

Cytotoxicity Evaluation of Eucalyptus camaldulensis leaf extract against Cholangiocarcinoma Cell lines (CL-6)

Asmare Amuamuta (✉ asmareamu2005@yahoo.com)

Bahir Dar University

Birhanu Berihun

University of Gondar

Research Article

Keywords: Cholangiocarcinoma, E. camaldulensis, cytotoxicity, in vitro

Posted Date: March 22nd, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2710703/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Cholangiocarcinoma(CCA) is an important public health problem in many tropical and subtropical parts of the world. *Eucalyptus camaldulensis* leaves as well as its essential oil extracts are well known for its traditional use and pharmacological actions including insect repellent/insecticidal activity, antimicrobial, cytotoxic/anticancer activity as well as for many other disorders in different parts of the world including Africa and Southeast Asia.

Materials and Methods

This study was conducted to investigate the cytotoxic activity of *E. camaldulensis* leaf extract preparations at *in vitro* against the CCA tumor (CL-6 cell line). *E. camaldulensis* leaves collected from Ethiopia (Western Gojjam and Southern Gonder area) was used for extract preparation in 95% ethanol using different extraction methods, *i.e.*,ultrasonication, heating reflux, and maceration (1 day, 3 days and 7 days). The extract and the standard anticancer drug preparation 5-fluorouracil (5-FU) were evaluated in triplicate for their cytotoxic activities against CL-6 cell line and normal human cell line (OUMS) using standard MTT cell proliferation assay at eight serial dilution concentrations (3.8–500µg/mL).

Results

A semi-solid brown black residue with a characteristic odor was obtained after solvent removal and extract concentration was done by using a rotary evaporator at 60°C. The highest (mean ± SD) percent yield (3.73 ± 0.61) was obtained by using maceration technique with the solvent for 7 days, followed by heating reflux (1.73 ± 0.80) and the least was obtained from 1day maceration method (0.90 ± 0.52). The extract was then stored at -20°C until use. The cytotoxic activity (mean ± SD IC₅₀ values) *E. camaldulensis* extract and 5-FU against CL-6 were 102.29 ± 5.87 and 106.75 ± 3.06 (mean IC₅₀ ± SD) µg/mL, respectively. The extract's IC₅₀ value observed in normal OUMS cell line was 165.9 ± 10.3 .

Conclusion

The extract of *E. camaldulensis* collected from the study area exhibited *in vitro* cytotoxic activity against human CCA tumor cell line (CL-6). Further *in vitro* and *in vivo* evaluation of the selected herbal extract preparation is recommended in the search for development of safe and promising drug options against CCA and other cancer types.

Introduction

Cholangiocarcinoma (CCA) is an important public health problem in many tropical and subtropical parts of the world. The highest prevalence of CCA in Northeast of Thailand was associated to the consumption of improperly cooked and fermented or preserved cyprinid fish species food which contains the fluke, *Opisthorchis viverrini* [1]. The major challenge for CCA control and treatment is due to the lack of early diagnosis and multidrug or radio-resistant nature of CCA tumor [2] which requires an ongoing and urgent need for the discovery and development of effective alternative diagnostic tools and chemotherapeutics. The promising therapeutic options in many types of cancers including CCA is the use of combination therapies of standard treatments (e.g. 5-FU) and in conjunction with alternative therapy with dietary phytochemicals are becoming [3].

Approximately 80% of the population in developing countries depend on TM (also known as complementary and traditional medicine or CAM) for their primary health care [4]. In addition to such therapeutic options, TM has also afforded a rich source of remedies or drugs clinically used currently for cancer therapy with diverse chemical structures and bioactivities against several health disorders including cancer and several modern anticancer drugs approved by FDA (including vinblastine, vincristine, etoposide, teniposide, paclitaxel, vinorelbine, docetaxel, topotecan, and irinotecan) have been developed from plant sources. A comprehensive discussion of these plant-derived and clinically approved anticancer agents is presented in a previous review [5].

Eucalyptus, with almost 900 species, is found worldwide; more than 300 species of this genus contain volatile oils in their leaves. There were three broad categories of uses for Eucalyptus oil (medicinal, industrial and perfumery/flavouring). *Eucalyptus camaldulensis* (*E. camaldulensis*) leaves are traditionally used to treat wounds and several other disorders [6]. The leaves as well as stems and immature flowers of *E. camaldulensis* essential oil extracts are well known for its action as insect repellent/insecticidal activity, analgesic/anti-inflammatory, cytotoxic or anticancer activity as well as for many other disorders in different parts of the world including Africa and Southeast Asia [6, 7, 8, 9, 10]. The leaf extract essential oils of Eucalyptus species showed an excellent antibacterial activity (inhibitory effect on *S. aureus* and *E. coli*) [11]. The main constituent of the volatile oil derived from fresh leaves of Eucalyptus species include 1,8-cineole (up to 54–95%). Beside 1,8-cineole, the oil contained monoterpene hydrocarbons (such as cymene, α -pinene, β -pinene and limonene, geraniol and camphene), aromadendrene, cuminaldehyde, globulol and pinocarveol according to the previous reports [6]. Monoterpenes hydrocarbons were predominant in the essential oil sample extracts of *E. camaldulensis* and the most abundant compounds in the three essential oils were γ -terpinene, as a principal oil component (57.4–72.5%) followed by o-cymene (14.6–26.3%) and terpinen-4-ol (6.6–16.2%) [7].

Up on reviewing the current literature, *in vitro* cytotoxic activity of the essential oil extract of *E. camaldulensis* was reported in some geographic location including against Ehrlich ascites carcinoma (EAC) cells [7], against colorectal cancer cell lines [8] and against breast cancer cell lines (MCF-7) [10]. However, there has no report found at *in vitro* and *in vivo* anticancer activity of the specific plant species in the study location and also little evidence for the cytotoxic activity of *E. camaldulensis* against CCA cell line (CL-6). Therefore, the aim of the study was to determine the leaf essential oil extract contents of

the specific plant and to evaluate the cytotoxicity (anti-CCA activity at *in vitro*) of the crude leaf extract of *E. camaldulensis* grown in Ethiopia and compare with other previous studies from other countries.

Materials And Methods

Reagents and cell lines

The solvent used for plant extraction (95% ethanol) was commercial grade and obtained from Labscan Asia Co. Ltd. (Bangkok, Thailand). The cell culture reagents including RPMI 1640, DMEM, fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin and streptomycin antibiotics were obtained from Gibco Life Technologies (NY, USA). CCA cell line (CL-6) used for both *in vitro* and *in vivo* study was kindly supplied by Professor Kesara Na-Bangchang, Chulabhorn International College of Medicine, Bangkok, Thailand. Methyl thiazol diphenyl tetrazolium (MTT) reagent was obtained from Life Technologies (CA, USA) and dimethyl sulfoxide (DMSO) was obtained from MP Biomedicals (CA, USA). 5-FU was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and tween-80 was obtained from Sigma-Aldrich (MO, USA).

Plant material collection and crude extract preparation

The dried leaves of the plant *E.camaldulensis* was obtained from the study areas (Northwest Ethiopia) in consultation with the traditional herbalists. Botanical identification and authentication of the plant material was carried out at the National herbarium, Addis Ababa University, Department of Plant Biology and Biodiversity, where voucher specimen was deposited with voucher number 0012 (*E. camaldulensis*). The plant leaves were collected from the study areas (Western Gojjam and Southern Gondar), Ethiopia, by informed personnel for collection along with the two investigator close supervision to obtain ample amount of leaves from several trees of the specific plant. Then the leaves were washed under running tap water to remove adhering dust, air dried under shade for 7 days and then powdered using a grinding mill. Extraction techniques including sonication, heating under reflux and maceration (1 day, 3 days and 7 days) were conducted by using the solvent 95% ethanol and the plant powder. An appropriate solvent (300ml) was mixed with a sample powder of about 10gm and sonicated with the ultrasonicator (Elma™, Meditop) for about 75 minutes. The plant rhizome of about 10gm in 300ml of ethanol was also used for heating under reflux by using water bath (Mettler, Germany) for about 50 minutes according to the previous methods with modification [12]. For maceration technique, about 10gm of the powdered leaves of *E.camaldulensis* were also mixed with in 300ml of ethanol with small flasks and allowed to wait for 1 day, 3 days and 7 days. Later on, about 1,000gm powdered plant material (rhizome) was added in each three large clean flask of containing 5 liters of 95% ethanol and allowed to wait for 7 days at 25–30°C by occasional shaking and stirring. The extracts were separated first (filtered) with gauze and then through Whatman no. 1 filter paper (GE Healthcare^R, England). The residues were re-macerated for another 24 h and then filtered as indicated above, followed by extract evaporation to remove the solvent by using a rotary evaporator (Heidolph, Germany) and then further concentration by water bath. The crude extract obtained by using the different methods was weighed for percentage yield calculations and stored at

-20°C until used (as shown below) to ensure the apparent stability and activity of the extract according to the previous methods [12, 13].

In vitro **cytotoxicity evaluation of** *E. camaldulensis* **leaves extract**

The crude ethanolic extract from *E. camaldulensis* was initially dissolved in 50% ethanol. The concentrated stock solution of about 5mgextract was prepared by adding in a known volume (500 µl) of solvent (50% ethanol) to prepare the initial highest concentration (500 µg/ml) of extract preparation in fresh medium, and then serially diluted (1:2) with the media to obtain the working solutions at eight final concentrations (*i.e.* 500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 µg/ml) according to the previous methods used [14]. Positive control agent 5-FU was also prepared as a stock solution by dissolving in 50% ethanol (500 µl) and then serially diluted with fresh medium. The concentration of 5-FU to be used also started from 500 µg/ml in fresh medium as an initial highest concentration and then serial dilutions of it was prepared as 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 µg/ml) according to according to the previous methods used with modification [14, 15].

The cholangiocarcinoma cell line (CL-6) was used for cytotoxic screening of *E. camalduensis* leaf extract. CL-6 cell line was cultured in T₂₅ and T₇₅ cm³ culture flasks (Corning Inc., NY, USA) with RPMI 1640 medium (Gibco Life Technologies, NY, USA) supplemented with 10% heated fetal bovine serum (FBS) and 100 IU/ml of penicillin-streptomycin solution. The cell cultures were maintained at 37°C in a 5% CO₂ atmosphere with 95% humidity according to the previous methods used [14]. When the optimum confluency of cultured cells was reached (90%), cells were removed from the culture flask by trypsinization, collected in a 15ml conical tube, centrifuged at 100 × g for 5 min at 25 °C, the supernatant was removed and cells were resuspended in 2 ml of complete media and the cell number was counted using a hemocytometer chamber.

The MTT colorimetric assay with some modification was used to screen for cytotoxic activity of the plant extract and standard drug preparations on cultured CL-6 cells in 96-well plates (Corning Inc., NY,USA). The cells were seeded at a density of 10⁴ cells/well in 100 µl culture medium and after 24-h incubation and attachment, the cells were treated with different concentrations (serial dilutions) of the plant extract and 5-FU prepared (as mentioned above). After 48h incubation, MTT solution (20 µl of 5 mg/ml in PBS) was added and then incubated at 37°C for 3 h, followed by removal of the media and then cells were lyzed with dimethyl sulfoxide (DMSO). Absorbance (OD) was measured at 570 nm using a Varioscan™ flash microplate reader machine (Thermoscientific, MA,USA) and finally the percentage of cytotoxicity compared to the untreated cells was determined with the equation according to the previous methods [16]

$$\% \text{ Inhibition of cells} = \left[\frac{1 - \frac{A - \text{Blank}}{B - \text{Blank}} \times 100 \right]$$

Where, A is absorbance (OD) of control cells and B is absorbance of treated cells.

The results were evaluated from three independent experiments (triplicates) and Calcsyn^R version 1.1 software (Biosoft, Cambridge, UK) was used to calculate the IC₅₀ value (concentration that inhibits cell growth by 50%). Similarly, another cultured normal fibroblast cell lines (OUMS) were cultured in Dulbecco's modified eagle's medium (DMEM) and treated with the *E. camaldulensis* extract, and 5-FU for MTT cell proliferation assay and estimation of selectivity index [14, 15]. Selectivity index was calculated from IC₅₀ ratio of normal fibroblast cell lines (OUMS) and CCA cell lines (CL-6).

Data analysis

Data was presented with Tables and Figures and quantitative variables were expressed as number (n), percent (%) and mean (mean ± SD) values.

Results And Discussion

The ethanolic leaf extract of *E. camaldulensis* was a semi-solid oily solution and with characteristic odor and amber colored. Other previous studies also reported essential oil extract from the plant with such characteristic odor although color was light in Malaysia [7]. The percent yields (w/w) obtained by using the different extraction methods in the present study (sonication, heating reflux, 1 day maceration, 3 days maceration and 7 days maceration) done in triplicates for each method was evaluated. The highest (mean ± SD) percent yield (3.73 ± 0.61) was obtained by using maceration technique with the solvent for 7 days, followed by heating reflux (1.73 ± 0.80) and the least was obtained from 1 day maceration method (0.90 ± 0.52) (Table 1).

Table 1
The % extract yields of *E. camaldulensis* leaf oil extract by using different techniques.

Extraction method	Oil yiled (% w/w)*
Heating reflux	1.73 ± 0.80
Ultrasonication	1.13 ± 0.61
Maceration for 1 day	0.90 ± 0.52
Maceration for 3 days	1.36 ± 0.30
Maceration for 7 days	3.73 ± 0.61
* Oil yield determination was carried out in triplicates and the mean ± SD values were determined for each extraction method.	

As compared to the oil yield from the leaf extract of *E. camaldulensis* using hydrodistillation method (1.40 ± 0.10) in a previous report [7], the present study showed moderately higher percent yield (1.73 ± 0.80) using heating reflux. The yield of medicinal plant extracts which contain the bioactive metabolites vary considerably with plant species, plant parts used and the extraction method or solvent used for extraction [17].The extract of *E. camaldulensis* and the standard anticancer drug preparation 5-

fluorouracil (5-FU) were evaluated in triplicate for their cytotoxic activities against CL-6 cell line and normal human cell line (OUMS) using standard MTT cell proliferation assay at eight serial dilution concentrations (3.8–500 µg/mL). The cytotoxic activity or IC₅₀ value of *E. camaldulensis* extract and 5-FU against CL-6 were 102.29 ± 5.87 and 106.75 ± 3.06 (mean IC₅₀ ± SD) µg/mL, respectively, as shown below on Table 2. The extract's IC₅₀ value observed in normal OUMS cell line was 165.9 ± 10.3 as compared to the value for 5-FU (140.4 ± 8.3) and hence the selectivity index for the herbal extract was about 1.63. The concentration (mean values) versus effect (% inhibition) of the plant extract and 5-FU on CL-6 and normal OUMS cell line cultures was described on Fig. 1.

The cytotoxic activities (calculated from the percent inhibitions) of the plant extract and the standard drug 5-FU showed direct relation (dose dependent increment) for both CL-6 and normal cell lines (OUMS) in the present study (Fig. 1). A previous study [8] on *E. camaldulensis* essential oil on colorectal cancer cell line (CaCo-2) also indicated cancer cell death in a time and concentration-dependent manner that also showed many morphological alterations of cell death in the colorectal cancer cells treated with *E. camaldulensis* essential oil. Another study on the ethanolic extract of *E. camaldulensis* treatment on breast cancer cell lines (MCF-7) with four different concentrations (6.125- 100 µg/mL) indicated a decrease in the percentage of MCF-7 cell viability or an increase in the cytotoxic activity with an increase in extract concentration [10].

The ethanolic leaf extract of *E. camaldulensis* in the present study indicated better cytotoxic inhibitory activity in CL-6 cell lines as compared to the standard anticancer drug 5-FU used ((IC₅₀ = 102.29 ± 5.87 µg/mL vs. 106.75 ± 3.06 µg/mL, respectively). The extract's inhibitory effect on CL-6 cell lines at *in vitro* which indicated moderate cytotoxic activity in the present study was in accordance with the previous ethnobotanical and *in vivo* anti-tumor activity reports [9]. Unlike the *in vitro* cytotoxicity activity of *E. camaldulensis* in this study (IC₅₀ = 102.29 ± 5.87 µg/mL), *E. camaldulensis* leaves essential oil demonstrated a better inhibitory/cytotoxic effects in three tested cancer cell lines; WEHI-3, HT-29 and HL-60 up on which WEHI-3 was the most sensitive (with IC₅₀ = 16.1), followed by less cytotoxic effects in HT-29 and HL-60 cells (IC₅₀ = 50.5, 42.1, respectively [7]. However, the cytotoxic activity of this plant extract in the same study was very low in RAW cells (IC₅₀ = 264.7) as compared with the effect found in present study. Factors including variations in geographic origin of the plant or seasonal changes and extraction methods used for extract preparation could contribute for the % yield variations, cytotoxic, and *in vivo* anti-CCA activity differences [17].

Since thousands of years ago, natural resources such as plants had been used to treatment of various diseases. As described in other previous reports [6, 7, 8, 10], *E. camaldulensis* leaves as well as its other plant parts essential oil extracts are well known for its ethnobotanical medicinal uses and pharmacological actions (including insect repellent/insecticidal activity, analgesic, antimicrobial and cytotoxic/anticancer activity as well as for many other disorders in different parts of the world. Furthermore, the plant has wider global distribution and easy availability for its use/actions. As observed in several other previous reports and the present study, the plant's leaf extract exhibited *in vitro* anticancer

activities and hence promising options for affordable and safe anticancer drug development from this plant against CCA and/or other cancers for use in humans.

Conclusions

It can be concluded that the extract of *E. camaldulensis* collected from the present study area exhibited *in vitro* cytotoxic (antiproliferative) activity against human CCA tumor (CL-6) cell line as compared with other previous reports done in other countries. Further *in vitro* and *in vivo* evaluation of the selected herbal leaf extract preparation, including safety and efficacy, is recommended in the search for development of safe and promising drug options against CCA and other cancer types

Abbreviations

CCA
cholangiocarcinoma
CL-6
cholangiocarcinoma cell line
DMEM
Dulbecco's modified eagle's medium
EC
Eucalyptus amaldulensis
5-FU
5-fluorouracil
IC50
inhibitory concentration by 50 percent
SD
standard deviation.

Declarations

Competing interests

The authors declare that there is no any competing interests.

Authors' contributions

AA and BB developed study concept, collected plant leaves and write-up. AA prepared crude ethanolic plant extract, conducted the *in vitro* experimental study, data collection and data analysis. AA and BB finalized the main manuscript write-up and AA prepared Table 1 and Figure1. AA and BB read and approved the final submitted manuscript.

Acknowledgements

The authors would like to thank Professor Kesara Na-Bangchang, Director of Graduate Studies of Chillabhorn International College of Medicine (CICM), Thammasat University, Thailand for her kind support of reagents and CL-6 cell line. The authors also would like to acknowledge Bahir Dar University, Ethiopia, for study leave and financial support during the study.

Availability of data and materials

All data generated or analyzed for this study are included in this published paper.

Ethics approval and consent

Not applicable.

Consent for publication

Not applicable.

References

1. Haswell-Elkins M, Satarug S, Elkins D. ***Opisthorchis viverrini* infection in northeast Thailand and its relationship to cholangiocarcinoma.** *J Gastroenterol Hepatol* 1992; 7: 538-48.
2. Na-Bangchang K, Karbwang J. **Traditional herbal medicine for the control of tropical diseases.** *Trop Med Health* 2014; 42: 3-13.
3. Vapiwala N, Mick R, Hampshire M, Metz J, DeNittis A. **Patient initiation of complementary and alternative medical therapies (CAM) following cancer diagnosis.** *Cancer J* 2006; 12: 467-74.
4. Zia-Ul-Haq M, Stankovic M, Rizwan K, Feo V. ***Grewia asiatica* L., a food plant with multiple uses.** *Molecules* (Basel, Switzerland) 2013, 18: 2663-82.
5. Martino E, Casamassima G, Castiglione S, Cellupica E, Pantalone S, Papagni F. **Vinca alkaloids and analogues as anti-cancer agents: Looking back, peering ahead.** *Bioorg. Med. Chem. Lett.* 2018; 28 (17):2816–2826 (doi: 10.1016/j.bmcl.2018.06.044).
6. Al-Snafi EA . **The pharmacological and therapeutic importance of Eucalyptus species grown in Iraq.** *IOSR J. Pharm.* 2017;7(3):72-91.
7. Mubarak E., Ali L, Ahmed I, Ahmed A, Taha M. **Essential oil compositions and cytotoxicity from various organs of *Eucalyptus camaldulensis*.** *Int. J. Agric. Biol.*, 2015; 17: 320–32.
8. Elham T, Saba G, Maryam S,, Nasrin S, Fatemeh F. Masoud H, Reza C, Saba H, Alireza I, Mansour H. **Inhibition of Colorectal Cancer Cell Line CaCo 2 by Essential Oil of *Eucalyptus camaldulensis* through induction of apoptosis.** *Acta Medica Iranica*, 2020; 58(6).
9. Farhadul I, Hasina K, Soby G, Ali M, Khanam J. **Bioassay of Eucalyptus extracts for anticancer activity against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice.** *Asian Pac J Trop Biomed*, 2012; 2(5): 394-398.

10. Mohammed W, Soud S, Hameed A, Hussein N. **Assessment of the Cytotoxic Activity of Alcoholic Extract of *Eucalyptus camaldulensis* on Breast Cancer Cell Line** . *Archives of Razi Institute*, 2021; 76(5),1307-1314.
11. Ghalem B, Mohamed B. **Antibacterial activity of leaf essential oils of *Eucalyptus globulus* and *Eucalyptus camaldulensis***. *Afr J Pharm and Pharmacol* 2008; 2(10):211-215.
12. Sasidharan S, Chen Y, Saravanan D, Sundram K, Yoga L. **Extraction, isolation and characterization of bioactive compounds from plants' extracts**. *Afr J Tradit Complement Altern Med* 2011; 8: 10.
13. Builders M, Wannang N, Aguiyi J. **Antiplasmodial activities of *Parkia biglobosa* leaves; *In vivo* and *In vitro* studies**. *Ann Biol Res* 2011; 2: 13.
14. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K. **Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro**. *BMC Complem and Alt Med*, 2010; 10: 55.
15. Amuamuta A, Plengsuriyakarn T, Na-Bangchang K (2017). **Anticholangiocarcinoma activity and toxicity of the *Kaempferia galanga* Linn. Rhizome ethanolic extract**. *BMC Complem and Alt Med* 2017; 17: 213.
16. Umar M, Asmawi M, Sadikun A. **Ethyl-p-methoxycinnamate isolated from *Kaempferia galanga* inhibits inflammation by suppressing interleukin-1, tumor necrosis factor-alpha, and angiogenesis by blocking endothelial functions**. *Clinics* (Sao Paulo) 2014; 69: 134-44.
17. Namwat N, Amimanan P, Loilome W. **Characterization of 5-fluorouracil-resistant cholangiocarcinoma cell lines**. *Chemotherapy*, 2008; 54: 343-51.

Tables

Table 2 is not available with this version.

Figures

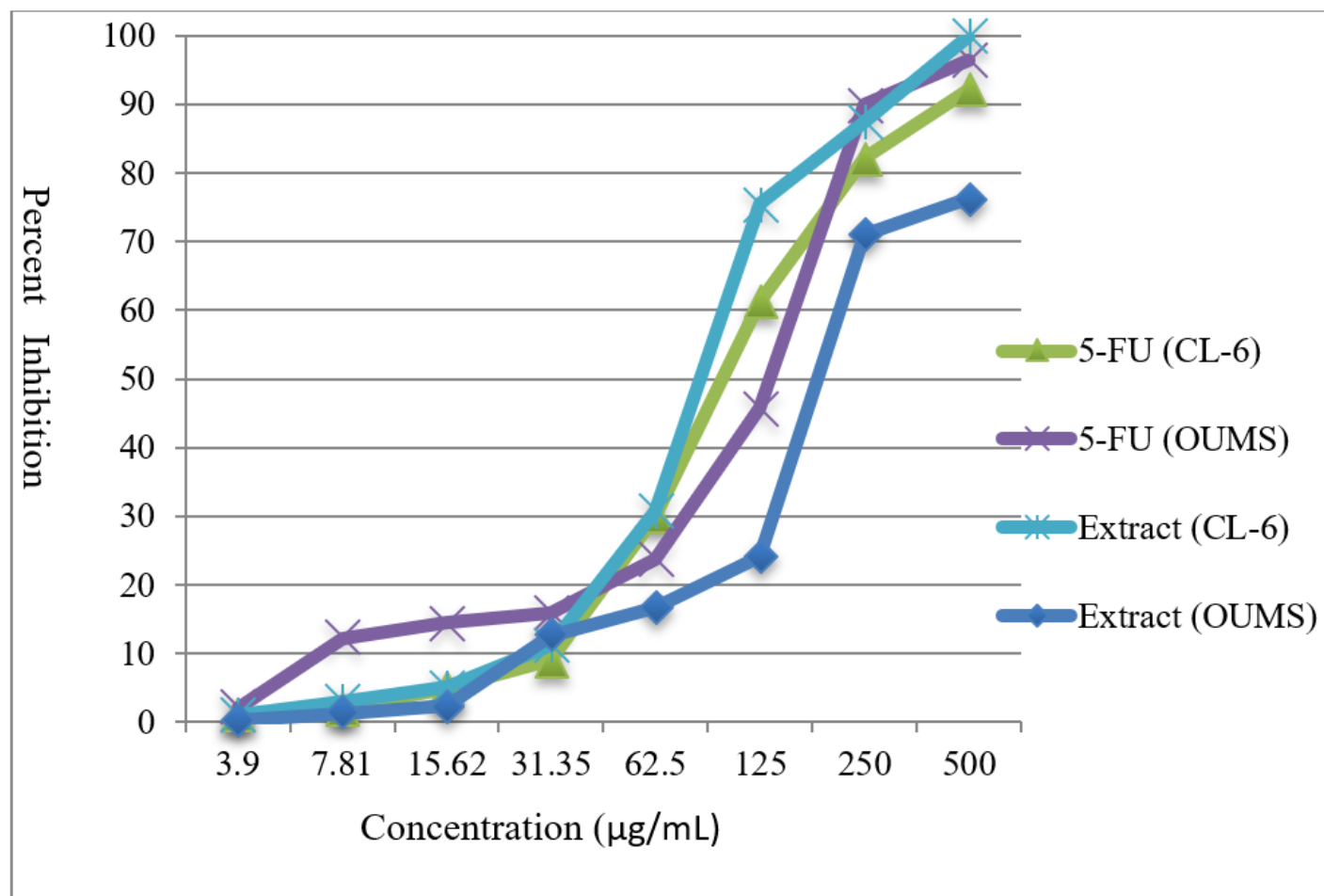


Figure 1

Cytotoxic activity (mean percent inhibition values) of *E. camaldulensis* leaf extract and 5-FU in CL-6 and normal OUMS cell lines at *in vitro*.

* Data was expressed as mean percent inhibition values performed in triplicates to calculate mean $\pm$ SD IC50 values.