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Short Communication

Toxicity Evaluation with Brine Shrimp Lethality Test and Phytochemical Analysis of Some Indonesian Plant Extracts As Potential Anti-Colon Cancer Agents

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ABSTRACT

Garlic (*Allium sativum*), red ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), tea (*Camellia sinensis*), tomato (*Solanum lycopersicum*), bitter melon (*Momordica charantia*), pearl grass (*Hedyotis corymbosa* (L.) Lamk.), soybean (*Glycine max*), grape (*Vitis venifera*) and Sambiloto (*Andrographis paniculata*) were studied for their anticancer potential in colon cancer. Using a combination of these plants as promising anticancer agents still requires further exploration from the cultivation and formulation. This research was aimed at the phytochemical analysis and evaluation of the anticancer potential of these plants in colon cancer. The Brine Shrimp Lethality toxicity test using *Artemia salina* Leach larvae was used to determine the LC_{50} value of the plant extracts. Two of the ten plants with the lowest LC_{50} values include turmeric and ginger, with cytotoxicity values of 15.995 ppm and 3.488 ppm, respectively.

***Keywords*:** Extract, colon cancer, Brine Shrimp Lethality Test, Phytochemical analysis

Introduction

Cancer, a disease characterized by pathophysiological changes in the normal process of cell division, has become a major health issue that kills a substantial number of people each year all over the world.[1,2] According to data released, there were more than 19.3 million new cases of cancer diagnosed and recorded recently. About 10 million deaths were due to cancer worldwide in 2020.[3] The necessity and demand for creating effective medications to treat various malignancies have arisen from the ongoing global increase in cancer cases, which results in millions of deaths each year.[4,5]

The most common methods of cancer therapy are radiation and chemotherapy. Radiation therapy and chemotherapy aim to destroy cancer cells, control tumour growth in primary cancer-affected tissues, and control tumour growth in other tissues not initially affected by cancer. Cancer development generally occurs through four stages: initiation, growth, survival, and metastasis. Oncogenic, oxidative metabolic production stress and mitochondria signalling can initiate cancer. Mitochondria play an essential role in the process of cancer development. Mutation in mitochondria will produce enzymes or oncometabolite material that triggers tumour occurrence. Oxidative stress and mitochondrial signalling may also be initiation triggers supporting cancer cell growth. Mitochondria biogenesis and homeostasis of oxidation-reduction in cells play a role in metastasis.[6]

Chemotherapeutic drugs like doxorubicin are frequently used to treat a variety of malignancies, and they have been shown to be very effective in treating these conditions.[7] Currently, the extensive clinical use of doxorubicin is strongly questioned due to its dose-dependent adverse side effects, such as cardiotoxicity, hair loss, impaired heartbeat, and decreased white blood cell count involved in the body's defence despite its high efficiency.[8]

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This condition poses a challenge in the effective treatment of cancer and can help reduce the adverse effects of the primary treatment. One approach to overcome the high cost of therapy and the magnitude of side effects caused by cancer therapy is the utilization of natural ingredients as an alternative to anticancer agents.[9]

In 2020, colorectal cancer (CRC) constituted 10% of all cancer incidences worldwide and resulted in 9.4% of cancer-related fatalities; lung cancer contributed to 18% of these deaths. It is estimated that by 2040, 3.2 million new cases of colorectal cancer will occur globally due to ageing, population growth, and advances in human development. The rise in the prevalence of colorectal cancer is primarily attributed to increased exposure to environmental risk factors resulting from a shift in food and lifestyle towards Westernisation.[10,11] Javed et al. (2017) reviewed plant extracts with potential as colon cancer drug candidates based on *in vitro* and *in vivo* studies done by researchers. [12]

The use of plants as an anticancer agent still requires further exploration concerning their cultivation and formulation. This research involves a selection from a pool of 10 plants studied for their potential as anticancer agents against colon cancer and their cytotoxicity screening using the Brine Shrimp Lethality Test (BSLT) bioassay employing *Artemia salina* Leach larvae to determine their LC_{50} value.

Materials nd Methods

Sample Preparation

The plant samples in different harvesting stages were collected from various plantations in July 2022. Each plant sample used for this study was identified at the Herbarium Bogoriense, Research Center for Biology - Indonesian Institute of Sciences, Botany Field, Bogor - West Java, and voucher numbers were assigned. All the samples were dried under the shade for a week and ground to powder, weighing 2 kg each.

Preparation of Plant Extracts

The dried powdered samples were macerated separately in 96% ethanol at 1:7 for 24 hours. Subsequently, the macerates were filtered using Whatman No. 1 filter paper, concentrated to dryness using a rotary evaporator at 40°C, and stored at 4°C before further analysis. The yields were computed in percentages from the weight of the extract obtained relative to the weight of the powdered material used.

Phytochemical Analysis
Phytochemical analysis was carried out to determine the type of secondary metabolites using an established method.[13]
Test for steroid/triterpenoid: The extract (1 mg) from each sample was dissolved in 2 mL DMSO in a test tube. Ten drops of acetic acid anhydrous and 3 drops of sulfuric acid were added to the mixture. A pred precipitate, which turns blue and green, was formed.

Flavonoid Test: The extract (1 mg) from each sample was added to 0.1 mg of magnesium powder, 0.4 mL of amyl alcohol, and 4 mL of alcohol, then shaken. An orange precipitate was formed on the amyl alcohol layer.

Alkaloid Test: The extract (1 mg) from each sample was dissolved in a few drops of 2 N sulfuric acid, after which it was tested with alkaloidal reagents (Dragendorff, Meyer, and Wagner). Brown, white, and orange-red precipitates were formed with Wagner's, Meyer, and Dragendorff reagents, respectively.

Saponin Test: The extract (1 mg) from each sample was dissolved in hot water and shaken. A stable foam that persists for 30 minutes and does not disappear after adding 1 drop of 2 N HCl was formed.

Hydroquinone Phenol Test: Each sample extract (1 mg) was added to 20 mL DMSO. 1 mL of the extract solution was added to 2 drops of 5% $FeCl_3$ solution. A green colour precipitate was observed.
Tannin Test: To each sample extract (1 mg), a few drops of 3% $FeCl_3$ reagent. A blue colour precipitate was formed.

Brine Shrimp Lethality (BSLT) Toxicity Evaluation
Preparation of Artificial Seawater: Artificial seawater was prepared by dissolving 15 g of sodium chloride in 1 L of distilled water.[14]
Hatching of *A. salina* Leach eggs: Shrimp eggs were hatched in about 36-48 hours using clear or transparent conical containers in the artificial seawater, with the pH adjusted accordingly. The receptacle was illuminated with a 40-watt lamp to maintain the temperature in the hatchery so that the hatching temperature of 25ºC-31ºC is maintained, and the hatching process was stimulated by using an aerator. *A. salina* Leach eggs, 50-150 mg, were washed first and soaked in a container filled with distilled water for 1 hour. After that, the eggs were transferred into a container filled with 500 mL of artificial seawater, and the aerator was turned on. Eggs were hatched within 18 - 48 hours and moved naturally towards bright areas to separate the shrimp larvae from the eggshells. Healthy larvae were autotrophic and ready to be used as test animals at 36-48 hours. Larvae were separated from the eggs by pipette and placed into a vial containing artificial seawater.
Preparation of Test Solutions: Eight vials were prepared for each extract concentration (0 ppm (negative control), 15.625 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL) in triplicates. Stock solutions for the test samples were obtained by dissolving 10 mg of the extract in 100 mL of artificial seawater. Each vial (10 mL) contained 10 *A. salina* Leach larvae, and one drop of yeast suspension (0.6 mg/mL) was added as food. The negative control test (blank) was treated the same as the test solution but without the extract. The vials were placed under lighting with a 40-watt lamp. The number of dead *A. salina* larvae in each vial was counted manually for 24 hours. The number of dead larvae indicated the level of toxicity.

Results and Discussion

The Brine Shrimp Lethality Test is a simple, fast, reproducible, and inexpensive method to evaluate the cytotoxicity of plant extracts, fractions, pure compounds, and other medicinal agents. In this study, ten medicinal plants (Table 1) used for centuries in different countries ethnomedicine to treat various diseases (cancers, inflammations, diabetes, infertility, bacteria and viral infects, etc.) were chemically profiled using standard methods to identify their secondary metabolites and evaluated for their cytotoxicity against the *A. salina* larvae. Results of the phytochemical screening, toxicity evaluation, and extraction yields are shown in Tables 2 and 3.
The mortality of *A. salina* shrimp larvae resulting from the addition of Leach concentrations of medicinal plant extracts was assessed in the Brine shrimp lethality test. Results of the study determined through probit analysis showed that larvae mortality was concentration dependent. As the test sample concentration increased, the corresponding linear increase in shrimp larvae mortality. Consequently, the LC_{50} value for each plant extract was determined through probit analysis. The LC_{50} values reveal insights into the potential anticancer activity of each studied medicinal plant. Of the ten plants evaluated, *Allium sativum*, *Curcuma longa*, and *Oldenlandia corymbose* exhibited strong cytotoxic potentials with LC_{50} values of 3.488, 15.995, and 25.49 ppm, respectively. The results of this study agree with a previous report of the cytotoxicity of bulbs of *Allium sativum* alcoholic and aqueous extracts with LC_{50} values of 10.840 and 8.180 mg/mL against brine shrimps.[15] In cytotoxicity studies involving *A. salina*, it has been reported that LC_{50} values lower than 1000 ppm are considered cytotoxic (active) while non-toxic (inactive) if it is greater than 1000 ppm.[16] Results of this study (Table 3) indicate that all the plant extracts examined were cytotoxic. The cytotoxicity of these plants can be attributed to different phytochemicals (alkaloids, flavonoids, triterpenes, saponins, and tannins) expressed in these plants, which have been proven in several studies to exhibit varied pharmacological activities.

Table 1: Plants Determination

No	Name of Plant	Part of the plant	Varieties	Family
1	*Curcuma longa* L	Rhizome	*Curcuma longa* L	Zingiberaceae
2	*Zingiber officinale* Roscoe	Rhizome	*Zingiber officinale* Roscoe	Zingiberaceae
3	*Allium sativum* L	Tubers	*Allium sativum* L	Amaryllidaceae
4	*Oldenlandia corymbosa* L	All parts of the plant	*Oldenlandia corymbosa* L	Rubiaceae
5	Glycine max (L.) Merr.	Fruit	Glycine max (L.) Merr.	Leguminosae/Fabaceae
6	*Vitis venifera* L	Fruit	*Vitis venifera* L	Vitaceae
7	*Andrographis paniculata* (Burm f) Nees	Leaf	*Andrographis paniculata* (Burm f) Nees	Acanthaceae
8	*Camellia sinensis* (L.) Kuntze	Leaf	*Camellia sinensis* (L.) Kuntze	Theaceae
9	*Solanum lycopersicum* L.	Fruit	*Solanum lycopersicum* L.	Solanaceae
10	*Momordica charantia* L.	Fruit	*Momordica charantia* L.	Cucurbitaceae

Table 2: Phytochemical Screening Results

Name	Chemical compounds				
	Alkaloid	**Flavonoid**	**Tanin**	**Saponin**	**Steroid/ Triterpenoid**
Curcuma longa L	+	+	-	+	+
Zingiber officinale Roscoe	+	+	+	+	-
Allium sativum L	-	+	-	+	-
Oldenlandia corymbosa L	+	+	+	+	+
Glycine max (L.) Merr.	-	+	+	+	-
Vitis venifera L	-	+	+	+	+
Andrographis paniculata (Burm f) Nees	-	+	-	-	+
Camellia sinensis (L.) Kuntze	+	+	+	+	+
Solanum lycopersicum L.	+	+	-	-	+
Momordica charantia L.	-	+	-	+	-

Table 3: Lethal Concentration and Extracts Yield of the Samples

No	Sample Name	LC_{50} (ppm)	Extracts Yield (%)
1	*Curcuma longa* L	15.995	50.32
2	*Zingiber officinale* Roscoe	232.27	14.60
3	*Allium sativum* L	3.488	15.32
4	*Oldenlandia corymbosa* L	25.49	38.20
5	Glycine max (L.) Merr.	117.679	22.41
6	*Vitis venifera* L	41.495	41.32
7	*Andrographis paniculata* (Burm f) Nees	719.50	5.33
8	*Camellia sinensis* (L.) Kuntze	118.304	65.82
9	*Solanum lycopersicum* L.	90.364	7.50
10	*Momordica charantia* L.	119.124	61.80

Conclusion

Results of the evaluation of red ginger rhizome, turmeric, and pearl grass extracts showed good potential for anticancer activity. These plants can further be exploited for potential anticancer agents through bioactivity-guided isolation and comprehensive *in vitro* and *in vivo* evaluation of the compounds for cytotoxic activity against different cancers.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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