
Duyen Thi My Huynh¹, Minh-Ngoc T. Le¹, Van De Tran², Viet-Hung Tran³*, Duy Toan Pham⁴*

¹Department of Pharmaceutical and Pharmaceutical Technology, Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, Can Tho, Vietnam
²Department of Health Organization and Management, Can Tho University of Medicine and Pharmacy, Can Tho, Vietnam
³Institute of Drug Quality Control-Ho Chi Minh City (IDQC HCMC), Ho Chi Minh City, Vietnam
⁴Department of Chemistry, College of Natural Sciences, Can Tho University, Can Tho, Vietnam

ABSTRACT

Cancer, one of the deadliest diseases worldwide, is projected to affect 30.2 million people by 2040. Among the various cancer types, rhabdomyosarcoma (RMS) is a unique tumor primarily impacting the muscular system of children. The current treatment for RMS has limited efficacy and numerous side effects, emphasizing the need for novel therapeutic approaches. This study investigates the potential treatment of the RMS cell line RD using extracts from five folklore-based medicinal plants in An Giang, Vietnam. The plants—*Moringa oleifera* Lam, *Brucea javanica* (L.) Merr., *Eclipta prostrata* (L.), *Callisia fragrans* (Lindl.) Woodson, and *Zingiber zerumbet* (L.) Smith—were extracted and fractionated using three solvents: ether, ethanol, and water. These fractions underwent phytochemical screening and cytotoxicity testing on the in-vitro RMS cell line RD. The results indicate that the ether fraction of *Eclipta prostrata* (L.) and the ether and ethanol fractions of *Zingiber zerumbet* (L.) Smith exhibit moderate cytotoxic effects on RD cell lines, with IC50 values of 37.08 ± 1.23 µg/mL, 23.15 ± 1.17 µg/mL, and 45.63 ± 2.39 µg/mL, respectively. These findings provide preliminary data for further in-depth research into the anticancer properties of these plants, which are widely grown in the South of Vietnam.

Keywords: rhabdomyosarcoma; *Moringa oleifera* Lam; *Brucea javanica* (L.) Merr.; *Eclipta prostrata* (L.); *Callisia fragrans* (Lindl.) Woodson; *Zingiber zerumbet* (L.) Smith; cytotoxicity; fractionation.

1. INTRODUCTION

According to the 2020 statistics from the Global Cancer Observatory (GCO), more than 19 million people worldwide have cancer, with Asia accounting for 49.3%, or about 9.5 million people¹. Predictably, by 2040, the number of people with cancer is expected to reach 30.2 million. Currently, cancer ranks as the second leading cause of death worldwide, significantly impacting both the mental and physical lives of patients²,³. Among the more than 100 types of cancers, rhabdomyosarcoma (RMS) stands out as a special and rare tumor, primarily affecting the muscular system of children, especially the skeletal (voluntary) muscles⁴. Most cases of RMS are diagnosed in children aged ≤6, with risk factors and etiology remaining unknown. RMS is often sporadic, associated with familial syndromes, and can be categorized into different types:
embryonal RMS (~60%), alveolar (~20%), pleomorphic (~10%), and spindle/sclerosing (~10%)\(^4\). This disease has been reported as the 3rd most common cancer and the most common soft tissue sarcoma in children\(^5\). Moreover, it can metastasize and develop into common cancers such as uterine cancer, stomach cancer, colon cancer, lymphoma, and limb cancer\(^6\). The current treatments for RMS, including surgery, radiation therapy, and chemotherapy (vincristine, actinomycin D, and cyclophosphamide/ifosfamide), yield poor and inadequate outcomes, especially in patients with metastatic and/or recurrent RMS\(^7,8\). For instance, the long-term event-free survival in metastatic RMS patients is <20\%\(^9,10\). Last but not least, these treatments often result in numerous side effects such as fatigue, hair loss, nausea/vomiting, and diarrhea\(^11\). Therefore, it is crucial and urgent to search for novel treatments that offer better oncological outcomes with long-term safety for RMS patients.

To this end, a potential source for finding novel RMS chemotherapeutic treatments is the medicinal/herbal plants that grow wildly or are cultivated across ASEAN countries. Specifically, in Vietnam, a country with a rich source of medicinal plants, with over 7,000 described species, of which 3,830 species possess therapeutic properties\(^12\). In fact, in most Vietnamese hospitals, medicinal plants have been comprehensively utilized in complement with modern medicine, with over 700 official medical products containing herbal ingredients\(^13,14\). In the Mekong Delta, a green area in the South of Vietnam, nearly 1,000 medicinal plant species have been exploited, with 500-700 species originating from the forests in provinces with mountainous terrain, such as An Giang. An Giang, a frontier province bordered with Cambodia, is famous for its extremely diverse and rich vegetation, harboring numerous precious medicinal herbs\(^15,16\). Among them, five particular plants have gained much interest, namely moringa (Moringa oleifera Lam, Moringaceae, Chum Ngay [Vietnamese], MO), Macassar kernels (Brucea javanica (L.) Merr., Simaroubaceae, Xoan rung [Vietnamese], BJ), ink plant (Eclipta prostrata (L.), Asteraceae, Co muc [Vietnamese], EP), basket plant (Callisia fragrans (Lindl.) Woodson, Commelinaceae, Luoc vang [Vietnamese], CF), and shampoo ginger (Zingiber zerumbet (L.) Smith, Zingiberaceae, Gung gio [Vietnamese], ZZ), due to their well-known ethnopharmacology (i.e., folk remedies) in supporting cancer treatments\(^17-21\). In the literature, these medicinal plants have been widely reported for their diverse pharmacological effects, including anti-inflammation, anti-plasmodial, antibacterial, anti-diabetic, and anti-oxidant\(^18,21-24\). Nevertheless, limited information on the chemotherapeutic properties of these plants has been published, especially for RMS treatment.

Therefore, this study, for the first time, investigated the ability of these five plants to treat RMS in vitro cell culture settings. Prior to the cytotoxicity tests, the plants were extracted, and their phytochemical compositions were determined accordingly. We hypothesized that the plants would possess potential action on RMS, significantly contributing to the literature on novel ethnopharmacological medicinal plants in An Giang, Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

The samples of MO leaves, BJ seeds, EP leaves, ZZ roots, and CF leaves were collected in Tinh Bien district, An Giang province, Vietnam, in February 2021. The plants were identified by a botanical specialist with specialized botanical documents provided by the An Giang Forest Protection Department. Voucher specimens (CTUMP-111, CTUMP-112, CTUMP-113, CTUMP-114, and CTUMP-115 for MO, BJ, EP, ZZ, and CF, respectively) were kept at the Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy. The collected plants were dried, ground, and sieved to appropriate sizes. The RD cell line (ATCC CCL-136\(^\text{TM}\)) was imported from ATCC.

Chemicals for determining plant compositions (i.e.,
diethyl ether, ethanol, and acetic acid) were imported from Xilong, China; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal calf serum (FCS), Eagle's minimum essential medium (EMEM), penicillin-streptomycin (Pen-Strep), trypan blue, trypsin-EDTA, L-glutamine, and dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich, Singapore. The positive control Anzatax® (paclitaxel 30 mg/5 mL) was purchased from Merck, Australia. All other chemicals were of reagent grade or higher.

2.2. Plant extraction
Fresh samples of MO leaves, BJ seeds, EP leaves, ZZ roots, and CF leaves, after being harvested, were washed, sliced, dried at ambient temperature, and finely ground to appropriate sizes. The plant powders with a moisture content of <13% were then extracted and fractionated with three solvents of different polarity, including diethyl ether, water, and ethanol, following the process demonstrated in Figure 1. Briefly, 100 g of the plant powders were macerated with 1200 mL of ether (plant:solvent ratio of 1:12 w/v) for 24 h. The product was divided into two parts: the solution and the plant residues. The solution was condensed with a rotavapor until the moisture content reached <20%, and then used to determine phytochemical compositions and investigate cytotoxicity on cancer cells. The residues were further fractionated with ethanol and water and then divided into two fractions similar to the ether solvent.

Figure 1. Plant fractionation procedure of 05 selected medicinal plants, including Moringa oleifera Lam (MO) leaves, Brucea javanica (L.) Merr. (BJ) seeds, Eclipta prostrata (L.) (EP) leaves, Callisia fragrans (Lindl.) Woodson (CF) leaves, and Zingiber zerumbet (L.) Smith (ZZ) roots
2.3. Phytochemical determination

The chemical constituents, in terms of the main compound groups, of the plant fractions (i.e., ether, ethanol, and water) were determined following standard procedures described in Figure 2. Each test was repeated in triplicate to confirm the results.

Figure 2. Phytochemical determinations procedures of the fractions (ether, ethanol, and water fractions) of 05 medicinal plants, *Moringa oleifera* Lam, *Bracea javanica* (L.) Merr., *Eclipta prostrata* (L.), *Callisia fragrans* (Lindl.) Woodson, and *Zingiber zerumbet* (L.) Smith.
2.4. In-vitro cytotoxicity MTT assay

The cytotoxicity of the plant fractions on RMS was tested using RD cell line. The cells were grown in EMEM medium, supplemented with 10% FCS, 2 mM L-glutamine, and 100 IU/mL + 100 µg/mL PenStrep. Cells were cultured in a 75-cm² flask, incubated at 37 °C with 5% CO₂ in humidified atmosphere, and the medium was changed every even day. Confluent cells (70-80% flask coverage) were washed with PBS, trypsinized with trypsin-EDTA, counted with trypan blue 0.4%, and the cell suspension was transferred into 96-well plates with a density of 12.5 x 10⁴ cells/mL (100 µL/well) for the testing experiments.

The plant fraction test samples were prepared in a DMSO solution at an initial concentration of 10 mg/mL and diluted in medium to reach the investigated concentrations of 100, 50, and 10 µg/mL. The negative control was DMSO at the same concentrations in the test samples (1%, 0.5%, and 0.1%, respectively). The positive control was the reference drug paclitaxel. All samples were filtered through a 0.22-µm membrane prior to cell treatments. The samples were subjected to the cells, incubated for 24 h, and the cytotoxic MTT assay was then conducted following the manufacturer’s protocol²⁵,²⁶. The formed formazan crystal was dissolved in isopropanol, and the solutions were UV-Vis spectroscopically measured at 570 nm with a microplate reader (Multiskan). All experiments were repeated four times. The %Cell inhibitory was calculated based on equation (1).

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%\text{Cell inhibitory} = 100\% - \frac{\text{OD sample} - \text{OD blank}}{\text{OD negative control} - \text{OD blank}} \times 100\% \tag{1}
\]

2.5. Statistical analysis

The results were processed using Microsoft Excel software and presented as mean ± standard deviation (SD). For statistical significance, the Mann-Whitney test was utilized on SPSS 20.0 software, with p<0.05 for meaningful comparisons.
3. RESULTS

3.1. Phytochemical determination

The phytochemical constituents of the five investigated medicinal plants (MO, BJ, EP, CF, and ZZ) are presented in Table 1. Each fraction contained different chemical groups, dependent on their polarity. Overall, the MO leaves contain major components of lipids, carotenoids, volatile oils, flavonoids, tannins, and polyuronides. The BJ seeds mainly possess lipids, alkaloids, coumarins, anthraquinones, and tannins. The EP leaves have volatile oils, terpenoids, alkaloids, flavonoids, saponins, and tannins as the main constituents. The ZZ roots mostly comprise volatile oils, alkaloids, anthraquinones, flavonoids, saponins, tannins, and polyuronides. The CF leaves contain terpenoids, alkaloids, coumarins, flavonoids, proanthocyanidins, saponins, tannins, and polyuronides.

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<th>Phytochemical group</th>
<th>Moringa oleifera Ether</th>
<th>Moringa oleifera EtOH</th>
<th>Moringa oleifera Water</th>
<th>Brueca javanica Ether</th>
<th>Brueca javanica EtOH</th>
<th>Brueca javanica Water</th>
<th>Eclipta prostrata Ether</th>
<th>Eclipta prostrata EtOH</th>
<th>Eclipta prostrata Water</th>
<th>Callisia fragrans Ether</th>
<th>Callisia fragrans EtOH</th>
<th>Callisia fragrans Water</th>
<th>Zingiber zerumbet Ether</th>
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3.2. In-vitro cytotoxicity MTT assay

The in-vitro cytotoxicity MTT assay on the RMS cell line RD of the ether fraction, the ethanol (EtOH) fraction, and the water fraction, at concentrations of 100, 50, and 10 µg/mL, of the five medicinal plants (MO, BJ, EP, CF, and ZZ) are presented in Table 2, in comparison with the reference compound, paclitaxel. The results demonstrated that the MO leaves, BJ seeds, and CF leaves did not possess significant cytotoxic effects on the RD cells (i.e., IC₅₀ > 100 µg/mL). On the other hand, the EP and ZZ fractions showed high efficacy. Specifically, the EP ether fraction, the ZZ ether fraction, and the ZZ ethanol fraction, had an IC₅₀ of 37.08 ± 1.23 µg/mL, 23.15 ± 1.17 µg/mL, and 45.63 ± 2.39 µg/mL, respectively. Compared to the well-known drug paclitaxel with an IC₅₀ of 6.96 ± 0.72 µg/mL, these fractions show potential efficacy on the RD cell lines.
Table 2. In-vitro cytotoxicity on the rhabdomyosarcoma RD cell lines of the ether fraction, ethanol (EtOH) fraction, and water fraction, at concentrations of 10, 50, and 100 µg/mL, of the 05 investigated medicinal plants, *Moringa oleifera* Lam (MO) leaves, *Brueca javanica* (L.) Merr. (BJ) seeds, *Eclipta prostrata* (L.) (EP) leaves, *Callisia fragrans* (Lindl.) Woodson (CF) leaves, and *Zingiber zerumbet* (L.) Smith (ZZ) roots. The results are expressed in terms of % Cell inhibitory (mean ± SD) and IC50 (mean ± SD) (n = 4). Note: the concentrations of the positive control (paclitaxel) were 10, 5, and 1 µg/mL, correspondingly.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>% Cell inhibitory</th>
<th>IC50 (µg/mL)</th>
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<td></td>
<td><strong>Moringa oleifera</strong></td>
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<td>Ether</td>
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<td>100</td>
<td>3.4 ± 0.5</td>
<td>10.8 ± 1.3</td>
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<td>50</td>
<td>7.3 ± 2.1</td>
<td>7.7 ± 1.8</td>
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<td>10</td>
<td>0.9 ± 0.1</td>
<td>6.3 ± 1.6</td>
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<th>Concentration (µg/mL)</th>
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<td><strong>Callisia fragrans</strong></td>
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<td>100</td>
<td>29.0 ± 2.5</td>
<td>22.2 ± 3.1</td>
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<td>27.7 ± 3.4</td>
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<td>≥ IC50 (µg/mL)</td>
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4. DISCUSSION

RMS, one of the most common cancers in children, has gained increasing attention due to its treatment difficulty. In fact, current RMS treatments involving surgery, radiation therapy, and chemotherapy possess poor outcomes with numerous side effects. Thus, novel chemotherapeutic agents are necessary. To this end, this study preliminarily investigated the efficacy of ether, ethanol, and water fractions of five potential medicinal plants, based on local folk remedies in An Giang, Vietnam, in the in-vitro cytotoxicity assay on the RD cell line. Ethanol and water were selected due to folklore wisdom (i.e., these plants are ethnopharmacologically used with ethanol and water as maceration solvents), whereas ether was chosen because it is a non-polar solvent that could possibly extract the non-polar therapeutic compounds in these plants.

Firstly, the MO plant has been ethnopharmacologically utilized for a long time in An Giang ethnic groups as an antioxidative, anti-inflammatory, antihypertensive, and immuno-regulatory agent. In addition, MO leaves are used in folk remedies for cancer treatments. In terms of phytochemical constituents, the main compounds in MO leaves are lipids, alkaloids, flavonoids, coumarins, and tannins, in agreement with a previous study. Interestingly, the phytochemical compounds in our research were different than the MO leaves in Nigeria, which could be due to geographical variations such as soil conditions, weather, and plant growth stages. Regarding its anticancer effect, previous studies have confirmed that the compound niaziminin (a water-soluble thiocarbamate glycoside) in MO leaves possesses high anticancer properties. Nevertheless, in all three fractions, with different polarities, the MO leaves did not show significant cytotoxicity on the RD cell line, indicating that this plant might not be potential for RMS treatment.

Secondly, the BJ plant is commonly used as food ("goi Sau dau" [Vietnamese] – a Vietnamese salad with a mixture of various vegetables and herbs).
Ethnopharmacologically, the BJ seeds could be ground, and its aqueous extract (i.e., tea) is used to treat diarrhea, appendicitis, and malaria. In BJ seeds, the main components are lipids, terpenoids, alkaloids, flavonoids, saponins, and tannins. Its anticancer property is acknowledged to be based on the compound bruceantin (C28H36O11), a quassinoid. Bruceantin has been proven to have high anticancer activity in various cancers such as lung cancer, myeloma, and gastric cancer.

Furthermore, in clinical tests with 68 lung cancer patients, BJ seeds extract demonstrates a complementary effect, in conjunction with radiotherapy, in enhancing the patients' quality of life and prolonging their lifespan from 10 months to 15 months compared to radiotherapy alone. However, in our study, the BJ seeds fractions did not show adequate action on the RD cell line.

Thirdly, the EP leaves are popularly utilized to make "tea" by the local people in An Giang. According to folklore, EP tea is believed to possess numerous effects, including antimicrobial, antiviral, pain relief, and anticancer properties. Phytochemically, the EP leaves in An Giang have similar constituents compared to those in other regions. In terms of cytotoxicity activity, dasycyphin C (C28H40O8), a saponin in EP, has been confirmed to possess various anticancer effects such as cervical carcinoma (IC50 = 50 μg/mL on HeLa cells). Interestingly, in our study, the ethanol and water fractions, which contain lots of saponin, did not yield significant effects on RD cells (IC50 > 100 μg/mL). On the other hand, the ether fraction, with no saponin, exhibited potential cytotoxicity on the RD cell line (IC50 = 37.08 ± 1.23 μg/mL). Further study is necessary to investigate in-depth the biological activities of the components present in the EP leaves ether fraction. Moreover, it is worth noting that the high concentration of 100 μg/mL of the ether extract significantly reduced the EP leaves' cytotoxicity on the RD cells (Table 2). This could be attributed to the fraction's cell proliferation effect. Therefore, the optimal concentration, in this case, was 50 μg/mL. Conclusively, it is necessary to investigate the suitable dose of herbal extracts to ensure their effectiveness in cancer treatment.

Fourthly, the CF leaves are commonly extracted with ethanol to produce a traditional pharmaceutics dosage form called herbal wine. According to the folklore, this wine is a good complementary medicine in treating liver cirrhosis, liver cancer, and other liver-related diseases, as well as acne, joint pain/inflammation, and gout. CF phytochemicals consist of alkaloids, flavonoids, glycosides, coumarins, and saponins, which are in well agreement with previous studies. In terms of the anticancer effects, to the best of our knowledge, no report (up to 2022) has been published on the CF extract action on the cancerous cells/tissues. Our results, for the first time, showed that CF leaves fractions might not be potent on RD cell line (IC50 > 100 μg/mL). Nevertheless, its anticancer effects need further analysis and evaluation on other cell lines, to fill in the literature gap.

Finally, the ZZ roots, which is generally used as “ginger tea” for various therapeutic effects, contain mostly volatile oils, flavonoids, saponins, and alkaloids, which are in correlation with previous work. Among these chemicals, zerumbone (C15H22O), a sesquiterpene volatile oil with a humulan-based carbon framework, possesses outstanding anticancer activity. For example, the anticancer activity of zerumbone against the human HeLa cell line was confirmed with an IC50 of 2.5 μg/mL. In our work, the IC50 values of the ether fraction and ethanol fraction of ZZ roots were 23.15 μg/mL and 45.63 μg/mL, respectively, indicating the potential effects of this plant on RMS cell line RD. The fact that the ether fraction was more potent than the ethanol fraction could be contributed to the higher amount of volatile oils (i.e., zerumbone) in the former (Table 1).

5. CONCLUSION
This study investigated the potency of five common medicinal plants (MO, BJ, EP, CF, and ZZ) in An Giang, Vietnam, for the treatment of RMS using RD cell line. The EP ether fraction, the ZZ ether fraction, and the GIN...
ethanol fraction, possess moderate cytotoxic effects on RD cell lines, with an IC_{50} of 37.08 ± 1.23 µg/mL, 23.15 ± 1.17 µg/mL, and 45.63 ± 2.39 µg/mL, respectively. These results provide preliminary data for further in-depth research on the RMS anticancer properties of these plants, especially the EP and ZZ plants, which are widely grown in the South of Vietnam.

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REFERENCES
1. Global Cancer Observatory.


Eclipta prostrata (L.) Merr., (Moringa oleifera Lam), (Brucea javanica (L.) Merr), (Callisia fragrans (Lindl.) Woodson), prostrata (L.)

The study was conducted in An Giang, Vietnam: Initial study on the potential of Moringa oleifera Lam in the treatment of Rhabdomyosarcoma (RMS) cell lines in the laboratory. The researchers investigated five medicinal plants (Moringa oleifera Lam, Brucea javanica (L.) Merr, Eclipta prostrata (L.), Callisia fragrans (Lindl.) Woodson, Zingiber zerumbet (L.) Smith) grown in southern Vietnam, which are known to have anti-tumoral properties. The researchers extracted the plants and tested their essential oils, methanol, and ethanolic fractions in a cell line assay.

The results showed that the ethereal fraction of Eclipta prostrata (L.) and ethanolic fractions of Zingiber zerumbet (L. Smith) and ethanolic fractions of ethereal fraction showed moderate cytotoxicity against RMS RD cell lines, with IC50 values of 37.08 ± 1.23, 23.15 ± 1.17, 45.63 ± 2.39 micrograms/ml, respectively. These findings suggest that further research is needed to explore the anti-tumoral potential of these medicinal plants.

The study highlights the potential of these plants in the treatment of RMS, which is a rare but severe form of cancer in children. Further research is needed to confirm these findings and to develop effective treatments for RMS.

Keywords: rhabdomyosarcoma, Moringa oleifera Lam, Brucea javanica (L.) Merr, Eclipta prostrata (L.), Callisia fragrans (Lindl.) Woodson, Zingiber zerumbet (L.), cytotoxicity, southern Vietnam.

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