Biotech Studies 33(2), 106-117
http://doi.org/10.38042/xx.xx

EARLY VIEW
RESEARCH PAPER

BIOTECH STUDIES

The antioxidant and cytotoxicity capabilities of the total methanol leaf extract of *Camellia yokdonensis*

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Article History
Received 11 June 2024
Accepted 22 October 2024
First Online 28 October 2024

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Keywords
Antioxidant
C. yokdonensis
Cytotoxicity
Leukaemia
Lung cancer

Abstract

Camellia yokdonensis was first described in 2006 and clarified as a Vietnamese endemic Camellia. Camellia species are often associated with strong biological activity and have high application potential. However, there is currently no research on the biological activity of C. yokdonensis. The experiment was performed using DPPH assay and PFRAP assay to investigate the antioxidant capacity of the leaf extract. Besides, a 72-hour cytotoxic assay experiment on cancer cells showed the toxicity of the extract. The results showed that C. yokdonensis leaf extract has the ability to reduce iron ions and scavenge DPPH free radicals with an EC50 of 19.37 ± 1.66 µg/mL. Anticancer activity was determined by IC50 values (µg/mL) on HCC-J5, A549, MCF-7, and K562 cells as 138.00 ± 11.60, 172.90 ± 22.31, 175.52 ± 16.70, and 48.82 ±12.59, respectively.

Introduction

Tea culture is popular in Asia countries; it has gone along with humans throughout history (Wang et al., 2022). Drinking tea is not only a culture or habit; it also brings many crucial benefits (Aboulwafa et al., 2019; Bakhriansyah, Sulaiman, & Fauzia, 2022). Many scientific reports showed the potent antioxidant effects of tea, which contribute to a healthy human life (Khan & Mukhtar, 2013). Tea leaves were used in folk remedies to treat human illnesses such as diarrhea, metabolic disorders, and cardiovascular diseases (Besra et al., 2003; Yang, Chen, & Wu, 2014). Tea extracts are essences with biological activity in modern medicine, typically as catechin derivatives (Chí et al., 2020). The tea family (Theaceae) gather many different individuals; some species are drinkable, and others may have toxins (Li et al., 2022; Nguyet Hai Ninh et al., 2020). *Camellia* is a vital genus that contributes the most regarding the number of species and potential for exploitation and application (Nguyet Hai Ninh et al., 2020). Although many species are used as herbs in traditional medicine, *Camellia* species still require scientific evidence to be applied easily in orthodox medicine. *Camellia sinensis* is the most popular type of tea, and it has been exploited and scientifically researched. The potent antioxidant capacity of *C. sinensis* extracts has been reported (Chí et al., 2020). Besides, it is also famous for its anti-inflammatory and proliferation-inhibiting properties in many different types of cancer (Chattopadhyay et al., 2004; Chaudhary et al., 2023; Novilla et al., 2017). Regular use of tea helps prevent the commencement of

cancer effectively (Boehm et al., 2009; Yang et al., 2007). However, tea research is still only conducted on common species such as *C. senensis*, *C. oleifera*, or *C. nitidissima*, and many other *Camellia* are still overlooked in research, especially local tea species. Recently, research on endemic tea species in Vietnam has shown the remarkable biological activity of endemic tea varieties, such as *C. cuongiana*, *C. quephongensis*, *C. tamdaoensis*, *C. tienii*; that indicating the potential of many untapped tea species (Nga, Oanh, & Linh, 2023; Nguyen et al., 2023). Although it was first discovered in 2005 and described in 2006 in Yokdon National Park in Vietnam, studies on *Camellia yokdonensis* are still scarce. This endemic tea is rarely approached due to its distribution in deep forests, so mining is challenging. The locals call it "pink tea" because the flowers are in carmine. According to them, these types of "pink tea" often have the ability to improve health and treat digestive diseases. Studies on the activity will be necessary to evaluate the medicinal value and better conserve this rare tea species. This study aims to investigate two major biological activities of tea leaves: antioxidants and anticancer.

Materials and Methods

Sample preparation

The mature leaves from *Camellia yorkdonensis* were harvested in July, and the tea was identified and tagged with voucher 073022CYO. The leaves were doubly washed with distilled water before draining and drying at 40 °C. The dry powder was obtained by grounding the dried leaves; the tea powder and pure solvent methanol mixture were added 10 times the solvent volume into the powder. The extract was filtrated every 24 h of shaking, and the process took place 5 times. The pooled extract eliminated the solvent by using a rotary evaporator. The crude extract was dissolved by DMSO (Dimethyl sulfoxide, Sigma-Aldrich, USA) to concentrate the final stock of 400 mg/mL, abbreviated as CYE.

Cell lines and cell culturing

The lung, chronic myeloid leukaemia and breast cancer cells, A549, K562, and MCF7, were derived from ATCC (American Type Culture Collection, USA), and the hepatocellular carcinoma cells, HCC-J5, were derived from the Cell Culture Center of the National Taiwan University (Taipei, Taiwan). Cells were cultured in RPMI-1640 Medium (Sigma-Aldrich, USA) added with 10% Fetal bovine serum (FBS, Sigma-Aldrich, USA) and 1% antibiotic (Penicillin-Streptomycin (100U/mL), Sigma-Aldrich, USA). Cells were refreshed medium every 72 h, and the sub-culturing was carried out as 80% of the culturing surface was occupied by cells. The initial cell density was 10^5 cells/mL.

Free radical scavenging assay

The DPPH (2,2-Diphenyl-1-picrylhydrazyl, Sigma-Alrich, USA) reagent was used as a nitrogen-centered radical for analysis. The extract in different concentrations reacted with DPPH 0.3 mM at a ratio of 1:1 in the dark for 30 mins at 37°C. The absorbance of the reacted solution was measured at 517 nm. The percentage of trapped DPPH was computed following the formula:

$$\%DPPH_{scavenging} = [1- (OD_{test} - OD_{blank})/(OD_{negative} - OD_{blank})] \times 100\%.$$

Non-linear regression was performed with model $Y=100*(X^{HillSlope})/[EC50^{HillSlope} + (X^{HillSlope})]$ to regress the half-maximal response dose (EC50).

Reducing power investigation

A volume of 1 mL of extract in different concentrations was diluted with 2.5 mL of 1X PBS solution and 2.5 mL of 1% $K_3[Fe(CN)_6]$ solution. The solution was mixed in 15 s by using a vortexer, and the tubes were incubated in the dark at 50°C for 20 min. Then, the reaction was stopped with 2.5 mL of 10% trichloroacetic solution and incubated at room temperature for 10 min. 2.5 mL of the solution was diluted with 2.5 mL of water; 1 mL of 0.1% $FeCl_3$ solution was added. After mixing, the absorbance at 700 nm was measured (Ly et al., 2019).

Cytotoxicity evaluation

Cells were seeded into 96-well plates at a density of 10^5 cells/mL and then incubated for 24 h. The medium supplemented with extract in different concentrations was added into wells to reach the final volume of 200 µL and the extract's 0 to 400 µg/mL range. The evaluation lasted for 72 h before replacing the medium with a basal medium with 10% MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Sigma-Aldrich, USA). After 4 h of incubation, the medium was removed, and the crystals were dissolved by 100 µL DMSO. The cell viability reflected as the absorbance at 490 nm; thus, the cell viability rate was declared as the percentage between the test well and the negative one (DMSO 0.01%). Non-linear regression was performed with model $Y=100/(1+(IC50/X)^{HillSlope})$to regress the half-maximal inhibitory dose (IC50).

Data analysis

The data accumulation was in triple. The data was stored in Microsoft Excel 365 and analyzed using GraphPad Prism version 9.0.0. The differences were determined by performing the one-way ANOVA and Turkey Post-hoc combination or Student's T-test with a confidence level of 95% (alpha value = 0.05).

Results and Discussion

CYE inhibits radical scavenging capacity

The CYE showed the ability to neutralize the DPPH, which was indicated in a dose-dependent impact. The reaction curves are shown in Figure 1. In the presence of the CYE below 50 µg/mL, the more concentrated extract, the more DPPH was electronically received. At

50 µg/mL of the extract, the reaction reached a saturation state with about 80% scavenged DPPH. In addition, the curves of the two positive controls, vitamin C and Trolox were also described, and the curves were observed to be asymptotic to each other. The similarity and proximity to the two controls showed that the free radical scavenging ability of CYE extract was comparable to vitamin C or Trolox.

Non-linear regression indicated the EC50 values of CYE, Trolox, and Vitamin C extracts on DPPH were 19.37 ± 1.66 µg/mL, 14.36 ± 2.71 µg/mL, and 12.22 ± 1.15 µg/mL, respectively (Figure 2). The lower the EC50 value, the stronger the antioxidant capacity of a compound. Therefore, Vitamin C and Trolox have a more remarkable ability to scavenge DPPH radicals than CYE (with lower EC50 values). However, CYE also demonstrates an excellent ability to scavenge DPPH radicals with an EC50 value of 19.37 ± 1.66 µg/mL, close to Trolox and Vitamin C, indicating that CYE still holds considerable antioxidant potential.

There was a statistically significant difference in the activity of CYE compared to Trolox (P-value = 0.0455) and Vitamin C (P-value = 0.0099). However, the Vitamin C and Trolox tested were essences while CYE was a total extract, and the effect noted on the total extract was notable. Therefore, CYE has the potential for application in health protection products or as an antioxidant ingredient in the food and pharmaceutical industries. CYE can be developed into health care products or functional foods to enhance antioxidant capacity and protect the body from harmful free radicals. Additionally, further research on other antioxidant mechanisms could unlock its diverse potential applications in anti-aging and disease prevention industries.

Moreover, considering cost factors, natural origin, and long-term safety, CYE could be a potential alternative or complementary to standard antioxidants like Vitamin C and Trolox.

C. yokdonensis has not been reported to scavenge DPPH, but there have been reports of other members of the same genus. The phenolic leaf extract of *C. sinensis* reflects the EC50 on DPPH of 7.60 ± 0.48 µg/mL (Chí et al., 2020). The antioxidant ability was found to be under the influence of ambient conditions, such as collection time, collection position, weather conditions, and other factors. Some Indigenous tea species in Vietnam, living under the same geographical conditions as *C. yokdonensis*, have also demonstrated antioxidant capacity through their ability to neutralize DPPH free radicals, with the following EC50 values: *C. kissi* (EC50 = 10.85 ± 0.65 µg/mL), *C. longii* (EC50 = 60.5 ± 1.19 µg/mL), and *C. tamdaoensis* (EC50 = 9.00 ± 1.72 µg/mL) (Nga, Oanh, & Linh, 2023; Tran-Trung et al., 2023).

CYE reduces cation iron in solution

The ability to balance the oxidation potential is also considered through the process of donating and accepting electrons, in which electron donors are considered to have antioxidant properties (Lobo et al., 2010). Functional groups and unsaturated structures allow many phytochemicals to act as reducing agents in redox reactions (Chen et al., 2020; Eggler & Savinov, 2013). Hence, investigating the reducing power of compounds helps reflect their antioxidant capacity (Eggler & Savinov, 2013). Reducing Fe^{3+} to Fe^{2+} in solution reflects the CYE's reducing power. The results showed that the reduction process depends on the CYE concentration. Figure 3 shows a curve of the reducing power correlating the concentration of Vitamin C, Trolox, and CYE in the PFRAP assay. The results indicate that the reduction process of Fe^{3+} to Fe^{2+} in the solution is correlated with the concentration of Vitamin C, Trolox, and CYE in the PFRAP assay. At a concentration of 1.6 mg/mL, the absorbance of CYE is only 50% of that of Trolox and Vitamin C, suggesting that CYE has a lower antioxidant activity compared to the two reference substances at this lower concentration. However, when the concentration increases to 3.2 mg/mL, the absorbance becomes nearly equal for all three substances, indicating that at higher concentrations, the antioxidant activity of CYE increases significantly and becomes comparable to Trolox and Vitamin C. This suggests that CYE has good antioxidant potential but may require higher concentrations to achieve the same effect as Trolox and Vitamin C. This antioxidant capacity could be related to the mechanism of action or the chemical composition of CYE, where certain compounds in the extract may need to reach a threshold concentration to exhibit their effects fully. Further research on CYE's chemical structure and mechanism of action could help optimize its use as a potential antioxidant.

CYE inhibits cancer cell proliferation

One popular research topic on *Camellia* is its ability to inhibit cancer cell proliferation. Studies show that *Camellia sinensis* extract has many inhibitory effects on cancer, such as breast, lung, liver, and other cancers (Esghaei et al., 2018; Koňariková et al., 2015; Mbuthia et al., 2017). Recently, Vietnamese endemic *Camellia* was indicated to express anticancer ability, namely *C. cuongiana*, *C. vuquangensis*, and *C. hatinhensis* (An et al., 2023; Linh et al., 2023). This study described the ability of *C. yokdonensis* to inhibit cancer cell proliferation for the first time. The results are illustrated in Figure 4.

The results indicate that the CYE extract inhibits the proliferation of tested cancer cells, including A549 (lung cancer), HCC-J5 (liver cancer), K562 (leukaemia), and MCF-7 (breast cancer). The inhibitory activity of CYE depends on the concentration of the extract, showing different levels of effectiveness across the various cell lines, which suggests a specific selectivity in the effects of this extract. At the highest tested concentration of 400 µg/mL, the survival rate of the cells decreased to about 20%, demonstrating a strong ability of CYE to inhibit the growth of cancer cells (Figure 4). Notably, the

IC50 value ranged from 48.82 ±12.59 µg/mL for K562 cells to 175.52 ± 16.70 µg/mL for MCF-7 (HCC-J5: 138.00 ± 11.6 µg/mL and A549: 172.90 ± 22.31) (Figure 5). Among these, CYE exhibited the most robust activity on the K562 cell line, with the lowest IC50 value (48.82 µg/mL), indicating its strong potential for application in leukaemia treatment. The activity is also notable on A549, HCC-J5, and MCF-7 cell lines but with higher IC50 values, suggesting that the inhibitory effects on these cell lines are weaker than K562.

There was a difference in the impact of CYE on suspension cells being more effective than on adherent cells, P-value = 0.0007. From the IC50 value, plant extracts can be divided into different effective groups, and based on that, research can further develop (Indrayanto, Putra, & Suhud, 2021). The effect of CYE on K562 cells was moderately effective, while the impact on the rest was weak (Indrayanto, Putra, & Suhud, 2021). In previous reports, some of *Camellia* showed cytotoxic on several cancer cells, such as *C. cuongiana* on HCC-J5 and K562 (IC50 > 100 and IC50 = 72.26 ± 5.75 µg/mL), *C. kissi* on K562 (IC50 = 40,01 ± 3,12 µg/mL), *C. hatinhensis* on A549 and MCF-7 (IC50 = 76.26 ± 1.68 and 72.27 ± 1.71 µg/mL) (An et al., 2023; Linh et al., 2024; Linh et al., 2023). These results demonstrate the potential of CYE as a natural anticancer agent, particularly in the K562 cell line. However, due to the differences in effects among the various cell lines, further research into the active components of CYE, specific mechanisms of action, and testing in animal models and clinical settings will be necessary to determine its application potential in cancer treatment. CYE could become a promising candidate for the development of anticancer therapies, especially when combined with other methods to optimize treatment efficacy.

The development of anticancer research from extracts is important in the context that cancer is gradually increasing rapidly, and current treatment methods still have many challenges in terms of effectiveness and specificity. In 2022, there were about 20 million incidents of cancer, most concentrated in Asia, with 9.8 million cases; China was the country with the highest number of cases, with 4.8 million cases. Lung, breast, liver cancer, and leukaemia, respectively, landed at the first, third, sixth, and 13th place in popularity (Bray et al., 2024). On the other hand, the first, third, fourth, and 10th positions were the peaks of the mentioned cancer types in terms of deaths (Bray et al., 2024). Thus far, the mechanism in action of the *Camellia* extracts on cancer cells has been investigated, such as BCR-ABL in K562, the P53 pathway in MCF-7, and Pi3K in HCT116, and it expressed the potential in cancer treatment research and applications (Chen, Chen, & Xu, 2013; Gao et al., 2020; Xuan et al., 2024).

The relationship between the antioxidant activity and the cancer cell inhibitory activity of CYE was considered to be closely related. The relationship was analyzed based on the DPPH scavenged rate and cell viability. The analysis results were recorded in the negative direction (Table 1), and the percentage of viable cells decreased as the amount of DPPH absorbed increased. The results suggested the supportive action of antioxidant and cytotoxicity effects of the *C. yokdonensis* extract. However, the connection between those impacts requires profound studies to be demystified. The evidence of the leaf extract's antioxidant and anticancer cell proliferation might positively support and contribute to the *C. yokdonensis* reservation, exploitation, and application.

Conclusion

The antioxidant and anti-proliferative properties of the *C. yokdonensis* leaf extract are reported for the first time in this study, thereby enhancing the existing scientific knowledge surrounding this rare Camellia species. Given its combined antioxidant and anti-cancer activities, CYE presents a promising opportunity for the development of natural cancer therapies. Nevertheless, additional research is essential to elucidate the underlying mechanisms of action and to identify the bioactive compounds responsible for these effects.

Funding Information

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Author Contributions

First Author: Design, Methodology, Writing -review and editing; Second Author: Data Curation, Formal Analysis, Investigation and Writing -original draft; Third Author: Funding Acquisition, Project Administration.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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Table 1. The correlation coefficient between the DPPH scavenging and anticancer effects of *C. yokdonensis*

Value	**HCC-J5**	**A549**	**MCF-7**	**K562**
Pearson r	-0,9255	-0,9386	-0,9044	-0,8509
P-value	0,1237	0,1121	0,1403	0,1761
Classification	Very strong correlation coefficient			

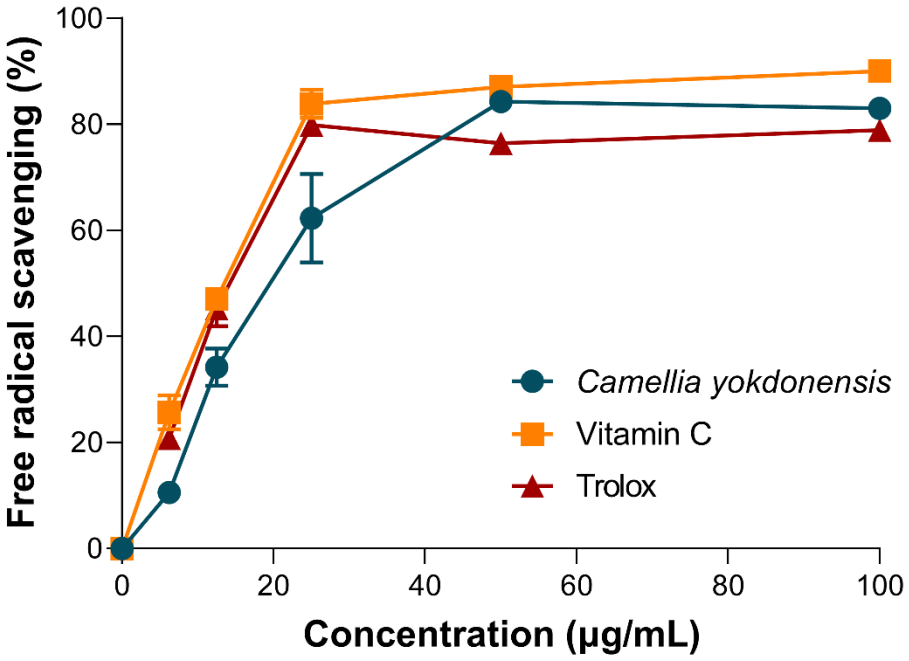

Figure 1. The DPPH scavenging effect of *C. yokdonensis* and positive controls (Vitamin C and Trolox).

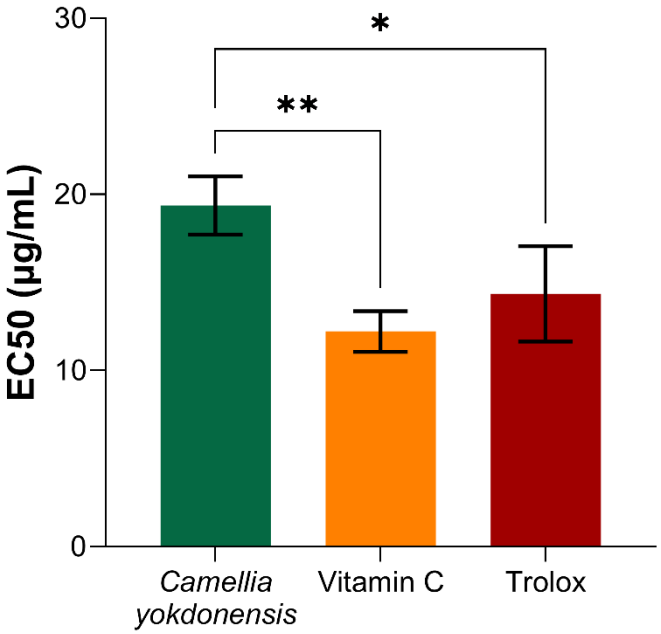

Figure 2. The EC50 values of *C. yokdonensis* and positive controls (Vitamin C and Trolox). *Note: p-value < 0.05 (*) and p-value <0.005 (**).*

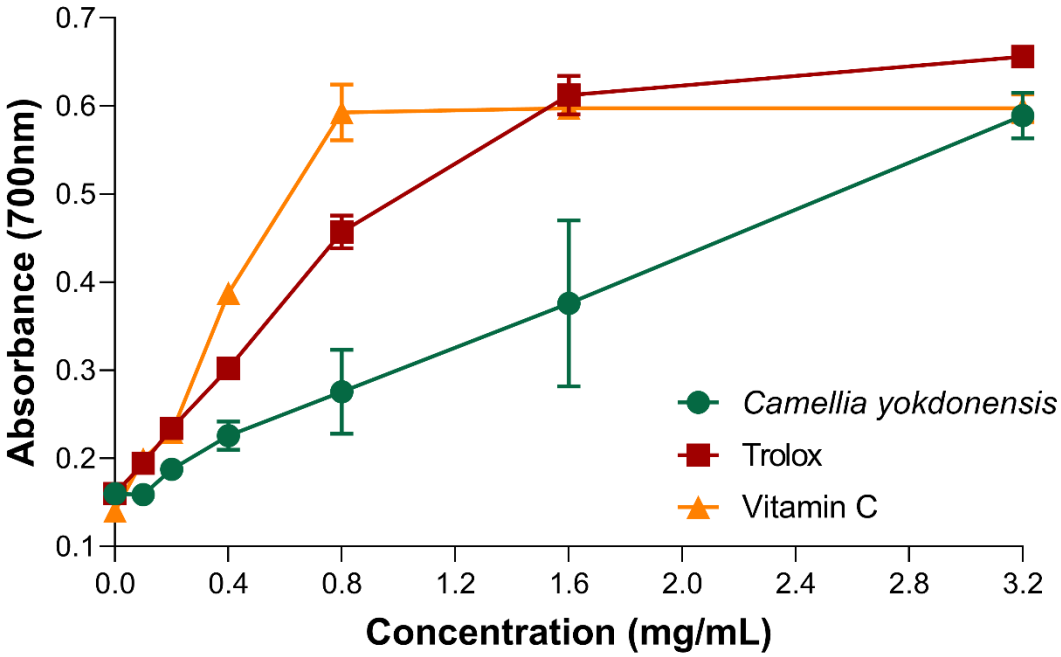

Figure 3. The reducing power of *C. yokdonensis* and positive controls (Vitamin C and Trolox).

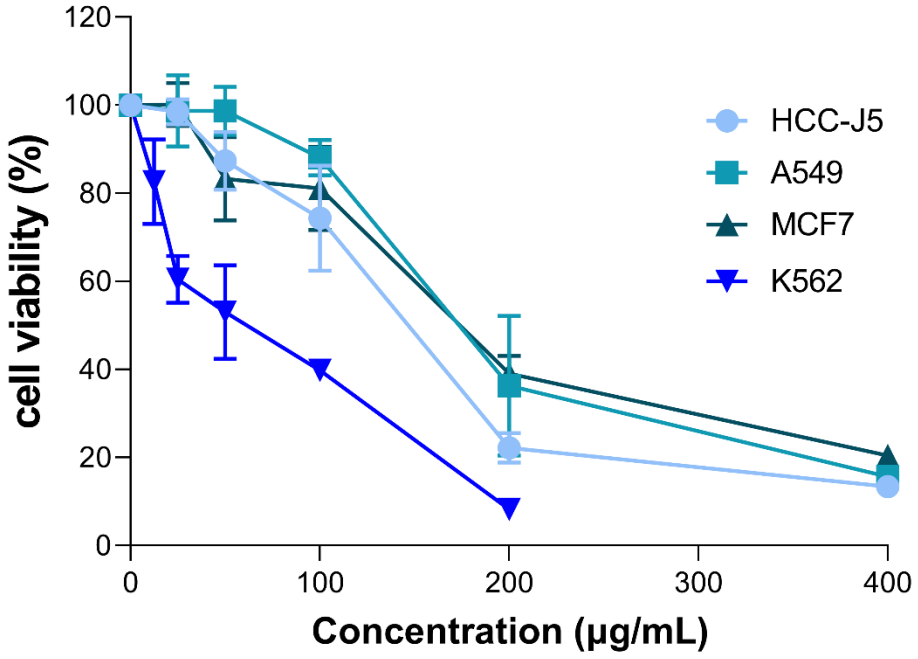

Figure 4. The cytotoxic effect of *C. yokdonensis* on cancer cell lines (HCC-J5, A549, MCF7, and K562).

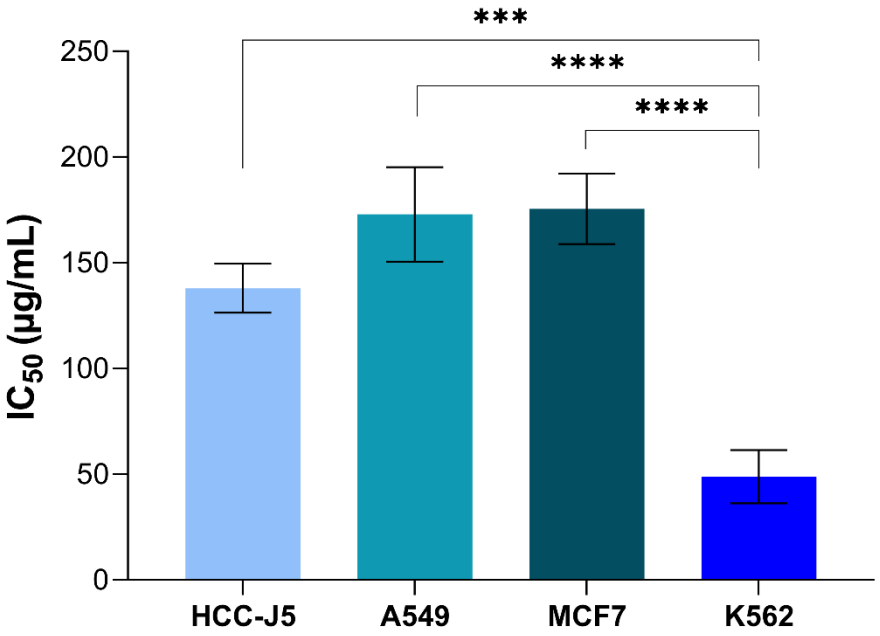

Figure. 5. The IC50 values of *CYE* on cancer cell lines (HCC-J5, A549, MCF7, and K562). *Note: p-value < 0.0002 (***) and p-value <0.0001 (****).*