**Investigation on the Cytotoxic Activity of Alcoholic Extract of Eucalyptus camaldulensis on Breast Cancer Cell Line**

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**Abstract**

The spread of different types of cancer has been increasing in the recent century. The use of chemical drugs, in addition to the development of drug resistance, also cause serious side effects. *Eucalyptus camaldulensis* (*E. camaldulensis*) is one of the most famous herbal remedies considered owing to its anti-inflammatory and boosting the intimate immune system, and has some anti-proliferative effects on cancerous cell lines. In the current study the cytotoxic activity of alcoholic extract of *Eucalyptus camaldulensis* (*E. camaldulensis*) at different concentrations 6.125, 12.5, 25, 50 and 100 µg/mL was investigated on breast cancer cell line MCF-7. To study the antioxidant efficiency of ethanolic extract of *Eucalyptus camaldulensis*, 2,2'-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS)) analysis were used. By FTIR analysis and Phytochemical screening, the results showed many active compounds found in this extract like Tannins, Saponins, Phenolic compounds, Reducing sugar, Terpenoids, Steroids, Glucosides, Alkaloids and Flavonoids. Also the results were demonstrated that this extract significantly inhibited the growth of MCF-7 cell line in a concentration dependent manner in comparisons to the control, and the cytotoxic activity of this extract increase with the increase of the concentration. The results indicate that *E. camaldulensis* can be considered as a particularly valuable source of effective anti-proliferative and cytotoxic agents. The experimental findings demonstrated that *E. camaldulensis* extract possessed significant antioxidant efficiency and anti-proliferative effects on cancerous cell lines.

**Keywords:** Antioxidant, Anti-proliferative, Breast cancer, Crud extract, Ethanol
1. Introduction

Herbs have been used since ancient times to improve the flavor and aroma of foods as well as maintain their nutritional value. One of medicinal plants is Eucalyptus, which are widely found in Mediterranean region. The Eucalyptus species used in many medical application as antipyretic remedies for the symptoms of infections of the respiratory system, like sinus congestion, flu, cold, astringent in dentistry analgesic, anti-inflammatory, and treatment of illness, bladder inflammation and diarrhea (Silva et al., 2003). There are many essential oils produced by Eucalyptus, which have high biological activities (Herzi et al., 2013). These oils used in medicinal and pharmaceutical purposes (Huang et al., 2015).

Leaves of Eucalyptus used traditionally to treat fungal infections and to heal wounds, expectorant, antimicrobial properties, analgesic and anti-inflammatory (Döll-Boscardin et al., 2012). In addition, traditional herbal medicines are of great importance in developing countries and 80 percent of the population depends on them for their primary health care needs (Garg and Paliwal, 2011). These compounds are known as secondary metabolites and have biological activities such as modulat in detoxification enzymes, prompt the immune system, reducing the aggregation of platelet, modulating the metabolism of hormone, as well as antioxidant, antimicrobial, and anti-cancer activities (Saidulu et al., 2014). Phytochemicals contain terpenoids, phenolics, and alkaloids (Dillard and German, 2000). Many of these compounds have cytotoxic effects on cancer cells like Alkaloids and Phenols and have antioxidants activities. Cancer is a malignant disease characterized by abnormal division and differentiation of cells, which leads to a large increase in the number of dividing cells that later collect to produce the tumor, or spread to the rest of the body tissue by blood or lymph. The cancer cell feeds itself through a method called the angiogenesis process in which the growth of a network of blood vessels will raise creation of angiogenesis activators and reduce production of angiogenesis inhibitors (Deshmukh and Sakarkar, 2011). Eucalyptus camaldulensis is an agricultural crop and has been used as raw resources in plywood sheets, epoxy putty and solar industry. Both the sleepers of railway trains, the shipbuilding, land, fuel and heavy buildings (Awan et al., 2012) are suited to Eucalyptus camaldulensis timber. It is an evergreen tree found in Pakistan, India, Australia, Nigeria and Egypt, Iraq and other areas of the country. In conventional treatments, Eucalyptus camaldulensis was commonly included as anesthetic, antiseptic and astringent in several different conditions (Musa et al., 2011). Its new and young leaves are boiled with water in Iraq and flu treatment is carried out using decoction (Sultana et al., 2006). Eucalyptus extracts and essential oils have been very concerned about the chemical structure and biological processes in nutrition and drug industries (Barra et al., 2010). Even though some of the research on Eucalyptus camaldulensis as an antioxidant compounds or cancer have been conducted (El-Ghorab et al., 2003), no studies about the use of
"Eucalyptus camaldulensis" extracts as an antioxidant compounds and anticancer have been reported. As scientists have reported, crown gall cancer reduction (induced by A. tumefaciens) on potatoes disks is obviously in line with natural products involved in anticancer test 3PS (leukemic mouse model). Several other study groups (Ashrafet al., 2015) have also confirmed that, considering the molecular mechanism, a potatoes disc test is a potent predictor of tumor activity. In spite of development in detection, determination and treatment, Breast cancer is one of the common threatening tumors in ladies. A comprehensive and efficient treatment required for these patients, endocrine treatment and counting systemic chemotherapy should focus on targeted treatment. For repetitive metastatic breast cancer (rMBC) one of the most viable treatment choices is the systemic chemotherapy (Bibi et al., 2010). There are some chemical compounds such as acetate, crystallization substance, valeric aldehyde and ethylic alcohol in small amount. The most important and main material found in most species of eucalyptus composed and 60-80% is cineole (Barrett-Lee et al., 2009). The only used part in the treatment is the Eucalyptus leaf. The leaves in the Middle-aged are more appropriate which have refrigerant, disinfectant effects and vasoconstrictor. Moreover, Eucalyptus leaves contain tryneol, aliphatic aldehyde, eucalyptol (cineol), phenols, sesquiterpene alcohols, isoamyl alcohols, terpenes, and flavonoids (Lopez and Hunson, 2002). This study aimed to evaluated to the cytotoxic activity of crud extract of "Eucalyptus camaldulensis" plant against to breast cancer MCF-7 cell line. In addition, this investigation also includes study of antioxidant activities of various concentrations of solvent ethanolic extract of Eucalyptus camaldulensis.

2. Materials and Methods

2.1. Plant materials: Preparation of extract

Dried "Eucalyptus camaldulensis" plant was extracted by the soxhlet with ethanol 70%. Using a rotary evaporator to completely remove the extracts and get a semi-solid mass, then transferred to an oven to produce the crude extract, stored at 4°C until use (Akin-Osanaiye et al., 2007).

2.2. Phytochemical Screening of Eucalyptus camaldulensis Extract

Phytochemical constituents such as tannins, saponins, phlobatanins, phenolics, reducing sugar, trepenoid, steroid, glycosides, alkanoids and flavonoids of the crude extracts were analyzed. "Eucalyptus camaldulensis" extracts (2 ml each) were utilized separately for each analysis, in a way that formation of precipitate, color change, or frothing indicates presences of the phytochemicals mentioned above (Ayepola and Adeniyi, 2008).

2.3. FTIR assay

Fourier transmission infra-red (FTIR), and UV Spectrum (Shimadzu) analyses were performed in the Lab of Iben Sena center/ University of Baghdad, Iraq.
2.4. Radical scavenging activity by DPPH

The evolution of scavenging activities by the utilization of the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical. The DPPH radical antioxidant activity of ethanolic extract was evaluated based on the technique which was published in the references (Nagpal et al., 2010 and Nehia, 2018). DPPH radical has a highest absorptance at 515 nm, that vanishes with degradation by a scavenging molecule. The DPPH• environment in methyl alcohol (0.006 mM) was synthesized every day, and 3.0 mL of this solution was combined with 100 µL of plant extract solution (6.125, 12.5, 25, 50 and 100 µg/mL). The incubation of eachsample was at 37°C for 20 min., absorbance was reduced at 515 nm. A solution of DPPH• (100 µL) of methyl alcohol as a blank solution was prepared for evaluation the absorption. The measurement was repeated triplicate. The antioxidant activity was calculated according to Equation (1):

\[
\text{Inhibition activity} = \frac{\text{Blank absorbance} - \text{Extract absorbance}}{\text{Blank absorbance}} \times 100 \quad (1)
\]

2.5. Radical scavenging activity by ABTS

The antioxidant efficiency of *Eucalyptus camaldulensis* extract was also investigated utilizing the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay (Miliauskas et al., 2004), according to reduce of ABTS+• radicals by scavengers of the plant *Eucalyptus camaldulensis* extract examined. The double deionized water was used to dissolve ABTS compound (0.007 M). ABTS+• was generated through the chemical reaction of ABTS medium with 0.00245 M K₂S₂O₈ and the solution allowed to be in the dark place for 16 h., at 303 K. In the current investigation, the ethyl alcohol was used to dilute the solution of ABTS+• to an absorbance 734 nm. Ethyl alcohol as a reading of blank solution was used. Then after 100 µL of Alcoholic extract of *Eucalyptus camaldulensis* to the solution ( 3 mL) of ABTS+•, at 303 K. All tested solutions have been utilized after 10-60 min., of preparation. Each experiment was conducted for three times and the mean was used. The inhibition performance of *Eucalyptus camaldulensis* solution against ABTS+• was evaluated regarding to Equation (1).

2.6. Detection of the toxicity: Maintenance of cell cultures

Cell line of Breast cancer MCF-7 cell line, from the unit of Cell Bank of Iraq biotech, reserved in RPMI-1640 supplemented by 10% fetal bovine serum, streptomycin and penicillin antibiotic’s (100 units/mL). Cells were passaged by Trypsin-EDTA re-seeded at 50% confluence for two weeks at 37 °C (Re et al., 1999).
2.7. Cytotoxicity Assays

Cytotoxic effects were determined by the MTT test. Ethanolic extract of *Eucalyptus camaldulensis* treatment by using different concentrations (6.125, 12.5, 25, 50 and 100 µg/mL). Cell line were cultured at $1 \times 10^4$ cells following overnight incubation, the monolayer was achieved, then the extract was applied to the cells. After 72 h the viability of the cells was measured by adding 28 µL of 2 mg/mL solution of MTT after removing the medium and incubating the cells for 1.5 h at 37°C. Then the MTT solution was eliminated, and solubilized the remaining crystals in the wells by adding 130 µL of DMSO then incubate at 37°C for 15 minutes with shaking (Al-Shammari et al., 2015). The absorbency was measured by a micro plate reader at 492 nm. The percentage of cytotoxicity determined as the following equation (2):

$$\text{Inhibition activity} = \frac{\text{Control optical density} - \text{Sample optical density}}{\text{Control optical density}} \times 100$$

3. Results and Discussion

3.1. Phytochemical Screening

Preliminary Phytochemical Screening of *Eucalyptus camaldulensis* extract Based on the preliminary screening it is revealed that the phlobatanninns, reducing sugar and steroids were absent in Ethanol extract of *E. camaldulensis*. While the active compound present in alcoholic extract of *Eucalyptus camaldulensis* are Tannins, Saponins, Phenolic compounds, reducing sugar, Terpenoids, Steroids, Glucosides, Alkaloids Wagner’s test and Flavonoids Ferric chloride test are positive as shown in table 1.

3.2. FTIR assay

Results in figure 1 showed that the high severe, band 3317-3340 cm⁻¹ which signify refer to (OH) groups, also band 1350,1037 cm⁻¹ refers to the incidence of a symmetrical patterns to the CH3 groups of alcoholic composite. 1618/cm⁻¹, band refer to the incidence of C=C group (Al-Shammari and Salman, 2016).

3.3. Anticancer activity of *E. camaldulensis*

The cytotoxic activity was examined as shown in figure 2. Ethanolic extract of *Eucalyptus camaldulensis* treatment with some different concentrations (6.125, 12.5, 25, 50 and 100 µg/mL) indicated there is a decrease in the percentage of MCF-7 cell viability and increase in the cytotoxic activity on the MCF-7 cells.
The cytotoxic activity of Ethanolic extract of *Eucalyptus camaldulensis* treatment increase with the increase of the concentration of the extract. The results illustrated that treatment of the cells with *Eucalyptus camaldulensis* significantly inhibit the growth of cells with the increase in extract concentration. The results showed that *Eucalyptus camaldulensis* is deemed to be valuable source of effective cytotoxic and anti-proliferative agents. Crud extract of *Eucalyptus camaldulensis* have the ability to apoptosis-inducing and proliferation-inhibiting effects in human body lead to killing cancer cells. In this study, the anti-tumor effect of breast cancer cells (MCF-7 cell line) was investigated using the crude alcoholic extract of *Eucalyptus camaldulensis* plant. Inducing apoptosis by anticancer drugs through exert their antitumor effects against cancer cells is a significant phenomenon in cancer chemotherapy (Adorjan and Buchbauer, 2010).

Table1. Preliminary Phytochemical Screening of *Eucalyptus camaldulensis* extract.

<table>
<thead>
<tr>
<th>Types of Active compound</th>
<th>Presence in alcoholic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>+ve</td>
</tr>
<tr>
<td>Saponins</td>
<td>+ve</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>+ve</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>-ve</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-ve</td>
</tr>
<tr>
<td>Steroids</td>
<td>+ve</td>
</tr>
<tr>
<td>Glucosides</td>
<td>-ve</td>
</tr>
<tr>
<td>Alkaloids Wagner’s test</td>
<td>+ve</td>
</tr>
</tbody>
</table>

In comparison to the typical round nuclei of the control, crud extract treated cells show up condensed and fragmented nuclei. It was noticed that level of apoptotic cell demise was maximum.
This study supports the usage of this plant which have significant effects on the cancer cell line, suggested that may be used to prevent the cancer while this is a good reason for further investigations in vitro and in vivo to understand more about the mechanisms of cell death (Ghassan et al., 2013). One of the most important reasons that cause death in the world is cancer. The Resistance to the drugs used as anticancer therapies has been recently shown which highlights the urgent need to conduct further research to discover plant-derived substances that act as anticancer. Many plants, vegetables, herbs and spices might have the potential use in medicine as a source to the prevention of cancer, which necessitates further studies of these plants to know their biological properties and therapeutic potential (Aggarwal and Shishodia, 2006). The results agreed with (Salomons et al., 1999) who revealed that the cell viability decrease with increase of the extract concentration. Also, *Eucalyptus camaldulensis* leaf ethanolic extract in vitro studies proved a cytotoxic effect on some types of leukemia like (k562 cells) human chronic myelogenous leukemia cells.

Many active compounds contained in Eucalyptus such as alkaloids, polyphenols, flavonoids, tannins, steroids, sterols, glycosides and fatty acids. Some of them like phenols and alkaloid have significant antioxidants activities and anti-cancer, apoptosis of cancer cells can be induced by phenol. At the same way, there are effects of the flavonoids as anti-tumors and anti-mutagens. Also by their effect on signal transduction in angiogenesis and cell proliferation so they have a protective effect against cancer (Mohamad et al., 2020). Also agreed with (Sun et al., 2015) who work on K562 cell line and found that 50 µg/ml had the highest cytotoxic effect and it is the best concentration in destroying the cancer cells.
Alcoholic Extract of *Eucalyptus camaldulensis* demonstrated the inhibitive performance (94.51% of DPPH inhibition), at the highest investigated concentration. The results of ABTS, were started from 67.6% to 97.1%, that were the various concentrations of Alcoholic Extract of *Eucalyptus camaldulensis* which was best than that of DPPH analysis. Alcoholic Extract of *Eucalyptus camaldulensis* possessed the highest scavenging activity 97.1%, regarding to the highest utilized concentration. Scavenging activities of Alcoholic Extract of *Eucalyptus camaldulensis* were depended on concentration of plant extract and also the conditions of the utilized measurements. Many reported techniques are available for the in vitro calculation of total antioxidant power, which could be divided into two kinds: hydrogen atom transfer testing (HAT) and electron transfer testing (ET). HAT-based testing, such as the ORAC test, uses a dynamic reaction system in which the scavenger and substrate compete for radicals generated thermally. It is known and certain that there is one sufficient and comprehensive technique to know the efficiency of a particular substance and study its ability as an antioxidant. Where more than one type of measurement method must be performed on the strength of antioxidants to take into account the types of action and mechanisms of different antioxidants (Naeimeh et al., 2014).

In this research, the free radical removal ability of a plant extract was determined using DPPH and ABTS techniques. DPPH and ABTS measurements have been used extensively and with multiple concentrations of plant extract to determine the viability of the plant extract as an antioxidant because it requires relatively standard equipment and delivers rapid and reproducible results. Indeed, comparative studies of six recently published methods of determining antioxidant capacity have shown that DPPH and ABTS techniques are faster, easier, and more real (Huang et al., 2005). The ABTS scale is a technique of particular interest in plant extracts because wavelength absorption at 734 nm eliminates color interference (Prior and Cao, 1999). Moreover, this test is easy to perform, like the DPPH and ABTS assays (Buenger et al., 2006).
It may be concluded from the present findings that Eucalyptus camaldulensis plant can be used as a probable source of innate cytotoxic potential. However, more research is needed for the identification of biologically activity for the compounds that present in this plant. The ethanolic extract showed a wide range of potentially promising antioxidant efficiencies. The ethanolic showed a significant scavenging effect with all measuring techniques.

4. ACKNOWLEDGMENTS
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5. CONFLICT OF INTEREST STATEMENT
We declare that we have no conflict of interest.
References


