 DOI: 10.31276/VJSTE.2024.0094

Extraction of saponin from *Calophyllum inophyllum* leaves by ultrasound-assisted extraction

Setiyo Gunawan[1, 2*], Miftahul Hidayah Nugrahini[1], Mochammad Ferdian Azizi[1], Safrina Hapsari[3], Raden Darmawan[1], Hakun Wirawasista Aparamarta[1], Firdaus[4]

[1]*Chemical Engineering Department, Institute Technology Sepuluh Nopember (ITS), Keputih, Sukolilo, Surabaya 60111, Indonesia*
[2]*Research Center for Agri-Food and Biotechnology, Institute Technology Sepuluh Nopember (ITS), Keputih, Sukolilo, Surabaya 60111, Indonesia*
[3]*Department of Chemical Engineering, University Brawijaya (UB), Ketawanggede, Lowokwaru, Malang 65145, Indonesia*
[4]*Faculty of Health, University Nahdlatul Ulama Surabaya (UNUSA), Jl. Raya Jemursari, Jemur Wonosari, Wonocolo, Surabaya 60237, Indonesia*

Received 8 September 2024; revised 18 September 2024; accepted 2 October 2024

Abstract:

***Calophyllum inophyllum* is a mangrove plant native to Indonesia, known to contain various bioactive compounds. Saponins, found in the leaves of *C. inophyllum*, exhibit anti-hypercholesterolaemic, anti-inflammatory, cardiac depressant, antitumour, antiviral, antioxidant, and anticancer activities. This study aims to investigate the effect of solvent type and extraction duration on the purity and yield of saponins using ultrasound-assisted extraction (UAE). The extraction was conducted at 40°C with a power input of 550 W and a solvent-to-sample mass ratio of 25:1 (w/w). The leaves of *C. inophyllum* were found to contain 0.2% saponins prior to extraction. Optimal results, achieving a saponin purity of 22.05% and a yield of 11.74%, were obtained using food-grade ethanol as the solvent for 30 minutes. Additionally, the antioxidant activity of the extract and its effect on bladder cancer cell viability were explored. The IC_{50} value for antioxidant activity was determined to be 19.99 ppm. Furthermore, at a concentration of 100 µg/ml, the extract reduced the viability of University of Michigan-Urothelial Carcinoma-3 (UMUC-3) bladder cancer cells by 29.50±1.94% and Black Foot Disease Transitional Carcinoma 905 (BFTC-905) cells by 30.26±5.83%.**

Keywords: **anticancer, antioxidant, *Calophyllum inophyllum*, saponins, ultrasound-assisted extraction.**

Classification numbers: **2.2, 3.3, 3.5**

1. Introduction

Cancer is one of the leading causes of mortality worldwide. In 2020, approximately 9.96 million people died from cancer, a figure projected to rise to 16.3 million by 2040 [1, 2]. Current cancer treatments, primarily chemotherapy and radiation, often have severe side effects [3]. Chemotherapy is frequently supplemented with anticancer drugs derived from the secondary metabolites of specific plants [4]. The utilisation of plants and animals in cancer treatment is a longstanding tradition, which continues to gain popularity despite advancements in modern medicine. According to the World Health Organisation (WHO), 80% of the global population uses herbal products for health maintenance, disease prevention, and the treatment of chronic, degenerative, and cancerous conditions [1, 2, 5].

In Indonesia, the national socioeconomic survey of 2017 reported a growing interest in traditional medicine, with 32% of Indonesians using it to treat ailments. Furthermore, 22.3% opt for plant-based treatments involving fruits, tubers, and roots. This trend, coupled with the increasing demand for raw medicinal materials in the pharmaceutical industry, underscores the importance of researching bioactive substances with medicinal potential. Indonesia's abundant and diverse biological resources remain underutilised, as 90-95% of the required medicinal raw materials are still imported [6].

Several studies have highlighted the potential benefits of mangrove plants, including their role in promoting Indonesia's economic transformation through blue economy development [7-14]. *Calophyllum inophyllum*, a mangrove species, has long been used in traditional medicine due to its bioactive compounds found in its leaves, seeds, and stems. The relevance of *C. inophyllum* in healthcare is particularly crucial, given the high cost of cancer treatments.

In traditional medicine, *C. inophyllum* has been used for various purposes. The water from soaking its leaves can treat inflamed eyes [7], and the oil from its seeds is used to heal skin ailments and stimulate hair growth. In Chinese medicine, the plant is employed to alleviate sore eyes, rheumatism, inflammation, and wounds [15].

Saponins, bioactive compounds in *C. inophyllum*, are known for their anti-hypercholesterolaemic, anti-inflammatory, cardiac

*Corresponding author: Email: gunawan@chem-eng.its.ac.id

depressant, antitumour, antiviral and anticancer properties [16]. Saponin extraction from *Acanthaster planci* using maceration has been reported [16]. Maceration is a simple, straightforward process that does not require specialised equipment, but it is time-consuming and yields low extraction efficiency. Conversely, microwave-assisted extraction (MAE) has been employed to extract saponins from *Phyllanthus amarus* [17], *Swietenia mahogany Jacq* [18] and *Furcraea* leaves [19]. MAE is more effective and efficient than conventional methods due to the combined effects of dipole rotation and ion conduction, resulting in shorter extraction times, higher yields, and lower energy consumption. However, MAE typically operates at higher temperatures.

Ultrasound-assisted extraction (UAE), a method that uses ultrasonic waves to enhance extraction efficiency at lower temperatures, has also been employed to extract saponins from *Sapindus rarak* [20]. UAE (operating at a high-frequency signal of 20 kHz) significantly reduces extraction duration while increasing yield [21, 22].

Despite these advances, optimal conditions for extracting saponins from *C. inophyllum* leaves using UAE have yet to be reported. Therefore, the present study aimed to evaluate the effect of extraction time and solvent type on the purity and yield of saponins from *C. inophyllum* leaves using UAE. Additionally, the antioxidant activity and the impact of the extract on the viability of bladder cancer cells were investigated.

2. Materials and methods

2.1. Materials

C. inophyllum leaves were purchased from Koperasi Jarak Lestari in Cilacap, Central Java. Bladder cancer cell lines (University of Michigan-Urothelial Carcinoma-3 (UMUC-3) and Black Foot Disease Transitional Carcinoma 905 (BFTC-905)) were obtained from the Biomedical Science Department at National Chung Cheng University (Chiayi, Taiwan, China). Chemicals, including methanol, denatured ethanol, food-grade ethanol, propanol, butanol, distilled water (aquadest), and hydrochloric acid (HCl), were sourced from commercial suppliers.

2.2. Saponin extraction by ultrasound-assisted extraction

The *C. inophyllum* leaves were dried in an oven at 60°C for one hour, ground into a powder using a mortar, and sieved through an 80-mesh sieve. Four grams of the powdered leaves were mixed with 100 ml of the chosen solvent in a beaker. The solvents tested were methanol, denatured ethanol, food-grade ethanol, propanol, and butanol, each with a water content of 20%. The sample was placed in the ultrasonic chamber, with power set to 550 W and temperature to 40°C, for varying extraction times (10, 20, 30, and 40 minutes). After extraction, the mixture was filtered using Whatman filter paper, and the filtrate was dried in an oven at 60°C. The dried extract was then weighed to determine the yield. The calculation was performed using the method described by N. Aryanti, et al. (2020) [20], as follows:

$$\%\text{Yield} = \frac{Crude\ saponin\ extract\ (g)}{C.\ inophyllum\ leaves\ powder\ (g)} \times 100\% \quad (1)$$

2.3. Foam analysis

Saponin presence in the samples was identified using a foam test. A 0.05 g sample was diluted in 1 ml of water at 80°C and shaken vigorously. Stable foam formation indicated the presence of saponins.

2.4. Ultraviolet-visible spectrometer analysis

Saponin content in *C. inophyllum* leaves and crude saponin extract was analysed using a UV-Vis spectrophotometer. Two millilitres of a standard saponin solution (CAS number 8047-15-2) at a concentration of 0.08 mg/ml was placed in a UV-Vis spectrophotometer cuvette to determine the absorbance of saponin at its maximum wavelength. Observations were made across a range of 200-700 nm [19]. The wavelength corresponding to the maximum absorbance was then used to prepare a calibration curve by diluting the standard solution to concentrations of 0.06, 0.03, 0.01, 0.005, and 0.001 mg/ml and measuring the absorbance of each concentration.

2.5. Antioxidant activity assay

The antioxidant activity of *C. inophyllum* leaf extract was measured following the method described by Q.D. Do, et al. (2014) [23], with modifications. The leaf extract was dissolved in methanol to prepare concentrations of 10, 20, 30, 40, and 50 ppm. One millilitre of each sample was added to 3 ml of 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution and vortexed for two minutes. The samples were incubated in the dark for 30 minutes, after which absorbance was measured at 517 nm using a spectrophotometer. Antioxidant activity (%) was calculated using the following formula:

$$\text{Antioxidant activity} = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100\% \quad (2)$$

The antioxidant activity was then plotted against extract concentrations to determine the IC_{50} value. These IC_{50} values were compared with those of gallic acid and ascorbic acid.

2.6. Cell viability assay

The effect of *C. inophyllum* leaf extract on bladder cancer cells was assessed using the Cell Counting Kit-8 (CCK-8) (Sigma Aldrich). UMUC-3 cells were cultured in MEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% sodium pyruvate. BFTC-905 cells were cultured in RPMI 1640 medium with 10% heat-inactivated FBS and 1% penicillin/streptomycin. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

A cell suspension of 100 µl (containing 10,000 cells per well) was seeded into a 96-well plate and pre-incubated for 24 hours under humidified conditions (37°C, 5% CO_2). The medium was then replaced with fresh medium (100 µl) containing various concentrations of *C. inophyllum* extract dissolved in DMSO (0.78-100 µg/ml). The plate was incubated for a further 48 hours.

Subsequently, the medium was replaced with fresh medium, and 10 µl of CCK-8 solution was added to each well. After incubating for three hours, absorbance was measured at 450 nm using an ELISA microplate reader. The effect of cisplatin (a standard chemotherapeutic agent) on cell viability was also evaluated using the same procedure, with cisplatin concentrations in DMSO ranging from 0.117 to 15 µg/ml.

2.7. Statistical analysis

The results were analysed statistically to ensure reliability. Mean differences were evaluated using analysis of variance (ANOVA), with significance accepted at $p<0.05$ [24].

3. Results and discussion

Mangrove ecosystems face significant challenges due to land conversion, largely stemming from a lack of public understanding of their benefits. Many people mistakenly believe that mangroves offer no tangible advantages. This situation is exacerbated by pollution, such as plastic waste, household refuse, and oil spills. Natural disasters also hinder efforts to expand mangrove vegetation, while illegal logging poses a further threat to mangrove survival. The Ministry of Environment and Forestry has undertaken various initiatives to manage mangrove forests and coastal areas, including Mangrove Forest and Land Rehabilitation (FLR) and collaborations with the private sector. The realised mangrove FLR in 2010 - 2014, 2015, 2016, and 2017 covered 31,675, 430, 497, and 500 hectares, respectively [25]. The involvement of multiple stakeholders has significantly increased these efforts. However, the application of appropriate technology is also essential for improving the success of mangrove rehabilitation. Isolating, identifying, and utilising valuable mangrove products, such as *C. inophyllum*, represent another approach to supporting mangrove conservation and development.

In this study, *C. inophyllum* leaves were dried and processed into a powder, which was then used for experimentation. A factorial design incorporating two-level and single-factor experiments was employed. Five solvents-methanol, denatured ethanol, food-grade ethanol, propanol, and butanol - were used to extract saponin compounds, with extraction times of 10, 20, 30, and 40 minutes. After extraction using UAE, the resulting mixtures were filtered and dried in an oven. Extraction yield and saponin purity were measured, as shown in Fig. 1.

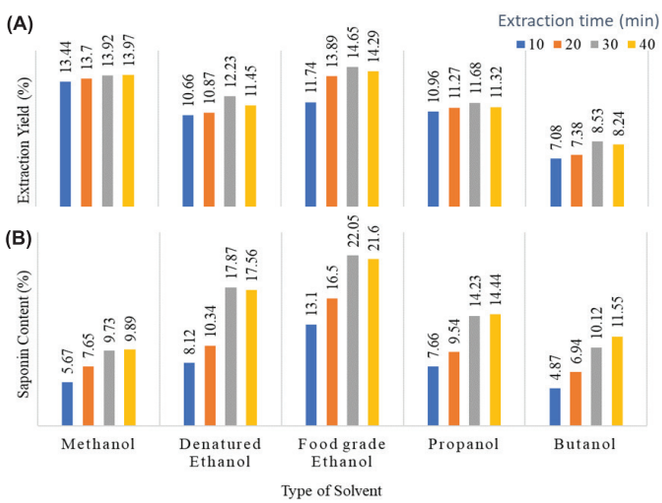

Fig. 1. Effect of solvent type and extraction time on (A) extraction yield and (B) saponin content.

After obtaining the *C. inophyllum* leaf extract, a qualitative foam test was conducted to confirm the presence of saponins. A stable foam persisting for 30 minutes indicated the presence of saponins, consistent with previous findings that saponins, as surfactants, efficiently produce foam when shaken [20]. Saponin content was further analysed using UV-Vis spectrophotometry, with wavelengths ranging from 200 to 700 nm. The saponin compounds from *C. inophyllum* leaves displayed a peak absorbance of 236 nm, which differs from the 220 nm wavelength reported for the Calbiochem saponin standard by S.E. Rikomah (2017) [26]. This discrepancy may result from variations in solution concentration, thickness, instrument calibration, and solvent polarity. Solvent polarity, in particular, influences the location of the maximum absorption.

3.1. Effect of solvent type on extraction yield and saponin content

Extraction is a process by which one or more substances are separated from a solid or liquid using a solvent. The solvent is either immiscible or only partially miscible with the solid or liquid, allowing the active agents to migrate from the solid/liquid mixture (raffinate) to the solvent (extract). In this study, dried *C. inophyllum* leaf powder was used with five different solvents: methanol, denatured ethanol, food-grade ethanol, propanol, and butanol. Choosing a safe, cost-effective solvent that maximises both saponin content and extraction yield is crucial. The choice of solvent was based on its Log *P* value relative to the Log *P* value of saponins, as shown in Table 1.

Table 1. Log *P* values for saponin and various solvents.

Parameters	Log *P*
Saponin (estimation) [27]	0.31
Water [28]	-1.38
Methanol 80% [28]	-0.77
Ethanol 80% [28]	-0.31
Propanol 80% [28]	0.25
Butanol 80% [28]	0.8

In physical chemistry, the partition coefficient (*P*) or distribution coefficient (*D*) can represent the ratio of compound concentrations between two immiscible phases at equilibrium. This coefficient measures differences in solubility across two phases. Log *P* is commonly used to represent the ratio of the concentrations of nonionised compound species. In contrast, the distribution coefficient refers to the ratio of the concentrations of all compound species (ionised and non-ionised). Log *P* for the partition coefficient assesses how hydrophobic or hydrophilic a compound is. The smaller the

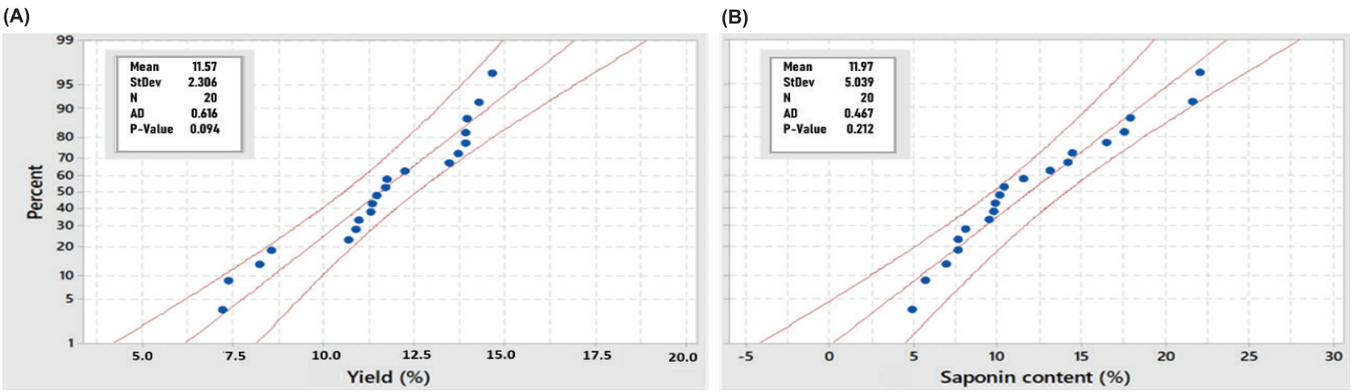

Fig. 2. Normal probability plot of (A) extraction yield and (B) saponin content.

Log *P* value, the more polar (hydrophilic) the compound. Conversely, a higher Log *P* value indicates a more non-polar (hydrophobic) compound that tends to dissolve in organic solvents.

The effect of solvent type and extraction time on the extraction yield and saponin content from *C. inophyllum* leaves using UAE is shown in Fig. 1. While propanol and butanol have polarities closest to the Log *P* of saponins, the saponin content extracted with these solvents was lower than that obtained with denatured and food-grade ethanol. This may be due to propanol's lower water solubility, which could limit its extraction efficiency. Methanol also produced suboptimal results, likely due to its low Log *P* value and incompatibility with water for extracting bioactive compounds.

Food-grade ethanol produced the highest saponin content (22.05%) and extraction yield (14.65%), significantly outperforming methanol, denatured ethanol, propanol, and butanol ($p<0.05$). Ethanol was chosen for its favourable solubility properties, as saponins dissolve readily in both water and ethanol [29]. The performance of food-grade ethanol was also superior to denatured ethanol. Food-grade ethanol, also known as ethyl alcohol, is derived from fermenting sugar with yeast and is used in alcoholic beverages. It is considered safe for human consumption due to its purity and lack of additives, unlike denatured ethanol, which contains substances like methanol and isopropanol. Food-grade ethanol is safe for human consumption. This is because of its purity and lack of additives. Moreover, denatured alcohol is not to be used for food, drug, or medical purposes, but, it is perfectly suitable for industrial use, general cleaning, and even as a solvent ingredient in various non-food products. The presence of additives (such as methanol and isopropanol) in denatured ethanol results in a lower extraction of saponins than that in food-grade ethanol. These additives lower the saponin extraction efficiency.

3.2. Effect of extraction time on extraction yield and saponin content

Ultrasound-assisted extraction is increasingly preferred over conventional methods due to its ability to shorten extraction time and process heat-sensitive bioactive compounds at lower temperatures. The mechanical effect of ultrasound enhances solvent penetration into cellular material, increasing mass transfer, disrupting the cell wall, and facilitating the release of internal compounds. As a result, UAE is more effective than conventional methods because it reduces extraction time and requires less solvent.

In this study, food-grade ethanol produced the highest yield. An extraction time of 10 minutes was found to be optimal, as longer extraction times damaged the powdered sample, leading to an increased amount of residue (Fig. 1). This is consistent with the theory that prolonged UAE generates more cavitation bubbles, damaging the cell walls and allowing solvent penetration. However, excessive extraction time can lead to stronger interactions between cavitation bubbles, potentially damaging bioactive compounds [30].

During the extraction process, the sample temperature increased due to cavitation and ultrasound waves. The UAE alarm sounded when the sample temperature exceeded 40°C and overheated. The UAE was then stopped until the temperature reached 27°C (about 15 min). The extraction process was continued until completion. The extraction process must be stopped prior to overheating as compound loss through oxidation can occur at temperatures above 50°C in bioactive compounds. Saponins are particularly sensitive to high temperatures, at which point they undergo structural changes, reducing extraction efficiency. Both excessively low temperatures and overly brief extraction times result in suboptimal extraction of bioactive components.

The normal probability plot (Fig. 2) was used to assess whether the data obtained were normally distributed. The data are considered reasonably well-distributed, as the plot follows a straight line with a p-value above 0.05 [24]. The results of the analysis of variance (ANOVA) for extraction yield and saponin content are presented in Tables 2 and 3, respectively. Both the solvent type and extraction time significantly influenced the extraction yield and saponin content ($p<0.05$).

Table 2. Analysis of variance for the extraction yield.

Source of variations	Degree of freedom	Sum of squares	Mean square	F-value	P-value
Model	7	98.264	14.038	59.92	0.000
Linear	7	98.264	14.038	59.92	0.000
Solvent type	4	92.759	23.189	98.98	0.000
Extraction time	3	5.505	1.835	7.83	0.004
Error	12	2.812	0.234		
Total	19				

Table 3. Analysis of variance for saponin content.

Source of variations	Degree of freedom	Sum of squares	Mean square	F-value	P-value
Model	7	464.030	66.290	43.36	0.000
Linear	7	464.030	66.290	43.36	0.000
Solvent type	4	278.590	69.648	45.56	0.000
Extraction time	3	185.440	61.813	40.43	0.000
Error	12	18.340	1.529		
Total	19				

Our results suggest that the following operating conditions were optimal: an extraction time of 30 minutes, food-grade ethanol as the solvent, an extraction temperature of 40°C, a power of 550 W, and a solvent-to-sample mass ratio of 25:1. Starting with *C. inophyllum* leaves containing 0.2% saponins, it was possible to obtain a fraction enriched with saponins (22.05% purity and 11.74% yield).

3.3. Antioxidant activity

Antioxidants are relatively stable molecules capable of neutralising free radicals, which are a known contributing factor to cancer development [31]. Antioxidant activity is a key parameter for assessing a substance's ability to neutralise free radicals. One of the most widely used methods for measuring antioxidant activity is the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. In this study, the antioxidant activity of *C. inophyllum* leaf extract was determined by preparing various extract concentrations (10-50 ppm) in methanol. The extract solution was then mixed with DPPH, and the absorbance was measured using a spectrophotometer at a wavelength of 517 nm.

Initially, the DPPH solution appears deep purple due to the delocalisation of odd electrons in the nitrogen atom. Upon accepting hydrogen atoms from antioxidants, the colour intensity decreases. According to the mechanism of action, antioxidants that donate hydrogen atoms are classified as primary antioxidants, as they provide hydrogen atoms to radical compounds (R*, ROO*) or stabilise these radicals. Their derivatives, such as antioxidant radicals (A*), are more stable than the initial radical compounds due to resonance within their aromatic structures [32]. Potent antioxidants change the DPPH solution from purple to yellow or transparent, depending on the number of free radicals neutralised [33]. The colour transformation caused by the reaction between *C. inophyllum* extract (10-50 ppm) and DPPH is shown in Fig. 3. Absorbance and antioxidant activity values are provided in Table 4.

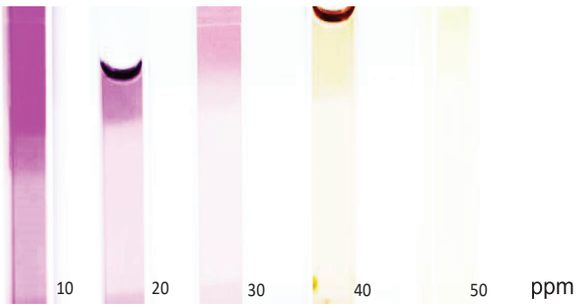

Fig. 3. Colour transformation due to *C. inophyllum* leaf extract (10-50 ppm) and 1,1-diphenyl-2-picrylhydrazyl reaction.

Table 4. Absorbance and antioxidant activity of each sample.

Concentration (ppm)	Absorbance	Antioxidant activity (%)
10	0.521	42.30
20	0.442	44.43
30	0.341	62.24
40	0.243	73.09
50	0.071	92.14

As the extract concentration increased, the solution became progressively more transparent (Fig. 3). Table 4 demonstrates that higher extract concentrations result in stronger suppression of free radical activity**.** The highest antioxidant activity (92.14%) was observed at a concentration of 50 ppm, meaning that 92.14% of DPPH radicals were neutralised.

Table 5. Antioxidant activity of gallic and ascorbic acids.

Concentration (ppm)	Antioxidant activity of gallic acid (%)	Antioxidant activity of ascorbic acid (%)
0.5	32.29	21.97
1	36.46	25.57
5	69.44	35.20
10	95.78	47.58
15	96.59	63.93
20	96.99	73.34
25	97.05	91.41

Gallic acid and ascorbic acid, two notable natural antioxidants belonging to the polyphenol group of secondary metabolites [34], were used as reference standards in this study. The antioxidant activity results for both compounds are shown in Table 5. Higher concentrations of gallic and ascorbic acids resulted in increased antioxidant activity. The IC_{50} values (half-maximal inhibitory concentration) for the extract, ascorbic acid, and gallic acid were calculated by plotting concentration against antioxidant activity, as shown in Fig. 4.

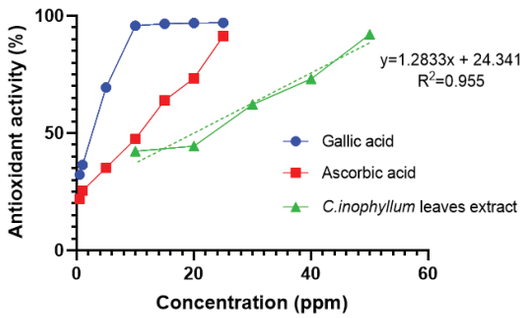

Fig. 4. Effect of concentration on antioxidant activity.

The IC_{50} value represents the concentration required to inhibit 50% of free radical DPPH. This value was obtained by plotting concentration (*x*-axis) against antioxidant activity (*y*-axis) and calculating the corresponding linear equation. The IC_{50} value for *C. inophyllum* leaf extract was approximately two-fold and nine-fold higher than that of ascorbic acid and gallic acid, respectively (Table 6). A higher IC_{50} value indicates lower effectiveness in inhibiting free radicals. These findings were compared with a previous study conducted by A.P. Faisal, et al. (2022) [35], who used *C. inophyllum* leaves extracted via maceration (a conventional method).

Table 6. IC_{50} values of various extracts.

Samples	IC_{50} (ppm)
This experiment	19.99
Gallic acid	2.28
Ascorbic acid	10.47
C. inophyllum leaves extract obtained by maceration [35]	66.47
Ciriguela extract obtained from the UAE [36]	190

Moreover, the IC_{50} value obtained in this study was three-fold lower than that of *C. inophyllum* leaf extract obtained via maceration. This difference is attributed to the ability of ultrasound waves to facilitate solvent penetration into the cell walls of *C. inophyllum*. As a result, bioactive compounds, including saponins, are extracted more efficiently and in a shorter time than through the maceration method. Additionally, the IC_{50} value of *C. inophyllum* leaf extract in this study was 10-fold lower than that obtained from ciriguela using the same method [36]. The IC_{50} value of *C. inophyllum* leaf extract classifies it as a potent antioxidant since it is less than 50 ppm (Table 7). Thus, the extract has potential as an antioxidant agent.

Table 7. Classification of antioxidant activity based on IC_{50} value.

IC_{50} (ppm)	**Classification** [37]
<50	Very strong
50-100	Strong
100-150	Fairly strong
150-200	Weak

3.4. Cell viability assay

The effect of *C. inophyllum* extract on bladder cancer cell viability was assessed using the CCK-8 assay with UMUC-3 and BFTC-905 cell lines. This *in vitro* method was chosen due to its ease of handling, unlimited replication capability, and the ability to replace contaminated cells with frozen stock [38]. Living cells contain NAD+ and NADP+, which are reduced to NADH and NADPH by dehydrogenase enzymes. NAD+/NADH regulates cellular energy metabolism, while NADP+/NADPH maintains redox balance and participates in the biosynthesis of fatty acids and nucleic acids [39]. The electrons carried by NADH and NADPH are transferred to 1-methoxy PMS, which subsequently transfers them to the tetrazolium salt WST-8, resulting in the formation of yellow/orange formazan. The intensity of the colour is directly proportional to the number of viable cells.

In this study, the efficacy of *C. inophyllum* leaf extract in reducing bladder cancer cell viability was compared with that of cisplatin, a platinum-based chemotherapy drug commonly used to inhibit bladder cancer cells [40]. The viability of bladder cancer cells is shown in Figs. 5 and 6.

The viability of UMUC-3 and BFTC-905 cells gradually decreased with increasing concentrations of the leaf extract (Fig. 5). At the highest extract concentration (100 µg/ml), UMUC-3 and BFTC-905 cell viability were 70.50±1.94 and 69.74±5.83%, respectively. By contrast, at the highest cisplatin concentration (15 µg/ml), UMUC-3 and BFTC-905 cell viability dropped to 20.96±1.97 and 11.54±2.23%, respectively (Fig. 6). In comparison, previous research on the herbal extract *Guizhi Fuling Wan* (GFW), a traditional Chinese medicine, demonstrated a 50% reduction in BFTC-905 cell viability at a concentration of 0.3564 mg/ml (356.4 µg/ml) after 24 hours [41]. Furthermore, *Rhodiola rosea* extract halved UMUC-3 cell viability at a concentration of 100 µg/ml [42].

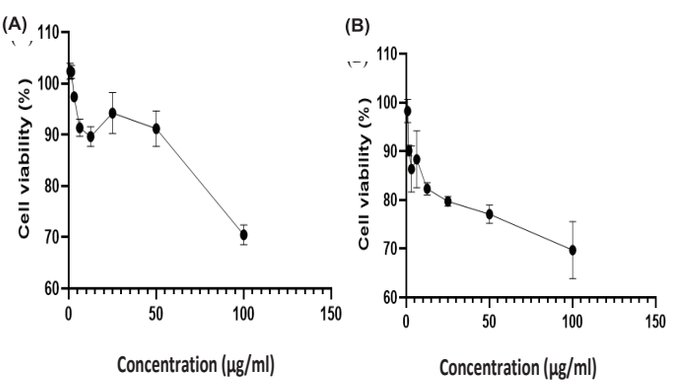

Fig. 5. Effect of *C. inophyllum* leaf extract on bladder cancer cell lines: (A) UMUC-3; **(B)** BFTC-905.

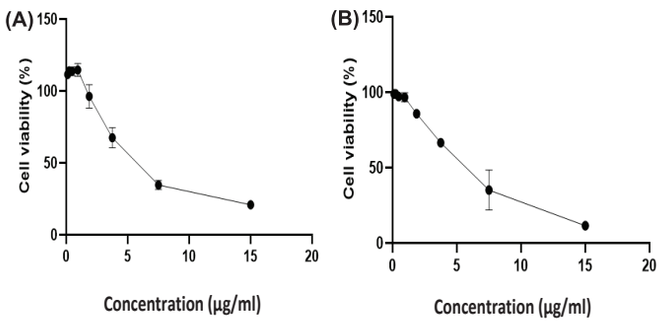

Fig. 6. Effect of cisplatin on bladder cancer cell lines: (A) UMUC-3; **(B)** BFTC-905.

The antioxidant activity of *C. inophyllum* leaf extract was inversely related to its effect on reducing bladder cancer cell viability. According to I. Grigalius, et al. (2017) [43], the correlation between these two properties is typically low. For instance, the compound 3,3,6-trihydroxyflavon exhibits negligible antioxidant activity but is highly effective at reducing lung cancer cell viability (A549). Additionally, a water extract from the roots of *Saururus* chinensis, despite having a high IC_{50} value (>100 µg/ml), shows promising activity against the breast cancer cell line MCF7 (Michigan Cancer Foundation-7) [44]. Therefore, *C. inophyllum* leaf extract, as obtained in this study, may function more effectively as an antioxidant rather than a bladder cancer-inhibiting agent. Further research is needed to elucidate the mechanisms involved in reducing bladder cancer cell viability.

4. Conclusions

The optimal *C. inophyllum* leaf extract, enriched with saponins (22.05% purity and 11.74% yield), was obtained through ultrasound-assisted extraction at 40°C, 550 W, with a mass solvent-to-sample ratio of 25:1 (w/w) for 30 minutes, using food-grade ethanol as the solvent. The antioxidant activity of the extract, which reduced the concentration of the free radical DPPH by half, was measured at 19.99 ppm. Additionally, at a concentration of 100 µg/ml, the extract decreased the viability of bladder cancer cells (UMUC-3 and BFTC-905) by 29.50±1.94 and 30.26±5.83%, respectively. Based on the findings, *C. inophyllum* leaf extract has potential as an antioxidant agent rather than a bladder cancer-inhibiting agent. Further research is required to better understand the mechanisms of bladder cancer cell viability reduction and to improve the saponin purity of the extract.

CRediT author statement

Setiyo Gunawan: Conceptualisation, Funding acquisition, Resources; Miftahul Hidayah Nugrahini: Software, Visualisation; Mochammad Ferdian Azizi: Investigation, Writing original draft; Safrina Hapsari: Project administration, Writing - Reviewing and Editing; Raden Darmawan: Data curation, Methodology; Hakun Wirawasista Aparamarta: Supervision, Validation; Firdaus: Formal analysis.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from the Institute Technology Sepuluh Nopember (ITS) for this work, under the project scheme of the Publication Writing and IPR Incentive Program (PPHKI).

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] World Health Organization (2018), "The top 10 causes of death", https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death, accessed 10 February 2023.

[2] World Health Organization (2020), "International Agency for Research on Cancer (IARC)", https://gco.iarc.fr/tomorrow/en/dataviz/isotype, accessed 17 February 2022.

[3] K.H. Schmitz, K.S. Courneya, C.E. Matthews, et al. (2010), "American college of sports medicine roundtable on exercise guidelines for cancer survivors", *Med. Sci. Sports Exerc.*, **42(7)**, pp.1409-1426, DOI: 10.1249/MSS.0b013e3181e0c112.

[4] A.P. Gupta, S. Khan, M.M. Manzoor, et al. (2017), "Anticancer curcumin: Natural analogues and structure-activity relationship", *Stud. Nat. Prod. Chem.*, **54**, pp.355-401, DOI: 10.1016/B978-0-444-63929-5.00010-3.

[5] Ministry of National Development Planning (2016), *Indonesian Biodiversity Strategy and Action Plan 2015-2020* (in Indonesian).

[6] S. Supardi, A.L. Susyanty (2010), "The use of traditional medicine in self-medication in Indonesia (Data analysis of SUSENAS 2007)", *Buletin Penelitian Kesehatan*, **38(2)**, pp.80-89.

[7] K. Heyne (1987), *Useful Plants of Indonesia Volume III*, Forest Research and Development Agency, Jakarta (in Indonesian).

[8] S. Gunawan, R. Darmawan, M. Nanda, et al. (2013), "Proximate composition of *Xylocarpus moluccensis* seeds and their oils", *Ind. Crops Prod.*, **41**, pp.107-112, DOI: 10.1016/j.indcrop.2012.04.010.

[9] S. Gunawan, H.W. Aparamarta, K. Kuswandi, et al. (2014), "The utilization of *Xylocarpus moluccensis* seed oil as biodiesel feedstock in Indonesia", *Ind. Crops Prod.*, **52**, pp.286-291, DOI: 10.1016/j.indcrop.2013.10.039.

[10] H.W. Aparamarta, T. Saputra, A. Claratika, et al. (2016), "Separation and purification of triacylglycerols from nyamplung (*Calophyllum inophyllum*) oil by batchwise solvent extraction", *Ind. Eng. Chem. Res.*, **55(11)**, pp.3113-3119, DOI: 10.1021/acs.iecr.5b04877.

[11] H.W. Aparamarta, S. Gunawan, S.I. Ihsanpuro, et al. (2022), "Optimization and kinetic study of biodiesel production from nyamplung oil with microwave-assisted extraction (MAE) technique", *Heliyon*, **8(8)**, DOI: 10.1016/j.heliyon.2022.e10254.

[12] D.F. Susanto, H.W. Aparamarta, A. Widjaja, et al. (2017), "Identification of phytochemical compounds in *Calophyllum inophyllum* leaves", *Asian Pac. J. Trop. Biomed.*, **7(9)**, pp.773-781, DOI: 10.1016/j.apjtb.2017.08.001

[13] S. Hapsari, I. Yohed, R.A. Kristianita, et al. (2022), "Phenolic and flavonoid compounds extraction from *Calophyllum inophyllum* leaves", *Arab. J. Chem.*, **15(3)**, DOI: 10.1016/j.arabjc.2021.103666.

[14] S. Hapsari, N. Jadid, H.W. Aparamarta, et al. (2023), "Impact of solvent type, solvent-water concentration, and number of stages on the extraction of coumarin mixture from tamanu (*Calophyllum inophyllum*) oil and its antioxidant activity", *Arab. J. Chem.*, **16(2)**, DOI: 10.1016/j.arabjc.2022.104449.

[15] M. Kainuma, S. Baba, H.T. Chan, et al. (2016), "Medicinal plants of sandy shores: A short review on *Calophyllum inophyllum* and *Thespesia populnea*", *Int. J. Pharmacogn. Pharm. Res.*, **8(12)**, pp. 2056-2062.

[16] A. Wijanarko, M.J. Ginting, M. Sahlan (2017), "Saponin isolation as the main ingredient of insecticide and collagen type I from Crown of Thorn-Starfish (*Acanthaster planci*)", *IOP Conf. Ser. Earth Environ. Sci.*, **89**, DOI: 10.1088/1755-1315/89/1/012032.

[17] V.T. Nguyen, M.C. Bowyer, I.A.V. Altena (2017), "Microwave-assisted extraction as an advanced technique for optimization of saponin yield and antioxidant potential from *Phyllanthus amarus*", *Sep. Sci. Technol.*, **52(17)**, pp.2721-2731, DOI: 10.1080/01496395.2017.1374972.

[18] E. Waziiroh, K. Kamilia (2018), "Microwave-assisted extraction (MAE) of bioactive saponin from mahogany seed (*Swietenia mahogany Jacq*)", *IOP Conf. Ser. Earth Environ. Sci.*, **131(1)**, DOI: 10.1088/1755-1315/131/1/012006.

[19] N.H. Ramli, S. Yusup, A.T. Quitain, et al. (2019), "Optimization of saponin extracts using microwave-assisted extraction as a sustainable biopesticide to reduce *pomacea canaliculata* population in paddy cultivation", *Sustain. Chem. Pharm.*, **11**, pp.23-35, DOI: 10.1016/j.scp.2018.12.002

[20] N. Aryanti, D.R. Heny, A. Nafiunisa (2020), "Optimization of ultrasound-assisted extraction of rarak saponin from *Sapindus Rarak* DC. using response surface methodology (RSM)", *AIP Conf. Proc.*, **2197(1)**, DOI: 10.1063/1.5140912.

[21] S. Rouhani, N. Valizadeh, S. Salimi (2009), "Ultrasonic assisted extraction of natural pigments from rhizomes of *Curcuma longa* L.", *Prog. Color Coat.*, **2**, pp.103-113, DOI: 10.30509/PCCC.2009.75754.

[22] M. Toma, M. Vinatoru, L. Paniwnyk, et al. (2001), "Investigation of the effects of ultrasound on vegetal tissues during solvent extraction", *Ultrason. Sonochem.*, **8(2)**, pp.137-142, DOI: 10.1016/s1350-4177(00)00033-x.

[23] Q.D. Do, A.E. Angkawijaya, P.L.T. Nguyen, et al. (2014), "Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica"*, *J. Food Drug Anal.*, **22(3)**, pp.296-302, DOI: 10.1016/j.jfda.2013.11.001.

[24] D.C. Montgomery (2005), *Design and Analysis of Experiments*, 6th ed., John Wiley and Sons Inc., 757pp.

[25] Ministry of Environment and Forestry (2021), "The condition of mangroves in Indonesia", https://www.kkp.go.id/djprl/kkp-akan-rehabilitasi-2400-hektare-hutan-mangrove-sepanjang-202165c2f1edf4151/detail.html, accessed 18 February 2022 (in Indonesian).

[26] S.E. Rikomah (2017), *Farmasi Rumah Sakit*, Deepublish Publisher, 156pp.

[27] United States Environmental Protection Agency (2022), "Estimation programs interface suite™ for Microsoft® Windows", https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface, accessed 18 June 2023.

[28] C.H. Hansch, A.J. Leo, D.H. Hoekman (1995), *Exploring QSAR: Hydrophobic, Electronic, Steric Constants*, DC: American Chemical Society, 368pp.

[29] T. Robinson (1995), *High Organic Content of Plants*, ITB Publisher, 285pp (in Indonesian).

[30] A.M. Goula (2013), "Ultrasound-assisted extraction of pomegranate seed oil - Kinetic modeling", *J. Food Eng.*, **117(4)**, pp.492-498, DOI: 10.1016/j.jfoodeng.2012.10.009.

[31] V. Lobo, A. Patil, A. Phatak, et al. (2010), "Free radicals, antioxidants, and functional foods: Impact on human health", *Pharmacogn. Rev.*, **4(8)**, pp.118-126, DOI: 10.4103/0973-7847.70902.

[32] M.H. Gordon (1990), "The mechanism of antioxidant action *in vitro*", *Food antioxidants/Elsevier Applied Science*, DOI: 10.1007/978-94-009-0753-9_1.

[33] G. Tirzitis, G. Bartosz (2010), "Determination of antiradical and antioxidant activity: Basic principles and new insights", *Acta Biochim. Pol.*, **57(2)**, pp.139-142, DOI: 10.18388/abp.2010_2386.

[34] A.A. Zanwar, S.L. Badole, P.S. Shende, et al. (2014), "Role of gallic acid in cardiovascular disorders, polyphenols in human health and disease", *Academic Press*, pp.1045-1047, DOI: 10.1016/B978-0-12-398456-2.00080-3.

[35] A.P. Faisal, P.R. Nasution, R.F. Wakidi (2022), "Antioxidant activity of starfruit leaves (*Calophyllum inophyllum L.*) against DPPH free radicals (1,1-Diphenyl-2-picrylhydrazyl), *Indonesian Journal of Pharmaceutical Research*, **4(1)**, pp.1-10, DOI: 10.33759/jrki.v4i1.200 (in Indonesian).

[36] M.E.S. Júnior, M.V.R.L. Araujo, A.A. Santana, et al. (2021), "Ultrasound-assisted extraction of bioactive compounds from ciriguela (*Spondias purpurea L.*) peel: Optimization and comparison with conventional extraction and microwave", *Arab. J. Chem.*, **14(8)**, DOI: 10.1016/j.arabjc.2021.103260.

[37] D.E. Gusungi, W. Maarisit, H. Hariyadi, et al. (2020), "Study of antioxidant and anticancer activity against breast cancer (mcf-7) of ethanol extract from langsat mistletoe leaves (*Dendrophthoe pentandra*)", *Tropical Bio. Pharmaceuticals*, **3(1)**, pp.166-174, DOI: 10.55724/j.biofar.trop.v3i1.274 (in Indonesian).

[38] S.E. Burdall, A.M. Hanby, M.R.J. Lansdown, et al. (2003), "Breast cancer cell lines: Friend or foe?", *Breast Cancer Res.*, **5(2)**, pp.89-95, DOI: 10.1186/bcr57.

[39] W. Xiao, R.S. Wang, D.E. Handy, et al. (2018), "NAD(H) and NADP(H) redox couples and cellular energy metabolism", *Antioxid. Redox. Sign.*, **28(3)**, pp.251-272, DOI: 10.1089/ars.2017.7216.

[40] M.A. Fuertes, J. Castilla, C. Alonso, et al. (2003), "Cisplatin biochemical mechanism of action: From cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways", *Curr. Med. Chem.*, **10(3)**, pp.257-266, DOI: 10.2174/0929867033368484.

[41] C.C. Lu, M.Y. Lin, S.Y. Chen, et al. (2013), "The investigation of a traditional Chinese medicine, Guizhi Fuling Wan (GFW), as an intravesical therapeutic agent for urothelial carcinoma of the bladder", *BMC Complem. Altern. M.*, **13**, pp.1-10, DOI: 10.1186/1472-6882-13-44.

[42] Z. Liu, X. Li, A.R. Simoneau (2012), "*Rhodiola rosea* extracts and salidroside decrease the growth of bladder cancer cell lines via inhibition of the mTOR pathway and induction of autophagy", *Mol. Carcinogen.*, **51(3)**, pp.257-267, DOI: 10.1002/mc.20780.

[43] I. Grigalius, V. Petrikaite (2017), "Relationship between antioxidant and anticancer activity of trihydroxyflavones", *Molecules*, **22(12)**, pp.1-12, DOI: 10.3390/molecules22122169.

[44] A. Alaklabi, I.A. Arif, A. Ahamed, et al. (2018), "Evaluation of antioxidant and anticancer activities of chemical constituents of the *Saururus chinensis* root extracts", *Saudi J. Bio. Sci.*, **25(7)**, pp.1387-1392, DOI: 10.1016/j.sjbs.2016.12.021.