

## Rose and eucalyptus essential oil as potent anti-liver cancer agents

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### Abstract

The present study was designed to investigate the anti-cancer potential of essential oil obtained from *Rosa indica* (REO) and *Eucalyptus citriodora* (EEO) against the liver carcinoma cell line (HepG2). Firstly, the cytotoxic activity was assessed using increasing concentrations ranging from 3.12 to 200 µg/ml via MTT assay. EEO showed only 2% cell viability while REO represented 18% at the highest concentration (200 µg/ml). The half-maximal inhibitory concentration (IC<sub>50</sub>) of EEO and REO was found to be 17.741 µg/ml and 18.55 µg/ml respectively. Additionally, evident morphological changes in HepG2 cells were observed after 24 hours of essential oil treatment compared to control or untreated cells. Furthermore, to strengthen the anti-cancer perspective of essential oils, the anti-metastatic potential was evaluated through the wound healing assay. EEO promisingly inhibited migration (4% wound closure, \*\*p > 0.01) in HepG2 cells after 24 hr treatment. Likewise, REO also exhibited good results (37% wound closure, \*\*\*p > 0.001). Conclusively, the present investigation provides preliminary results which suggest that REO and EEO are potent anti-cancer agents against hepatocellular carcinoma.

**Keywords:** Essential oil, Metastasis, HepG2, Cytotoxicity, Liver cancer

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## Introduction

For many years, agro-food science and folk medicine have been aided by the use of plant byproducts, such as essential oils, to fight several diseases and food preservation. Essential oils are highly concentrated materials, having complex chemical compositions characterized by terpenes/terpenoids, and low-molecular-weight aroma chemicals of a strong odor

and flavor extracted from different plant parts grown in tropical and subtropical countries (Kumari et al., 2014). Currently, around 3000 essential oils have been identified of which 300 are being used in numerous industrial applications such as in food as flavoring and preservatives, insecticides, medicine, and cosmetics, and also have allelopathic properties (Cutro et al., 2021). Among various essential oils, rose essential oil extracted from petals of *Rosa indica* (rose) has always



been considered as most fascinating due to its delicate, elegant, soft, and sweet aroma, which besides acting as calming and anti-inflammatory agent, also holds the ability of cells to regenerate relaxing effect (Javed et al., 2021). Moreover, it also has various biological activities such as anticancer (Nowak et al., 2014) antibacterial (Mohebitabar et al., 2017), antioxidant and has proven pharmacological very important (Javed et al., 2021). It has been shown that patients with dysmenorrhea may be treated with rose essential oil for pain (Uysal et al., 2016). Likewise, significant repression of Alzheimer's disease symptoms of hypersensitivity has been noticed because of the rose essential oil effect (Zhu et al., 2017). The effect of rose essential oil by inhalation aromatherapy is also very effective in decreasing anxiety, depression, and stress in hemodialysis patients (Fazlollahpour-Rokni et al., 2019). Over and above, eucalyptus essential oils extracted from leaves of *E. citriodora* are in great demand due to their broad spectrum of therapeutic properties and bioactive properties, used in the pharmaceutical and cosmetic industries. They contain biologically active compounds that play important roles as antiseptic, antimicrobial, antioxidant, chemotherapeutic, wound healer, gastrointestinal disorder treatment, insecticidal/insect repellent, and as herbicidal, acaricidal, nematocidal (Dhakad et al., 2018).

Essential oils may be taken as anticancer agents due to the presence of high amounts of thymol, carvacrol, 1,8-cineole, and limonene against different cancer lines (Bakkali et al., 2008). Essential oils act on cancer cells by inducing cell death, arresting the cell cycle, and losing key organelles' function. Moreover, as antioxidants, essential oils can also affect the cellular redox state (Tuttolomondo et al., 2013). The antiviral activity of essential oils (*Melaleuca alternifolia*, *Rosa damascene*, *Mentha piperita*, *Eucalyptus* sp., and *Thymus vulgaris*) and their constituents have been documented in several studies. Most recently, it was revealed that eucalyptus essential oil exhibited inhibitory effects on mumps virus and adenovirus (Mieres-Castro et al., 2021). Likewise, volatile oil from *Cynanchum stauntonii* possessed direct inhibitory activity against the influenza virus (Mieres-Castro et al., 2021). One of the most recent studies shows that *Rosa alba* essential oils can significantly reduce the viral reproduction of the Victoria strain (Vilhelmova-Ilieva et al., 2021). In Asia diversity of medicinal plants, are usually used by local people as pharmacopeia for many years, among them, *Fumaria*

*indica* and *Berberis lyceum* are used in Pakistani folk medicine, and also play a very important role to treat hepatic diseases such as hepatitis C virus (HCV) infection (Parra et al., 2018). For instance, the essential oil of *E. citriodora* contained monoterpenes followed by sesquiterpenes and has shown substantial antioxidant potential (Zhou et al., 2021) due to the presence of 1,8-cineole, which may act against hsubstantialepatic diseases (Horvathova et al., 2014). Likewise, in rose petal essential oil, the occurrence of  $\beta$ -caryophyllene (sesquiterpene), acts as a calcium channel blocker and produces a significant reduction in blood pressure, diarrhea and gut spasms (Bigliani et al., 2012), and isosteviol has also been reported to have a vasodilator activity (Rasheed et al., 2015). Different natural compounds (e.g. polyphenols) present in the essential have been known to interfere with HCV replication, facilitate cancer cell response to chemotherapy, modulate immune cell function, and anti-platelet activity (Jardim et al., 2018). Therefore, plants' essential oil could be an effective option to treat the hepatitis C virus (HCV), a small, enveloped RNA virus, which causes liver disease in humans (Moniruzzaman et al., 2020). The study was performed to assess the cytotoxic effect of essential oil extracted from leaves of *E. citriodora* and petals of *R. indica* for percent cell viability in the HepG2 cell line.

## Material and Methods

### Extraction of essential oil

The petal of *R. indica* and leaves of *E. citriodora* and were collected by handpicking. About 2 to 3 kg of samples were collected from the vicinity of Lahore, Pakistan. Essential oil extraction from leaves of *E. citriodora* and petals of *R. indica* was carried out by the hydro-distillation method using the Clevenger apparatus for one hour. Briefly, the steam was passed through a glass pipe to the flask containing fresh samples. The resultant volatile compounds in the form of vapors were passed over a distillation column where they condensed and captured in a receiver as a distillate. The oily layer was separated from the aqueous layer by adding diethyl ether to the steam distillate. To separate volatile and nonvolatile compounds from the collected mixture by separating funnel, the mixture was shaken for 30 min, and then it was allowed to stand for an hour to separate layers of water and organic layer (oil + solvent). Water traces were removed from the organic layer by Sodium sulfate as a desiccant. Oil and diethyl ether in the upper



layer were heated at low temperature to evaporate the solvent and to obtain pure oil. The oil sample was stored in sealed vials at 4 °C for further analysis (Javed et al., 2021). The stock solution of essential oils was prepared in ppm (10,000 ppm=10,000 µg/ml) by dissolving 1 g of oil in 100 ml of DMSO, while only DEMO alone was taken as control.

### Cell cytotoxicity assay

The MTT assay was carried out to assess the cytotoxic potential of REO and EEO as formerly described by Tariq et al. (2021). Briefly, HepG2 cells were seeded in 96-well plate at the density of  $2 \times 10^4$  cells/well and incubated at 37 °C with 5% CO<sub>2</sub>, overnight. The next day, cells were treated with increasing concentrations of essential oils (3.125 to 200 µg/ml) or DMSO (0.1%) for 24 hours. After the treatment, the culture medium containing the tested compounds was removed and cells were washed with PBS. Then, cells were added with 20 µl of MTT solution and incubated for 3 hours finally after incubation 100 µl of DMSO were added. After keeping 30 min in dark at room temperature, absorbance was measured at 570 nm and 650 nm. The percentage cell viability was calculated capered to untreated cells (control) using the following formula: Percentage Cell Viability = (absorbance at 570-absorbance at 650/absorbance Control) × 100

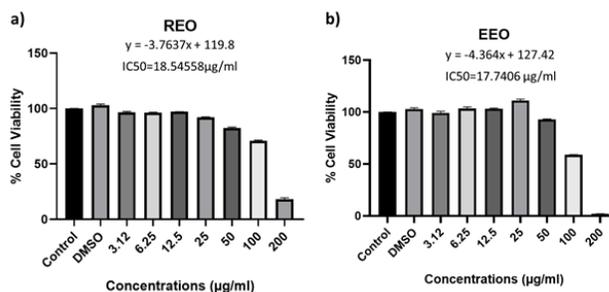
### Wound healing assay

Anti-metastatic potential of REO and EEO was evaluated by wound healing assay as previously described by Zhong et al. (2012). In brief, HepG2 cells were seeded in a 12-well plate. After 90% confluency, a scratch was made on the monolayer of cells using a pipette tip. The cells were washed with PBS and followed by the addition of fresh DMEM (Dulbecco's Modified Eagle medium) containing 1% FBS (Fetal Bovine Serum) and effective concentrations of essential oils (200 µg/ml and 100 µg/ml). Afterward, the photographs of the scratch were taken at different intervals of 0, 12 and 24 hours. Further, the average area of the wound was calculated via image J software and plotted as percentage wound closure vs time intervals using GraphPad prism 8.0 software.

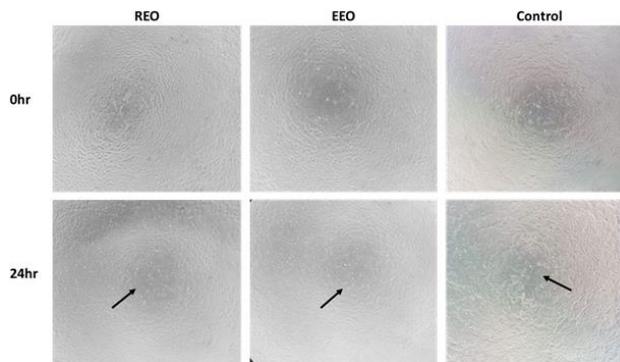
## Results

HepG2 cells were treated separately with increasing concentrations (3.125 to 200 µg/ml) of REO and EEO. Cell viability was calculated compared to the control (untreated cells), corresponding to 100% proliferation.

Generally, 200 µg/ml concentration of both essential oils was significantly more toxic against HepG2 cells than the others. The percentage cell viability of HepG2 cells at 200 µg/ml was restricted to 18% with application of REO and to 2% with EEO. Furthermore, 100 µg/ml concentrations of REO and EEO were also toxic causing 30% and 41% cell death in HepG2 cells, respectively (Figure 1). However, all other concentrations (3.12 to 50 µg/ml) of both essential oils were non-toxic.



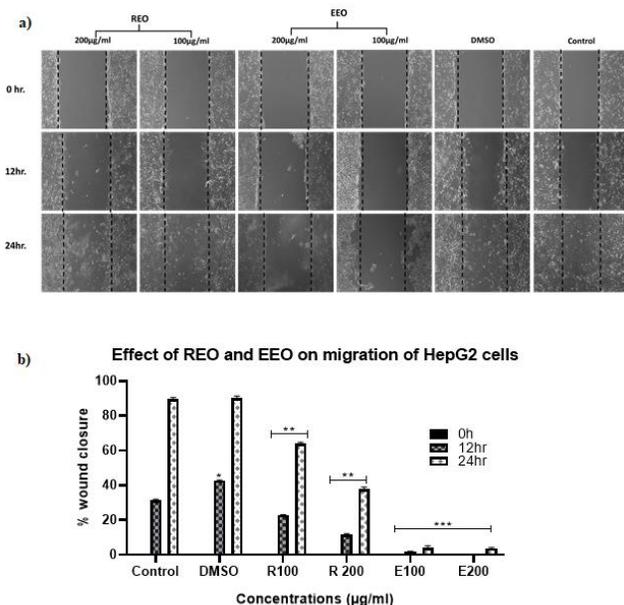
**Figure 1. Cytotoxicity assessment by MTT assay in HepG2 cells following the exposure of various concentrations of a) Rose essential oil (REO) and b) Eucalyptus essential oil (EEO) for 24 hrs. Each value in the table is represented as mean ± SD (n = 3).**



**Figure 2. Morphological analysis of control and treated HepG2 cells. The morphological changes were observed in HepG2 cell after 24 hrs of rose essential oil (REO) and eucalyptus essential oil (EEO) treatment in comparison to control (non-treated cells).**

The half-maximal inhibitory concentrations (IC<sub>50</sub>) of EEO and REO were projected from the concentration-response curve. REO was found to be active exhibiting an IC<sub>50</sub> value of 18.55 µg/ml, whereas IC<sub>50</sub> value (17.74 µg/ml) of EEO depicts it as an active anti-cancer agent against HepG2. In addition to the cytotoxicity analysis, the morphology of HepG2 cells was also observed at the highest (200µg/ml) and most effective concentration of REO and EEO. The results revealed a considerable change in the shape of hexagonal HepG2 cells compared to untreated or

control cells. These changes were blebbing of the membrane, shrinkage of cells and disruption of the cell membrane, and apoptotic body formation (Figure 2). To strengthen the anti-cancer perspective of REO and EEO, anti-metastatic potential was also evaluated in HepG2 cells via wound healing assay. Results revealed that both essential oils hold anti-metastatic potential. EEO exhibited the most promising results by restricting wound closure at only 4% at both potent concentrations after 24 hours of treatment compared to the control. REO also represented significant anti-metastatic properties with 37% and 63% wound closure at 200 µg/ml and 100 µg/ml concentrations, respectively as shown in Figure 3.



**Figure 3.** Effect of REO and EEO on cell migration of HepG2 cells evaluated by wound healing assay. a) A scratch was made in the monolayer of HepG2 cells. The cells were treated with two effective concentrations (100 and 200 µg/ml) of rose essential oil (REO) and eucalyptus essential oil (EEO) treatment

## Discussion

Results revealed that the lower concentrations (3.12 to 50 µg/ml) of REO and EEO were ineffective against the viability of HepG2 cells, while higher concentrations (100 and 200 mg/mL) significantly decreased cell viability. Studies have shown that essential oils stimulate cell death in an apoptotic manner and possess great anti-cancer potential (Al-Sheddi et al., 2019). Besides, enhanced production of reactive oxygen species and reduction in the levels of

cellular antioxidants like glutathione is the most encountered phenomenon in cancer cells in response to the treatment with essential oil that leads to cell death (Paik et al., 2005; Gautam et al., 2014; Mohammed et al., 2021). Many antioxidant compounds (e.g. oxygenated mono- and sesquiterpenes), as well as free radical scavenging properties, have been documented in REO and EEO (Javed et al., 2021; Mohammed et al., 2021; Zhou et al., 2021). The cytotoxic effect of higher concentrations of essential oil might reflect synergism between different oil components as described previously by Shala and Gururani (2021). Jamali et al. (2021) also ascribed synergistic and additive interactions between oxygenated monoterpenes or phenolic monoterpenes of *Oliveria decumbence* essential oil which might act as radical oxygen quenchers, inhibit lipid oxidation and therefore reduce damage in the biological cell membrane and protect tissues and cells against oxidative damage against A549 lung cancer cells. Moreover, literature indicated that  $IC_{50} \leq 20 \mu\text{g/ml}$  as an active,  $IC_{50} > 20\text{-}100 \mu\text{g/ml}$  as moderately active,  $IC_{50} > 100\text{-}1000 \mu\text{g/ml}$  as weakly active, and  $IC_{50} > 1000 \mu\text{g/ml}$  inactive values of any drug (Baharum et al., 2014). In this regard,  $IC_{50}$  of EEO (17.75 µg/ml) and REO (18.55 µg/ml) may enlist these oil among the active anti-cancer agent against HepG2.

The observation concerning all cytological features (blebbing of the membrane, shrinkage of cells and disruption of the cell membrane, and apoptotic body formation) with the highest (200 µg/ml) and most effective concentration of REO and EEO are considered the hallmarks of apoptotic cell death. As a recent study has also noticed these changes in HepG2 cells when treated with turmeric essential oil (Lu et al., 2021). Similar, morphological changes were also observed in HepG2 cells after the treatment of *Moringa oleifera* seeds' essential oil (Elsayed et al., 2015). Furthermore, metastasis is a vital characteristic of tumor cells that help escape and spread from the primary tumor site to distant organs (Asif et al., 2016). Therefore, an effective drug should control the migration of cancer cells (Fares et al., 2020). Besides, both essential oils hold anti-metastatic potential. Reliably, the active phytochemical compounds in plant extract inhibit certain cell signaling pathways to restrict cell migration. For instance, *Artemisia argyi* essential oil inhibits the Wnt/β-catenin signaling pathway, involved in hepatocarcinoma cell (HepG2 cells) migration and

invasion (Li et al., 2021). Similarly, a Daucosterol, a phytosterol inhibits HepG2 cell migration via regulating the Wnt/ $\beta$ -catenin signaling pathway (Zeng et al., 2017).

## Conclusion

REO and EEO both showed vowing cytotoxic activity against HepG2 cells at higher concentrations (100 and 200 $\mu$ g/ml). The IC<sub>50</sub> of EEO and REO characterized them as active anti-cancer agents against the liver cancer cell line. Furthermore, these essential oils possess great anti-metastatic properties required in a potent anti-cancer drug.

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**Conflict of Interest:** None.

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### Contribution of Authors

Javed S: Extraction of essential oil and data collection.  
Shoaib A: Wrote and edited manuscript.  
Malik A: Cytotoxic and wound healing assays.  
Ijaz B: Supervised cytotoxic and wound healing assays.  
Perveen S: Formatted & edited manuscript.

