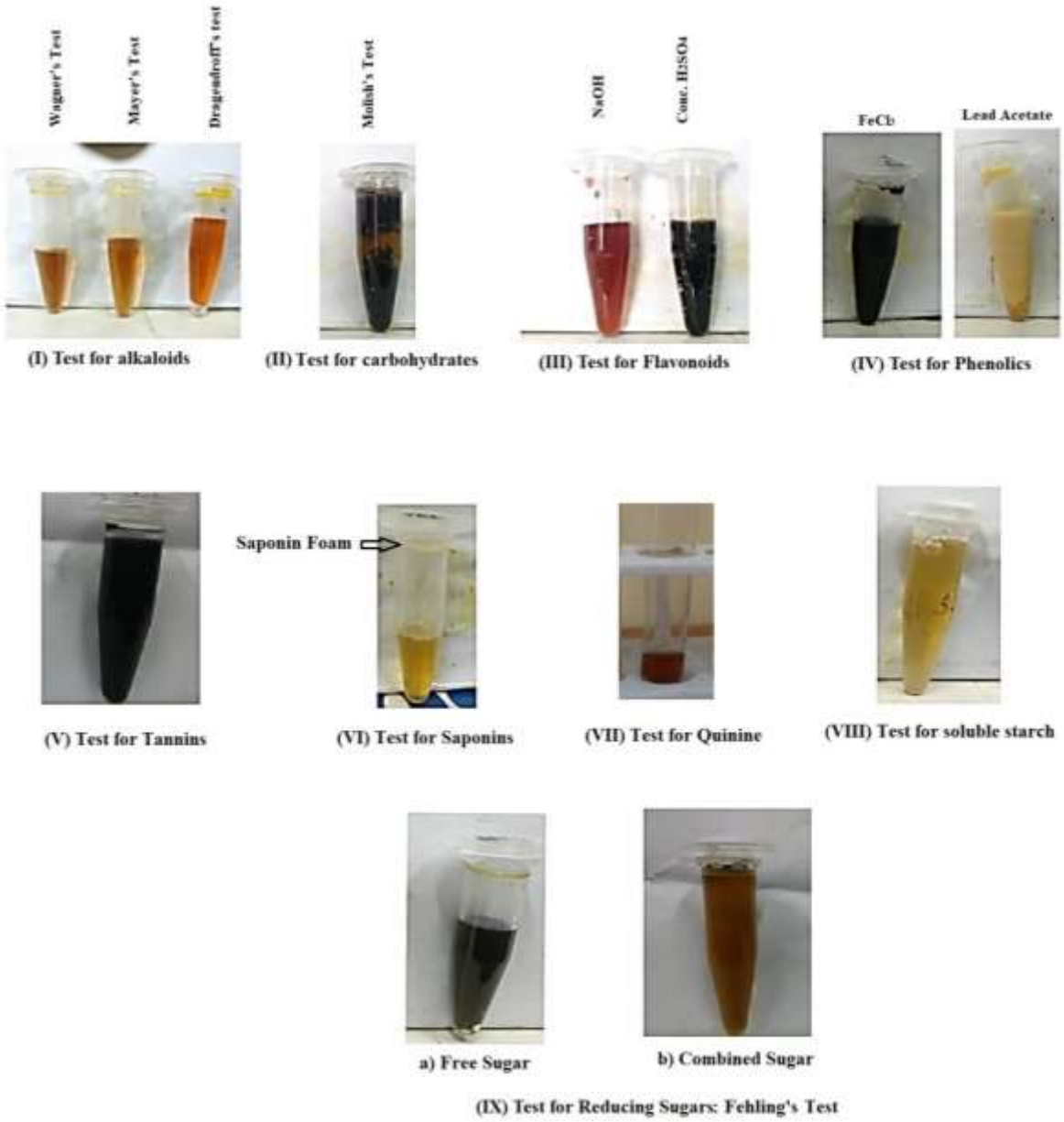

Figure 1: Different phytochemical test to confirm the presence of (I) Alkaloids, (II) Carbohydrates, (III) Flavonoids, (IV) Phenols, (V) Tannins, (VI) Saponins, (VII) Quinine, (VIII) Soluble starch, (IX) Sugar

The total phenolic content (TPC) of EaTCE was found to be 179.51 ± 0.11 mg gallic acid equivalent/g dry weights by Folin-Ciocalteau method. The total flavonoid content was found to be 17.71 ± 0.87 mg of quercetin equivalent/g dry weight. The total tannin content (TTC) of EaTCE was found to be 32.69 ± 0.52 mg tannic acid equivalents (TAE) per gram of dry extract (mg/g). This test signified the amount of total hydrolysable tannins present in the MTCE.

Isolation and loading of exosome

To extract exosomes from lemon juice, the ultracentrifugation method was employed. Exosomes were isolated, collected, and then reconstituted in 1XPBS solution for additional use. EaTCE was loaded in exosomes for efficient drug delivery. The results for in vitro study in HepG2 cell line showed that Ex-EaTCE had better therapeutic potential against HCC compared to bare EaTCE.

MTT Assay

The effect EaTCE at different concentrations ranging from 10–100 µg/mL on the cell viability of HepG2 cell line was evaluated by means of methyl thiazolyldiphenyltetrazolium bromide (MTT) assay (**Figure 2**). The result indicated that EaTCE and Ex-EaTCE were capable of notably suppressing the cell viability of HepG2 cells in a dose-dependent manner. However, the inhibition of cell viability was more prominent in the cell lines treated with Ex-EaTCE, due to increased intracellular delivery of EaTCE by exosomes. The IC_{50} values of EaTCE and Ex-EaTCE were found 34.3µg and 30.9 µg respectively in HepG2 cell line.

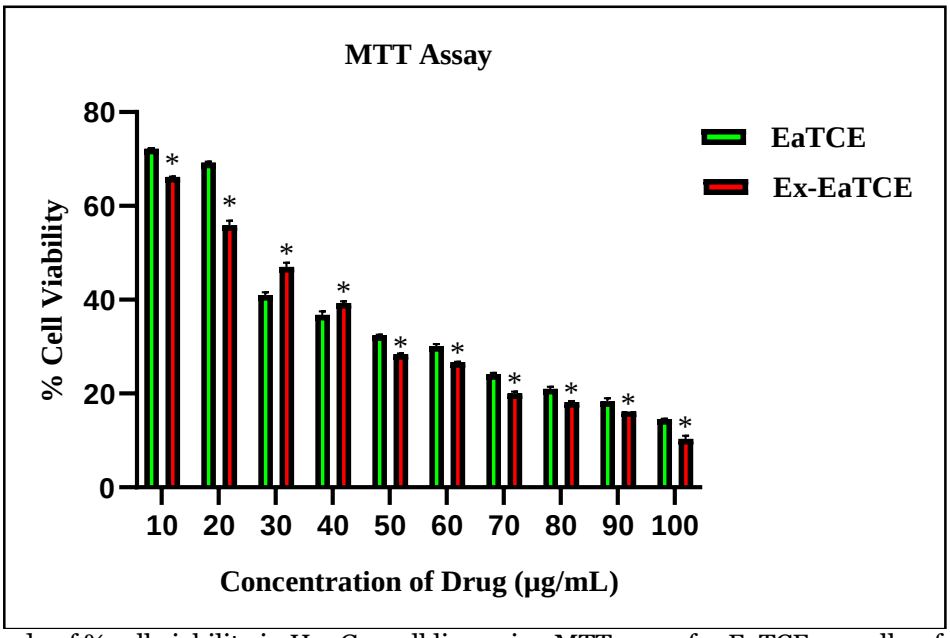

Figure 2: Study of % cell viability in HepG 2 cell line using MTT assay for EaTCE as well as for Ex-EaTCE

The images of MTT assay were also shown in **Fig. 3.** after treating with EaTCE and Ex-EaTCE in dose dependent manner. The results showed that treatment with EaTCE and Ex-EaTCE decreased the cell viability in HepG2 cell line. However decrease in cell viability was more visible in Ex-EaTCE compared to EaTCE. The untreated control HepG2 cells exhibited full proliferation and growth with high viability.

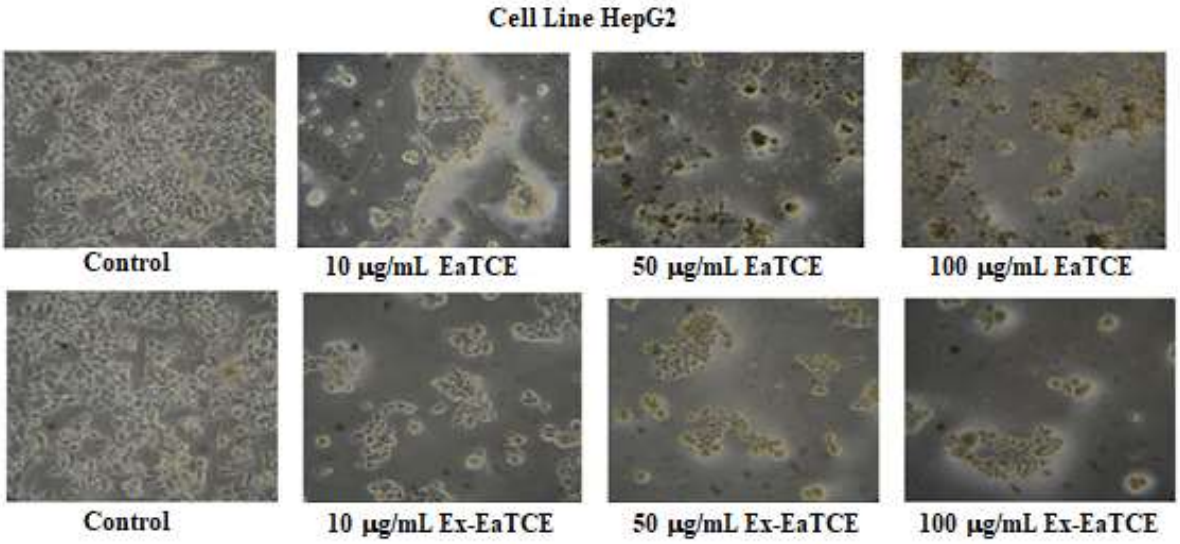

Figure 3: Images of cell viability studies in HepG2 cells using MTT assay method after treatment of EaTCE and Ex-EaTCE with dose dependent manner. Treatment decreased the cell viability in HepG2 cell line. The untreated control HepG2 cells showed full proliferation with high viability of cells.

Apoptosis Study

Acridine orange-ethidium bromide fluorescence labeling was used to identify the morphological change of apoptotic cells under the impact of EaTCE and Ex-EaTCE as shown in **Fig. 4.** Increased levels of mitochondrial oxidative stress are strongly associated with the process of apoptosis. This event is followed by release of cytochrome C and activation of caspases ultimately leading to cell death (Cadenas, 2004). Therefore, it can be assumed that plant-based antioxidant compounds have the potential to be an effective treatment for heptatocellular carcinoma. Furthermore, the images obtained from the apoptotic study suggested that Ex-EaTCE performs better than bare EaTCE in inducing apoptosis in the HepG2 cell line.

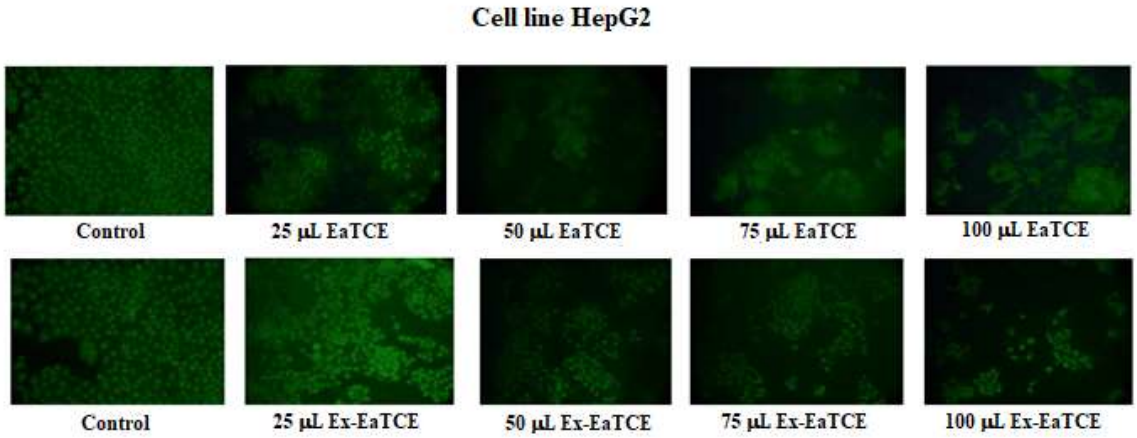

Figure 4: Apoptosis studies in HepG2 cells using florescence dyes with treatment of EaTCE and Ex-EaTCE with dose dependent manner. Treatment increased the apoptotic cell death in HepG2 cell line. The untreated control HepG2 cells showed green fluorescence that indicates high viability of cells. Magnification - 40 ×.

Conclusion

This investigation suggested that the phytochemical found from ethyl acetate extract of ripe fruits of *Terminalia chebula Retz.* (EaTCE) possess potent anti-cancer activity as observed in HepG2 cell line. Moreover, the exosomal drug delivery system was demonstrated to be a more effective therapeutic agent due to increased bio-distribution and targeted delivery for treating malignant cells as suggested by the images from the apoptosis study and the cell viability tests.
Future research will undoubtedly lead to the development of exosome-mediated delivery to not only in cancer but other diseases also i.e.; cardiovascular diseases, neurodegenerative diseases, diabetes, which will provide personalized medicine to combat deadly ailments. It is anticipated that this study may offer a preliminary framework and direction for creating novel tools for diagnosis and successful treatment of HCC.

Ethical approval
Not applicable

Declaration of competing interest
The authors declare that there is no conflict of interest regarding the publication of this paper

Data Availability Statement
Data will be made available on reasonable request

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