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## Research article

# Antioxidant and Cytotoxic Effects of *Chrozophora rotleri* Fruit Ethanol Extract

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## ABSTRACT

Liver cancer, particularly hepatocellular carcinoma (HCC), occurs from liver cell abnormalities influenced by factors such as viral hepatitis, obesity toxins and drugs. Non-alcoholic fatty liver disease (NAFLD) exacerbates liver disorders globally, marked by fat accumulation, whereas liver fibrosis raises cancer risk in NAFLD and alcohol-induced liver disease (ALD). Herbal medicine gains attraction for liver health, offering hepatoprotective benefits. Medicinal plant metabolites, particularly polyphenols, display antioxidative effects, possibly suppressing cancer cell development. This work focuses on evaluating the possible antioxidant and cytotoxic effects of *Chrozophora rotleri* fruit ethanol extract (CRFEE) using *in-vitro* experiments. Hydroxyl radical and nitric oxide scavenging experiments examined the antioxidant capacity of CRFEE. CRFEE revealed considerable scavenging efficacy against both radicals, suppressing hydroxyl radicals by 48.25% with an  $IC_{50}$  of  $35.08 \pm 1.62 \mu\text{g/mL}$  and nitric oxide by 49.35% with an  $IC_{50}$  of  $307.44 \pm 2.28 \mu\text{g/mL}$ . Notably, CRFEE displayed better antioxidant efficiency compared to the standard antioxidant, butylated hydroxyl toluene (BHT), in neutralizing hydroxyl radicals. Additionally, the *in-vitro* cytotoxicity of *C. rotleri* fruit ethanol extract (CRFEE) was examined using the MTT test on HepG-2 cell line cultures. CRFEE had strong detrimental effects on HepG-2 cells, with higher dosages resulting to a major loss in cell viability. The  $IC_{50}$  value for CRFEE was determined  $63 \pm 0.08 \mu\text{g/mL}$ , suggesting its efficacy in reducing HepG-2 cell proliferation. The results demonstrate substantial antioxidant activity and cytotoxic effects against liver cancer cells (HepG-2 cell line). The bioactive chemicals contained in CRFEE show potential for future pharmaceutical applications, underscoring the relevance of natural-based research in cancer therapy and the necessity for further exploration *in-vivo*.

## INTRODUCTION

Liver cancer, an uncommon but severe disease, arises in liver cells. It impairs the liver's important activities of filtering toxins, generating bile, and storing nutrients, which might cause it to expand to other places of the body.<sup>[1]</sup> Liver cancer exists in several distinct forms, with the most common being hepatocellular carcinoma (HCC), which originates in the primary liver cell type known as hepatocytes. Other kinds of liver cancer include intrahepatic cholangiocarcinoma, which develops in the bile ducts inside the liver, and angiosarcoma, which grows in the blood vessels of the liver.<sup>[2]</sup> Globally, hepatocellular carcinoma (HCC) is the fifth most common global liver

cancer. The factors associated with developing HCC include viral hepatitis, chemical agents, aflatoxin exposure, obesity, liver cirrhosis, and geographical location, which impact the occurrence of instances.<sup>[1,3]</sup> Approximately 25% of the worldwide population is plagued with non-alcoholic fatty liver disease (NAFLD), the primary cause of persistent liver disorders, contains excessive fat accumulation, not from alcohol consumption.<sup>[4]</sup> Excessive intake of alcohol leads to ALD, resulting in a spectrum of liver illnesses that vary from steatosis (fatty liver) to steatohepatitis, fibrosis, and cirrhosis.<sup>[5]</sup> Liver fibrosis, a common feature of severe NAFLD and ALD, is distinguished by excessive scar tissue formation. This impairs liver function, resulting

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in hepatic failure, fibrosis, cirrhosis, increased HCC risk, and eventually death.<sup>[5,6]</sup> Early identification boosts liver cancer treatment effectiveness, with frequent monitoring and hepatitis vaccines being vital. Screening blood transfusions for hepatitis also greatly decreases liver cancer risk through infection prevention.<sup>[6,7]</sup> Patients with hepatocellular carcinoma have many therapeutic options, which include liver transplantation, surgical resection, percutaneous ablation, radiation, and early-stage treatments.<sup>[8]</sup> Advanced treatments, including targeted medicines, radiation, immunotherapy, and chemotherapy, have significantly enhanced patient life quality and disease-free survival rates. However, chemotherapeutic medicines have limitations such as solubility, instability, and systemic toxicity, which may contribute to adverse consequences.<sup>[9]</sup> As such, the usage of complementary and alternative herbal medicine for liver health is increasing attention owing to its ability to supply hepatoprotective substances and alleviate liver damage with minimal adverse effects.<sup>[10]</sup>

Natural-origin medications, especially those derived from medicinal plants, are increasingly employed in liver cancer therapy owing to their biological activities.<sup>[11]</sup> Secondary metabolites from medicinal plants indicate anti-cancer, liver protection, antioxidant, anti-allergic, anti-inflammatory, and anti-mutagenic activities.<sup>[12]</sup> Polyphenols, essential secondary metabolites in medicinal plants with antioxidant activities, have been proven to protect liver cells from injury and maintain liver function. Antioxidants present in fruits, vegetables, whole grains, and plants neutralize free radicals, reducing their detrimental effects. They maintain biological components, and their richness in antioxidants may greatly prevent cancer.<sup>[13]</sup> Antioxidants neutralize free radicals, reducing DNA damage and mutations. Polyphenols, a crucial group, act *via* benzene-ring-bound hydroxyl groups. These groups have the capacity to give hydrogen or electrons to free radicals there by stabilizing them and avoiding injury to biological components. The B ring of polyphenols is vital in reducing oxidative damage.<sup>[14]</sup> Certain antioxidants may influence cell signaling pathways, possibly limiting the development and spread of cancer cells by reducing cell proliferation and survival.<sup>[15]</sup> Certain antioxidants present in plant extracts have proven anti-cancer potential, notably in green tea extract, which protects the liver and decreases inflammation.<sup>[16]</sup> Studies suggest that *Phyllanthus niruri* extracts decrease the progression of NAFLD.<sup>[17]</sup> Whereas *Picrorhiza kurroa* extracts reduce lipid levels and cholesterol concentrations.<sup>[18]</sup> Curcumin's potential benefits in managing and reducing inflammation in NAFLD is well established.<sup>[19]</sup> *Silybum marianum* extracts also have hepato-protective properties by lowering oxidative stress and inflammatory reactions.<sup>[20]</sup>

*Chrozophora rottleri* (Geiseler) A. Juss. ex Spreng., known as "Suryavarti" in Sanskrit and "Lingamenasu" in Kannada, is an annual plant of the Euphorbiaceae family, native to

Malaysia, Thailand, Saudi Arabia, Sudan, Ethiopia, Senegal, East Africa, India, and Nepal.<sup>[21]</sup> In traditional medicine, its different components are applied to manage colds, coughs, wounds, digestive difficulties, and skin infections due to its bitter, emetic, cathartic, and caustic properties.<sup>[22]</sup> This small, erect plant includes deeply lobed leaves with serrated edges, growing up to 50 cm and producing clusters of small yellow flowers, releasing milky white latex when harmed.<sup>[23]</sup> Rich in bioactive components like as phenols, alkaloids, flavonoids, and glycosides, *C. rottleri* has possible medicinal advantages. Notable chemicals include rutin, saponins, steroids, apigenin 7-o-b-d-[6-(3,4-dihydroxy benzoyl)], glucopyranoside (chrozophorin), and acacetin 7-orutinoside, which contribute to its therapeutic characteristics. Studies have shown that extracts from *C. rottleri* may be useful for treating infections because they are antibacterial and antifungal.<sup>[24]</sup> They also have anti-inflammatory effects, perhaps helping to control illnesses like arthritis.<sup>[25]</sup> Its antioxidant effects minimize oxidative stress, facilitate healing, and perhaps prevent carcinogens.<sup>[21,26]</sup>

This study investigates the antioxidant potential and cytotoxic effects of *C. rottleri* fruit ethanol extract (CRFEE). No reports are available in the literature about the use of this plant to explore the effects on cancer. To bridge this gap, this study uses CRFEE to perform *in-vitro* antioxidant and anti-cancer activities. The study aims to identify the therapeutic advantages of CRFEE by investigating its effects on oxidative stress and cancer cell growth.

## MATERIALS AND METHODS

### Plant Collections

*C. rottleri* specimens collected from Seernakatte in Chitradurga, India, were identified by Dr. V. Krishna, a taxonomist at the Department of Biotechnology, Kuvempu University. The plant parts were processed through drying, crushing, and successive soxhlet extraction. Using a soxhlet apparatus, about 50 g of powdered *C. rottleri* parts were used to make a crude extract. This was done with petroleum ether, chloroform, and ethanol, which are all solvents with decreasing polarity. A rotary evaporator was applied to vaporize the solvent, and the resulting crude dried extracts were collected in sterile petri plates, labeled, and stored in a desiccator for future use.<sup>[27]</sup>

### *In-vitro* Antioxidant Activities

#### Hydroxyl radicals ( $\cdot\text{OH}$ ) assay

The hydroxyl free radical scavenging ability was evaluated utilizing a slightly modified approach provided by Zhou, *et al.* (2020).<sup>[28]</sup> About 0.2 mL of the crude ethanol extract sample, 0.5 mL of PBS buffer (pH = 7.4), 9, 0.1 mL of phenanthroline (5 mmol/L), 0.1 mL of 7.5 mmol/L FeSO<sub>4</sub>, 0.1 mL of 0.05% H<sub>2</sub>O<sub>2</sub>, and 1.2 mL of ethanol were gradually added to the tube furthermore kept for 37°C for 20 minutes

incubation process. Subsequently, assess the absorbance at 536nm. Each sample was examined three times, and the results were averaged. The hydroxyl radical scavenging activity was evaluated by applying the formula below.

$$\text{Hydroxyl Radical Activity(\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}$$

#### Nitric oxide scavenging assay

A significantly modified approach was used for the nitric oxide radical scavenging experiment.<sup>[29]</sup> A solution of 10 mM sodium nitroprusside was dissolved in 0.2 M phosphate-buffered saline (pH 7.4) was made, and varying CRFEE doses (ranging from 100–500 µg) were applied. For 150 minutes, this mixture was incubated at room temperature. After the incubation time, 50 µL of Griess solution was added to the mixture to develop color. Subsequently, this reaction mixture absorbance was measured at 546 nm. Butylated hydroxyl toluene (BHT) served as the standard reference for comparison. For each sample, the findings from three different tests were calculated and averaged to assure accuracy.

$$\text{Nitric oxide(\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}$$

#### In-vitro cytotoxicity MTT assay

The HepG-2 cell line was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution for 24 hours to promote growth. Subsequently, an MTT experiment was done where cells were treated with various dosages of formulations. Following a 24-hour treatment time, MTT solution was added to each culture and it was incubated for a period of time 2 hours to allow for formazan crystal formation. The resultant cell layer matrix was then dissolved with DMSO. Analyzed optical density at 540 and 660 nm.<sup>[30]</sup> Which was determined using the Graph Pad Prism-8 program. Microscopic pictures of the cells were obtained via an inverted microscope fitted with a Scope Digital Camera, permitting extensive investigation and documenting of cellular shape and density changes after treatment.

#### Statistical Analysis

Graph Pad Prism version 8 was used for the analysis of the experimental data, and computing statistical differences using the standard deviation and standard error of the mean. ANOVA tests were employed to analyze differences between groups, enabling a rigorous examination of outcomes and an accurate interpretation of findings.

## RESULTS

### Hydroxyl Free Radical Scavenger Activity

The hydrogen peroxide scavenging test was conducted to look at the CRFEE antioxidant capacity. It demonstrated increased scavenging activity. Table 1 shows that the

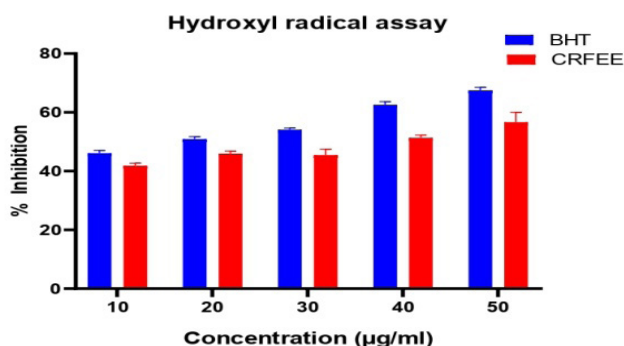


Fig. 1: Hydroxyl radical scavenging activities of BHT and CRFEE

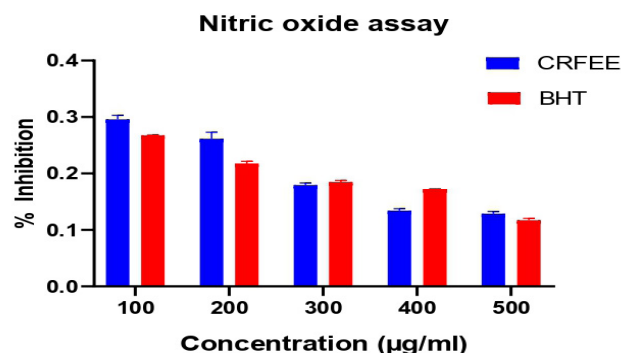


Fig. 2: Nitric oxide activities of CRFEE and BHT

Table 1: Antioxidant activities of CRFEE and BHT

Activity	Hydroxyl radical assay		Nitric oxide assay	
	IC <sub>50</sub> (µg/mL)	% of Inhibition (%)	IC <sub>50</sub> (µg/mL)	% of Inhibition (%)
CRFEE	35.08 ± 1.62	48.25	307.44 ± 2.28	49.35
BHT	15.47 ± 0.88	56.23	236.25 ± 0.57	55.77

NO Scavenging (Nitric oxide) test.

CRFEE extract had significant antioxidant activity at 48.25% (IC<sub>50</sub> = 35.08 ± 1.62 µg/mL) and BHT at 56.23% (IC<sub>50</sub> = 15.47 ± 0.88 µg/mL). CRFEE exhibited superior antioxidant activity compared to standard BHT, as shown in Fig. 1.

A nitrite radical-scavenging assay was conducted using CRFEE. The results demonstrated that CRFEE possesses significant scavenging activity, with the highest efficacy of 49.35% and an IC<sub>50</sub> value of 307.44 ± 2.28 µg/mL. The findings illustrated in Fig. 2 and outlined in Table 1 emphasize the effectiveness of both standard and CRFEE in neutralizing nitrite radicals. CRFEE exhibited the highest efficacy compared to BHT, effectively neutralizing nitrite radicals at lower concentrations.

### In-vitro Cytotoxicity MTT Assay

The study found that the CRFEE had significant harmful effects on the HepG-2 cell line. Exposure to higher doses reduced cell viability significantly compared to the control group. The IC<sub>50</sub> values for CRFEE were 63 ± 0.08 µg/mL,



indicating CRFEE has a greater efficacy in suppressing HepG-2 cell growth. Microscopic examination of cell morphology showed that changes in cell shape, such as cell shrinkage, rounding, and membrane blebbing, were dependent on the dosage of CRFEE shown in Fig. 3. The  $IC_{50}$  is the concentration of an inhibitor, sample, or formulation at which viable cells are reduced by half. Sample CRFEE was found to be more cytotoxic, as illustrated in Table 2. The MTT test demonstrated strong and proportional harmful effects of the ethanol extract of *C. rotleri* showed

strong harmful effects on the HepG-2 cell line, with higher doses resulting in a substantial decrease in cell viability in comparison of the control group with the experimental group, can be seen in Fig. 4.

## DISCUSSION

Plant extracts are increasingly used in cancer treatments as an eco-friendly alternative to synthetic drugs. Certain bioactive plant components have been revealed to contain strong anti-cancer effects.<sup>[31]</sup> Organic anti-cancer drugs are preferred over synthetic ones owing to its lesser side effects and affordability, making them a popular choice for cancer treatment. Developing alternative treatments with reduced side effects is important.<sup>[32]</sup> As long as we know, plants are well known to have anti-cancer properties. The National Cancer Institute conducts research on many plant species to identify possible characteristics that might be used in the development of anti-cancer treatments, with roughly 3,000 indicating proven anti-cancer activity.<sup>[33]</sup> Herbal medicines, such as *Catharanthus roseus*, *Taxus brevifolia* Nutt, and *Taxus baccata*, are extensively utilized as supplemental or alternative choices in cancer.<sup>[34]</sup> Secondary metabolites, including alkaloids, flavonoids, and terpenes, exhibit geno-protective properties, with alkaloids being commonly employed in cancer therapy. Paclitaxel, derived from Pacific yew tree bark, disrupts microtubules, promoting cell death. Vinca alkaloids inhibit microtubules and cell cycle arrest, which is crucial in cancer treatment and chemotherapy.<sup>[35]</sup> Curcumin, found in turmeric, helps those with NAFLD by lowering inflammation, protecting cells, and controlling lipid metabolism and insulin sensitivity.<sup>[19]</sup>

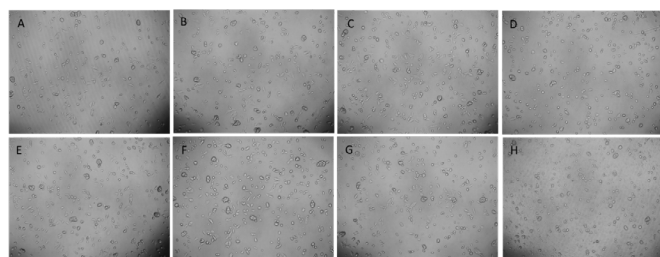
Among the members of the Euphorbiaceae family, *Phyllanthus niruri*, known as "stonebreaker," has promising actions against liver illnesses such as hepatitis and liver stones and perhaps reduces liver cancer cell growth.<sup>[17]</sup> The genus *Chrozophora*, comprising species like *C. tinctora*, *C. senegalensis*, and *C. plicata*, is renowned for its diverse biological effects, including as antioxidant antibacterial and anti-cancer properties. Phytochemical and pharmacological investigations have revealed numerous components contributing to its antioxidant and antibacterial activities.<sup>[36]</sup> Furthermore, species of *Chrozophora* have gained attention for their antioxidant and cytotoxic properties due to their phytochemical composition, which includes flavonoids and phenolic. Prior research consistently highlights the cytotoxic effects of *C. tinctoria* extracts, suggesting their potential as anticancer agents.<sup>[37]</sup> Further, it is reported that *C. rotleri* leaf extract is a natural anti-mutagenic agent that works well against EMS-induced mutations. This is because it contains antioxidants like polyphenols and flavonoids.<sup>[21]</sup>

The results of the current investigation coincide with many previous findings. Where plant extracts' cytotoxicity

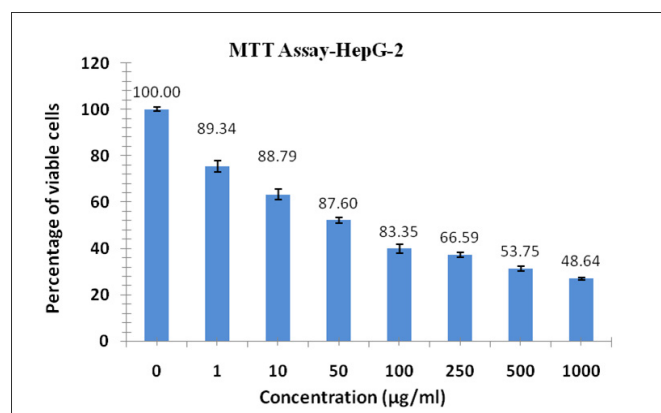
**Table 2:** Efficacy of CRFEE in eradicating liver cancer cells (HepG-2 cell line)

S. No	Sample conc. ( $\mu\text{g/mL}$ )	Percentage of cell viability	$IC_{50}$ value ( $\mu\text{g/mL}$ )
1	0	100 $\pm$ 0.71	63 $\pm$ 0.08
2	1	89.34 $\pm$ 2.45	
3	10	88.79 $\pm$ 4.64	
4	50	87.60 $\pm$ 2.11	
5	100	83.35 $\pm$ 1.05	
6	250	66.59 $\pm$ 4.55	
7	500	53.75 $\pm$ 3.49	
8	1000	48.64 $\pm$ 1.77	

Values are presented as mean  $\pm$  standard error of mean; the experiments were replicated (n = 2).



**Fig. 3:** Cytomorphological variations caused by different concentrations of CRFEE on HepG-2 cell line. Concentrations of extract (A) control, (B) 1- $\mu\text{g}$ , (C) 10  $\mu\text{g}$ , (D) 50  $\mu\text{g}$ , (E) 100  $\mu\text{g}$ , (F) 250  $\mu\text{g}$  and (H) 500  $\mu\text{g}$



**Fig. 4:** Cytotoxicity of CRFEE against HepG2 cell line through MTT assay

on cancer cell lines has been shown in various species of *Chrozophora*. Similarly, *C. rottleri* leaf extracts have also shown antioxidant properties.<sup>[21, 26]</sup> In this study the research focused on fruit extract showed comparable or even better antioxidant activity. Since there were no earlier reports on the anti-cancer properties of *C. rottleri* fruit extracts, this research was undertaken to examine the antioxidant and cytotoxic properties of ethanol extracts from this fruit.

*C. rottleri* fruit ethanol extract exhibited significant scavenging efficacy against both radicals, suppressing hydroxyl radicals by 48.25% with an IC<sub>50</sub> of 35.08 ± 1.62 µg/mL and nitric oxide by 49.35% with an IC<sub>50</sub> of 307.44 ± 2.28 µg/mL. Rich in polyphenols and flavonoids, CRFEE displays powerful antioxidant capabilities, reducing oxidative stress and DNA damage associated to cancer formation. Furthermore, its cytotoxic effects on liver cancer cells exhibited an IC<sub>50</sub> of 63 ± 0.08 µg/mL, showing potential efficacy in suppressing HepG-2 cell growth. Key components include rutin, saponins, steroids, apigenin 7-o-b-d-[6-(3, 4-dihydroxy benzoyl)], glucopyranoside (chrozophorin), and acacetin 7-orutinoside may contribute to its therapeutic attributes. The presence of these bioactive chemicals likely adds to its impact of cytotoxicity on the HepG-2 cell line, highlighting its potential as a therapeutic intervention for liver cancer. However, further work is necessary to identify the particular molecule responsible for this cytotoxic effect.

## CONCLUSION

Studies show that *C. rottleri* fruit ethanol extracts have The plant contain various bioactive compounds and show strong antioxidant properties, protecting cells against oxidative stress and DNA damage that may lead to cancer. These extracts reduce free radical levels, preventing cancer initiation and progression. Additionally, their antioxidant properties might increase the efficiency of common cancer therapies like chemotherapy and radiotherapy by reducing side effects. Future research on *C. rottleri* should focus on identifying and characterizing its bioactive compounds, their impacts on human health, and their potential for developing new, natural, and effective therapeutic agents.

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