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Original Research Article

Phytochemical, Antioxidant, and Cytotoxic Activity of Water Hyacinth (*Eichhornia crassipes*) Ethanol Extract

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ABSTRACT

Water hyacinth (*Eichhornia crassipes*) is a wide-spreading and damaging aquatic plant in Indonesia, especially near lake areas. The use of water hyacinth as a drug candidate has yet to be carried out systematically and comprehensively. This study aims to investigate the relation between the content of secondary metabolites from plant parts, and the *in vitro* antioxidants, and cytotoxic activities. This research was carried out by extracting the plant's leaves, stems, and roots using 96% ethanol and then evaporating the extract using a vacuum rotary evaporator and nitrogen gas. Each part of the plant extract was tested for antioxidant activity using ABTS and FRAP methods. In contrast, the cytotoxic assay was performed against human breast cancer cell lines, namely MCF-7, T47D, and MDA-MB 231. In addition, the secondary metabolite content, such as total tannin content (TTC) and total alkaloid content (TAC), was measured with colorimetric method using a visible spectrophotometer. The IC_{50} value was used to evaluate the activity of the extract in µg/mL units. The leaf extract had the best ABTS cation radical scavenging activity with an IC_{50} value of 42.91 ± 5.42 µg/mL. The high tannin content in the leaves (2.823 ± 0.139 mg TAE /g extract) is predicted to cause antioxidant activity. The best cytotoxic activity was found in leaf extracts against MCF-7 cells (IC_{50} 38.27 ± 19.17 µg/mL). Tannins are considered the main secondary metabolites that have a role in water hyacinth leaf antioxidant and cytotoxic activity.

Keywords: tannin, antioxidant, water hyacinth, *Eichhornia crassipes*, cytotoxic, cancer

Introduction

Plants are used for medicine in various countries and are a potential source of medicinal substances.[1] The mechanism of action of most plant drugs is due to secondary metabolites.[2] Products of natural plant origin have an important role in the treatment process during this time. As many as 50% of circulating drugs, including cancer drugs, are made from natural ingredients and their derivatives. Secondary metabolites of plants are largely responsible for the drug's bioactivity.[3] Bioactive compounds are useful in medicine, such as alkaloids, flavonoids, phenolics, essential oils, and polyphenols.[4] Water hyacinth is a plant that can damage aquatic ecosystems in the world but has various types of potential secondary metabolites that have not been utilized for therapeutic purposes yet. Phenolic compounds are detected in the leaves of water hyacinth. 4-Methylresorcinol, 2-methylresorcinol, catechol, pyrogallol, and genetisic, p-hydroxybenzoic, syringic, vanillic, and salicylic acids have been detected by TLC in the ethanolic shoot extract, whereas 4-methylresorcinol, 2-methylresorcinol, resorcinol, catechol, and genetisic and salicylic acids were present in rhizomes. 1(2,4-Dihydroxyphenyl) 2(4-methoxy-3-nitrophenyl) ethanone was identified in the ethanol extract by GC-MS.[5]

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Research related to the isolation of secondary metabolites of water hyacinth has been carried out, including isolation of tannin from aqueous extracts and methanol extracts,[6] as well as alkaloids, namely cytisine and tomatine in the shoots and rhizomes; codeine, thebaine, and quinine are present in the shoots; and the rhizome contains nicotine.[7] GC-MS analysis of the methanol extract showed the presence of 18,19-acid-19-socoyohimbanoic acid and 6,17,20,21-tetradehydro-16-(hydroxymethyl)-methyl ester,[8] while pipradrol, and 1H-pyrrole,1-phenyl were detected in the ethanol extract.[9]

Phenols, flavonoids, tannins, alkaloids, and glycosides are good antioxidants that prevent or control oxidative stress-related disorders. Results from a study suggested that water hyacinth is a potential source of antioxidant activity and could be used as an antioxidant.[5] Phenolic compounds have redox properties, which allow them to act as antioxidants. As their hydroxyl groups facilitate their free radical scavenging ability, the total phenolic concentration could be used to screen antioxidant activity rapidly. Flavonoids, including flavones, flavonols, and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Plant flavonoids have antioxidant activity *in vitro* and act as antioxidants *in vivo*. Phenolic and flavonoid content has shown a good correlation with antioxidant activity. This may be due to structural differences. The IC_{50} value in milligrams per milliliter of each extract correlated with higher concentrations of polyphenols and flavonoids. Extracts of different parts of plants having lesser IC_{50} values are more potent antioxidants and simultaneously showed an increased concentration of polyphenols and flavonoids.[10] Alkaloids were tested for Trolox equivalent antioxidant activity (TEAC) using the synthetic stable radical cation ABTS•+ /2.2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/. The results of these two assays

are usually in good accordance.[11-13] Antioxidants are also reductants, but the relationship is not always direct.[14-15] For this purpose, the ferric-reducing antioxidant power (FRAP) assay was ordinarily used for alkaloids.[16-17]

The antioxidant ability of several secondary metabolites also impacted their anticancer activity. Polyphenols, for example, hydroxy groups in the polyphenol structure that can block the formation of reactive oxygen species (ROS), such as hydroxyl radicals, through chelation of redox-active transition metal ions have a positive effect on cancer prevention by minimizing phenomena such as oxidative damage to DNA. In breast cancer cell lines, polyphenols exert a strong inhibitory effect due to their antioxidant properties that oppose H_2O_2 oxidizing activity by scavenging ROS or inhibiting ROS production. The mechanism of polyphenols as anticancer is related to their effects on estrogen production and signaling pathways have been attributed to their role as AI and their interactions with ER and estrogen-metabolizing enzymes. They act as BCRP inhibitors and interact with CYP1 as both inhibitors and substrates. Research has also demonstrated their ability to induce apoptosis and cell cycle arrest and to alter many signaling pathways involved in cancer-related phenomena such as inflammation and proliferation.[18] Several studies have shown that the water hyacinth plant has cytotoxic activity against some breast cancer cells. Water hyacinth leaves have cytotoxic activity against MCF-7 cells[3,19] and T47D[20]. Alkaloids exhibit a promising role as anticancer agents by restraining the enzyme topoisomerase, which is associated with the replication of DNA, instigating apoptosis, and modulating various other intracellular targets and signaling pathways. These alkaloids with diverse chemical structures showing varied cytotoxicity against various cancer cell lines can only be best understood through future molecular study and molecular docking analysis. Based on published results, alkaloids with anticancer activities showed ample diversity. The source of alkaloids with anticancer activity is very broad. Most of the alkaloids belong to various families, and the biosynthesis of these agents is also varied. It is daunting to indicate common mechanisms of action for alkaloids since compounds, even within a particular structural class, exhibit differential cellular and molecular mechanisms.[21] So far, there has been no research on the antioxidant and anticancer activity of the extracts of three parts of the *Eichhornia crassipes* plant, namely leaves, stems, and roots, that have been carried out together. In addition, this study also investigated the relationship between the content of secondary metabolites on antioxidant and/or anticancer activity.

Materials and Methods

Extraction

Water hyacinth was collected in August 2021 and obtained from Rawa Pening Lake, Ambarawa, Semarang Regency, Central Java, Indonesia. The plant species were identified at Plants Taxonomy Laboratory, Universitas Negeri Semarang, and the voucher specimen (No. 233) was deposited at Herbarium Semarangense. Initially, the plant parts (leaves, stems, and roots) are separated before drying using indirect sunlight. The plant parts are placed on woven bamboo, covered with black cloth, and then dried in sunlight. The dried part of the plant was then ground into powder. Extraction of water hyacinth leaf, stem, and root powder was conducted by maceration method using 96% ethanol (one gram of powdered material requires 10 mL of ethanol) for three days with daily solvent replacement. Each ethanol extract was then concentrated with a vacuum rotary evaporator at a temperature of 60°C. The concentrated extract was then dried using nitrogen gas to produce a dry ethanol extract.

Determination of total tannins content (TTC)

The total tannin content was determined using tannic acid colorimetric method,[22] with slight modifications using tannic acid as the standard. One mL of the extract was transferred to a 50 mL volumetric flask containing 2 mL of distilled water. To the mixture, 0.5 mL of Folin-Denis reagent followed by 1 mL of sodium carbonate solution were added and diluted to 10 mL with distilled water. The mixture was shaken well and kept for 30 minutes at room temperature. The developed blue color was read at 765 nm using a UV-Vis spectrophotometer. Total Tannin Content (TTC) was calculated using a standard tannic acid chart of 9 - 23 µg/mL (with r^2) and the results were expressed as mg of tannic acid equivalents (TAE) per gram of sample. All experiments were performed three times. The results were averaged and reported in the form of Mean ± SD.

Determination of total alkaloids content (TAC)

The total alkaloid content was determined using Shamsa *et al*.[23] method. Ten mL of the sample (1 mg/mL in water) was added with 5 mL of phosphate buffer pH 4.7 and 5 mL of BCG (Bromocresol green 10^4) solution in a separating funnel. The mixture was shaken with 10 mL of chloroform, and the chloroform phase was separated (three times extraction). The chloroform phase was added to a 10 mL volumetric flask. The absorbance of the complex in chloroform was measured at 470 nm using a UV-Vis spectrophotometer. Total Alkaloid Content (TAC) was calculated using a standard caffeine chart of 1 - 5 µg/mL (R^2 = 0,9943) and the results were expressed as mg of caffeine equivalents (CE) per gram of sample. All experiments were performed three times. The results were averaged and reported in the form of Mean ± SD.

ABTS Free Radical-Scavenging Activity

In the ABTS free radical assay, the method El Jemli *et al*.[24] adopted with minor changes. Briefly, a fresh ABTS reagent solution was prepared by mixing 2 mM ABTS solution with 70 mM potassium persulfate and stored in the dark at room temperature for 16 h before use. The ABTS •+ solution was diluted with methanol to obtain an absorbance reading of 0.8 – 1.0 at 743 nm. One hundred µL of sample solutions of various concentrations were added to 100 µL of ABTS solution. The absorbance was measured at 734 nm using spectrophotometer after 1 min of the reaction mixture. All measurements were repeated three times. Standard curves were obtained using sample solutions at various concentrations. Radical scavenging activity at different sample concentrations against ABTS radicals was also measured to calculate IC_{50}.

Ferric Reducing Antioxidant Potential (FRAP) Assay

The FRAP assay was measured by spectrophotometric assay as described by El Jemli *et al*.[24] Samples of 100 µL at different concentrations, 100 µL of phosphate buffer (0.2 M, pH 6.6), and 100 µL of potassium ferricyanide $K_3Fe(CN)_6$ (1%) were mixed and incubated at 50°C for 20 min, to reduce ferricyanide to ferrocyanide. The reaction was stopped by adding 100 µL of 10% (w/v) trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 min. Finally, 100 µL of the top layer was mixed with 100 µL of distilled water and 25 µL of 0.1% $FeCl_3$ solution. The absorbance at 710 nm was calculated by plotting the absorbance against the appropriate sample concentration. All determinations were made three times.

Cytotoxic Assay

The MCF-7, T47D, and MDA-MB 231 cells were obtained from the American Type Culture Collection. Cells in 80-90% confluent conditions for harvest were taken from the CO_2 incubator. The number of cells was counted, and the cells were diluted with complete medium. Cells with a density of 5 $x10^3$ - 10^4 cells/well were transferred into wells, 100 µl each. The cells were resuspended gently to remain homogeneous before adding to 96 well plate. The state of the cells was observed with an inverted microscope to see the distribution of cells. Cells were incubated in a CO_2 incubator overnight (for cells to recover after harvest). The cells were treated with samples after the cells returned to normal conditions. After normal cells returned for 1 x 24 hours, a series of sample concentrations (10 – 500 µg/mL) were made for treatment (including cell control and media control). The plate that already contains the cells is taken from the CO_2 incubator, then the cell media is discarded. A series of sample concentrations of 100 µL were put into wells (triplicate) and incubated in a CO_2 incubator for 24 hours (there was a cytotoxic effect). MTT reagent was prepared for treatment (0.5 mg/mL) by taking 1.0 mL of MTT stock in PBS (5 mg/mL), diluted

with complete medium to 10.0 mL. The cell media was discarded, and 100 µl of MTT reagent was added to each well. Cells were incubated for 4 hours in a CO_2 incubator. Incubation was carried out until formazan was formed. Cell conditions were examined with an inverted microscope. If formazan was clearly formed, 100 µl of DMSO was added. The plate was wrapped in aluminum foil, shaken, and incubated in the dark at room temperature for 15 minutes. The absorbance of each well was read with a plate reader at a wavelength of 595 nm.[25] The absorbance data obtained from the cytotoxic test was then converted into the percentage of cell viability which was calculated using the formula:

$$\%\ cell\ viability = \frac{treated\ absorbance - medium\ absorbation}{control\ absorbance - medium\ absorbance}\ x\ 100\%$$

Data Analysis

All the assays were carried out in triplicate. The means and standard deviation (SD) were determined using SPSS version 20. The total tannins content, total alkaloids content, antioxidant activities, and cytotoxic evaluation are expressed as mean values ± SD, and the analysis of variance was performed to determine significant differences. P values less than 0.05 were considered statistically significant.

Results and Discussion

This study examined the potential of water hyacinth to be explored for the source of secondary metabolites having antioxidant and anticancer activities. The study used ethanol as a solvent in the extraction process. Ethanol can extract secondary metabolites, both polar and non-polar compounds. In addition, ethanol is easily separated by evaporation so that it becomes a solvent-free dry extract. Furthermore, ethanol is non-toxic and easy to obtain, in which the resulting extract can then be used as a candidate for further drug development. A previous study showed the antioxidant activity of water hyacinth tested using ferric thiocyanate method. This study observed that ethanol extract (at a concentration of 100 µg/mL) caused 85.6% lipid peroxidation inhibition of linoleic acid emulsion, greater than that of aqueous extract (28.8%) and chloroform extract (64%).[26] Studies related to water hyacinth have reported that whole extract induces cytotoxicity in MCF-7 with an IC_{50} value of 1.2 ± 0.2 µg/mL, while more than 80% of cells died after being given leaf extract with a concentration of 100 µg/mL. The extraction method used by the two results was maceration using methanol and 80% ethanol, respectively. These studies reported that nine pure fractions separated from the whole extract (fraction A-I) had lower cytotoxic activity against MCF-7 cancer cells than the whole extract, which was between 11.1 to 69.1 ppm.[19-20]

Total Secondary Metabolites

This study examined two secondary metabolites belonging to tannic acid and alkaloid compounds. The values of TTC expressed in mg tannic acid equivalent (mg TAE) and TAC expressed in mg caffeine equivalent (mg CE) per g of raw extracts are presented in Figure 1. The tannin content in water hyacinth leaves is 2.823 ± 0.139 mg TAE/g extract. This value is the highest compared to the stems and roots. This is similar to previous studies using 80% methanol as a solvent for extraction, resulting in a TTC value of 0.74 mg/kg powder.[27] The study showed that the tannin content in the leaves was three times greater than that of the stems (0.777 ± 0.006 mg/g extract) and thirteen times greater than the roots (0.204 ± 0.002 mg/g extract). The higher tannin content in the leaves than roots is in concordance with research by Takshak and Agrawal[28], who reported that UV solar radiation induces the production of ROS and promotes the biosynthesis of tannin better than in root in *Withania somnifera*. This is corroborated by previous research on the content of other polyphenol groups besides tannins, namely flavonoids, which have a greater content in leaves than other plant parts.[29-30] However, the synthesis and accumulation of secondary metabolites are very complex which are affected by many factors, including internal developmental genetic circuits (regulated gene, enzyme) and by external environmental factors (light, temperature, water, salinity, etc.).[31] The quantitative analyses showed that the leaf and stem were rich in tannins, while the root showed the lowest value. This agrees with the general knowledge that secondary metabolites are mostly concentrated in the leaf.[32] The tannin content in water hyacinth leaves has the highest amount compared to other parts of the plant. It can be explained that the content of hydroxyl groups is high in the leaves because they are where photosynthesis occurs.[33] This study also found that alkaloid content in the stems showed higher yields than in the leaves and roots. A study reported that some alkaloids, i.e., pipradrol and 1H-pyrrole,1-phenyl were found in high levels in water hyacinth stems.[9]

Antioxidant Activities of Extract

The study conducted by Schlesier *et al*.[34] showed the analysis of antioxidant activity, which was carried out by at least two methods. In this study, an analysis of antioxidant activity was carried out on the roots, stems, and leaves of water hyacinth extracts using ABTS and FRAP. This method differs in its mechanism of action.[35] The ABTS method uses ABTS radical formed by oxidation of ABTS with potassium persulfate. Thus, the testing takes time for the ABTS radical to be generated. ABTS radicals are soluble in water and organic solvents, enabling the determination of the antioxidant capacity of hydrophilic and lipophilic compounds/samples.[36-37] The FRAP method has the principle of reducing iron ion complexes (Fe^{3+})-ligands to iron complexes (Fe^{2+}) by using antioxidants in an acidic environment.[38]

The results are presented in Table 1. The antioxidant activity of water hyacinth leaves extract had the highest activity based on the two methods with a value of 42.91 ± 5.42 µg/mL (ABTS) and 381.98 ± 15.49 µg/mL (FRAP) when it was compared with two other parts. The antioxidant activity value can be attributed to the higher tannin content in the leaves. Tannin is the polyphenol group that may play a role in the extract's antioxidant properties.[39] The antioxidant activity was higher in leaves extract, followed by stems and roots extracts. This is in line with the amount of tannin contained in each part of plant (Figure 1). According to observations by Surendraraj *et al*.[33], extracts with high total phenolic content are known to have the ability to reduce Fe^{3+}. Transition metals, such as Fe^{2+} and Cu^{2+} can catalyze the formation of reactive oxygen species such as hydroxyl radicals (•OH) and superoxide anions O_2^{-}.[40]

Tannins (commonly referred to as tannic acid) have effective DPPH radical • scavenging, ABTS radical •+ scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, Fe^{3+} reducing power, and metal chelating on ferrous ion activity. Some groups reported various antioxidant activities and were compared with BHA, BHT, α-tocopherol, and Trolox as reference antioxidant compounds. The more tannin content, the greater the antioxidant activity because tannins are composed of polyphenolic compounds with free radical scavenging activity. Compounds that react as radical scavengers will reduce DPPH to form reduced DPPH-H.[11]

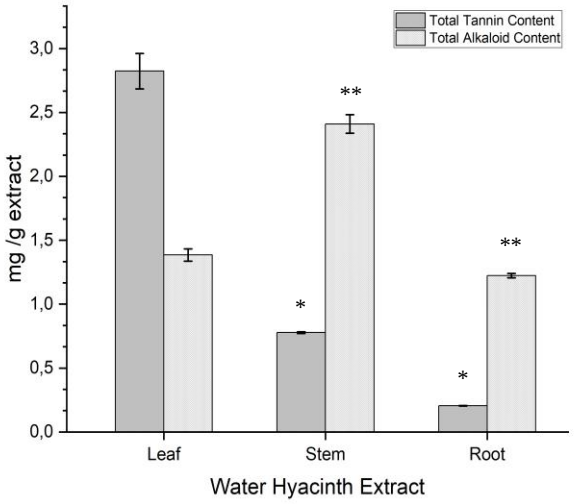

Figure 1: Total secondary metabolites of Water Hyacinth (*Eichhornia crassipes*) extract (mg/g extract).
Data were analyzed using *Kruskal Wallis, **One-way ANOVA; pos hoc LSD: P<0.05 (comparative analysis between leaf and the other extracts).

Relationship between Total Secondary Metabolites and Antioxidant Activity

Tannin is suggested to associate with antioxidant activity observed by both FRAP and ABTS assays because the basic mechanisms of antioxidant activity of tannins are free radical scavenging activity, chelation of transition metals, and inhibition of prooxidative enzymes.[41] This is in line with this study, where Total Tannin Content (TTC) has a higher value than Total Alkaloid Content (TAC) in water hyacinth leaf extract. The higher tannin content in water hyacinth is in line with the increase in its antioxidant activity.[42] This research is also supported by previous research reported by Naz *et al*.[43] on the methanol extract of *Jacaranda mimosifolia* leaves. The analysis of the antioxidant activity revealed the phenomenon that the higher the tannin content, the greater the antioxidant activity. This can be seen from the highest tannin content in the leaf extract of 2.6 ± 0.16 mg/g and the highest antioxidant activity in the leaves of 48 ± 1.3 µg/ml compared to other extracts. Other research also supports similar findings on parts of the *Mangifera indica* leaf plant.[44] The antioxidant activity of the leaves is higher than the stems, as well as the tannin content. A similar study by Formagio *et al*.[45] regarding the antioxidant activity of *Psychotria* leaf extracts showed the ability to clean up ABTS radical cations, which were effective on *P. carthagenensis* and *P. capillacea* with values of 92.5 ± 7.43% and 87.34 ± 8.32% (respectively at a concentration of 100 µg/mL) when compared to *P. leiocarpa* and *P. deflexa* extracts. Meanwhile, in the same study, the analysis of the highest levels of condensed tannins was found in *P. carthagenensis* (632.39 ± 5.63 mg catechin equivalents (CE)/g extract) and *P. capillacea* (571.95 ± 7.22 mg CE/g extract). In comparison, the lowest concentrations were in *P. deflaxa* and *P. leiocarpa* (194.67 ± 9.02 and 60.97 ± 10.45 mg CE/g extract, respectively).

Based on the category of antioxidant activity that has been stated by Blois[46], the antioxidant activity of the ABTS method on water hyacinth stems was considered strong (IC_{50} 66.83 ± 4.30 µg/mL). It is known that the smaller the IC_{50} value, the greater the antioxidant activity. The antioxidant capacity is said to be very strong if the IC_{50} value is < 50 ppm, strong is 50-100 ppm, medium is 101-150, and weak is > 150 ppm. Unlike water hyacinth leaves, the antioxidant activity of the stem extract may be contributed by alkaloid contents, which showed higher levels compared to tannin levels. In addition, this phenomenon is supported by the fact that the alkaloid content in the stem is the highest compared to other plant parts. Authors argue that the possibility of alkaloid compounds in stems is the main compound that influences their antioxidant activity. Another study that supports this argument has been reported by Tyagi *et al*.[22] regarding the total alkaloid content in the leaves and stems of the *Pistia stratiotes* plant. The alkaloid content in the leaves was 0.163 ± 0.041 mg alkaloid/gram dry weight, while in the stems, it was 0.096 ± 0.041 mg alkaloid/ gram dry weight. This is consistent with the antioxidant activity of the DPPH method with the best IC_{50} value in the leaves (2.463 ± 0.018 mg/ml) compared to the stems (4.098 ± 0.030 mg/ml). Similar research on the isolation of alkaloid compounds as antioxidants has also been carried out by Dalimunthe *et al*.[47] on *Litsea cubeba* plants. The antioxidant compounds DPPH and ABTS were in the chloroform fraction at pH 7 with IC_{50} values of 23.81 ± 0.01 and 56.43 ± 0.06 µg/mL, respectively. Other studies have been reported by Yin *et al*.[48] They successfully isolated the alkaloid compound (+)-orientaline, which was shown to have antioxidant activity against DPPH and ABTS with IC_{50} values of 6.64 ± 0.19 and 1.53 ± 0.05 µg/mL, respectively.[48] Alkaloids can scavenge radicals and cationic radicals because they have free hydroxyl groups, an abundance of hydroxyl groups, and the hydroxyl groups attached to the structure are in a state or position that is not steric. The higher the hydroxyl groups and the more positions of the hydroxyl groups that other groups do not block will increase the antiradical activity of the alkaloids.[49]

Cytotoxic Activities of Extract

Cytotoxic activity test was conducted to study potential anticancer activity of the plant extracts. The cytotoxic activity of each part of the plant showed significant differences upon examination using three different breast cancer cell lines. The leaf extract generally had the best cytotoxic activity compared to the other two parts (Table 2). The best cytotoxic activity in the leaf extract was observed for MCF-7 cells (38.27 ± 19.17 µg/mL), followed by MDA-MB 231 cells (104.54 ± 39.44 µg/mL) and T47D cells (183.22 ± 15.98 µg/mL).

The MCF-7 cells are ER and PR-positive cells. The high tannin content in leaf extract suggested the correlation between tannin structure and the hormone receptors within this cell line. Tannin compounds which are polyphenols, modulate the activity of signaling pathways involved in cancer cell proliferation, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositide 3-kinase (PI3K) pathways.[50] Inhibition of cyclic Adenosine Mono Phosphate (cAMP) response element binding protein (CREB) and/or extracellular signal-regulated signal kinases 1 and 2 (ERK1/2) is also a mechanism usually influenced by polyphenols to induce cell cycle progression. The chemical structure of polyphenols determines their binding affinity for ER.[51]

The IC_{50} value showed strong toxicity against MCF-7 cells (IC_{50} value is ≤ 100 ppm) while weak toxicity to MDA-MB 321 and T47D cells (IC_{50} value is 100-500 ppm).[52-53] A similar study by Taqi *et al*.[19] found the anticancer activity of *E. crassipes* leaf extract on MCF-7 cancer cells. They showed that treatment with leaf extract could significantly inhibit cell growth. Leaf extract at concentrating 100 µg/mL showed more than 80% of dead cells. Leaf extracts are considered antiproliferative and cytotoxic agents. Apoptogenic properties were investigated through morphological changes in MCF-7 cell lines using reverse-phase contrast microscopy. The difference in cytotoxic activity could be related to the status of p53 and ER cells, MCF-7 (p53+ and ER+), MDA-MB-231 (p53 mutant and ER-), and T47D (p53 mutant and ER+). The correlation of secondary metabolites (especially tannins and alkaloids) with cytotoxic activity is still to be investigated to determine which compounds are most effective and facilitate studying the mechanism.

Morphological differences occurred in MCF-7 cells after adding leaf extract with a concentration of 25 µg/mL (Figure 2). This study found the morphology change of MCF-7 cells, possibly due to the damage after exposure to the test compound or as an indication that apoptosis had been induced. Indications of apoptosis are indicated by cell shrinkage, as an early characteristic of apoptosis.[53] Membrane

blebbing is the last stage of apoptosis when cells experience irregular protrusions on the cell plasma membrane caused by local separation of the cytoskeleton from the plasma membrane.[54]

Relationship between Total Secondary Metabolites and Cytotoxic Activity

Water hyacinth leaves have the best cytotoxic activity among other plant parts. This anticancer activity has a good response in all three breast cancer cells, MCF-7, T47D, and MDA-MB 231.

Table 1: IC_{50} values (µg/mL) of leaf, stem, and root extract antioxidant activity.

Assays	Water Hyacinth Extract		
	Leaf	Stem	Root
ABTS	42.91 ± 5.42	66.83 ± 4.30	267.94 ± 29.63
FRAP	381.98 ± 15.49	2354.54 ± 234.72	7379.19 ± 1077.52

Note: Values represent means ± SD for triplicate experiments

Table 2: IC_{50} values (µg/mL) of leaf, stem, and root extract for cytotoxic activity.

Cell Lines	Water Hyacinth Extract		
	Leaf	Stem	Root
MCF-7	38.27 ± 19.17	403.08 ± 21.93	516.49 ± 15.50
T47D	183.22 ± 15.98	916.85 ± 23.12	862.59 ± 53.22
MDA-MB 231	104.54 ± 39.44	414.61 ± 46.92	170.75 ± 40.76

Note: Values represent means ± SD for triplicate experiments

Tannins are suggested as secondary metabolites that play a role in the anticancer activity of water hyacinth leaves. The presence of tannins in water hyacinth leaves is in line with previous studies which have found tannins qualitatively using the Braymer's test. The test was carried out by reacting the extract with a 10% ferric chloride solution in alcohol to form a green-brown or blue-black solution. Positive results were shown for shoots and rhizomes of water hyacinth treated with methanol[55] and for all parts of the plant treated with water.[56] Quantitative presence of tannins has been carried out by Lara-Serrano *et al*.[57] on leaf ethanol extract of 6.9 ± 0.05 wt.%. The tannin content was confirmed to be higher than that of the stems and roots. Tannins were also detected in the leaves using the TLC method with the mobile phase of chloroform: water (3:2), followed by the appearance of vanillin–sulfuric acid spots.[58] The tannin fragments were also found in the ethanol extract of water hyacinth leaves using the HPLC method, namely in the form of gallic, protocatechuic, gentisic, and p-hydroxybenzoic acid.[33] Tannin fragments were also found in the ethyl acetate extract of leaves using the GC-MS method in the form of Phenol, 3,5-bis(1,1-dimethylethyl)-.[59]

Previous studies that strengthen tannins as cytotoxic agents in breast cancer cells have been reported by Forman *et al*.[60] on *Cornus* L. Species. The study reported that the aqueous extracts of *C. alba*, *C. officinalis*, and *Cornus mas* leaves showed significant antiproliferative activity against MCF-7. These three species are known to have high levels of total tannins. Condensed tannins that were successfully separated from *L. leucocephala* plants had high cytotoxic activity against MCF-7 cells (IC_{50} value 38.33 ± 2.08 µg/mL). The inhibitory value of MCF-7 cancer cells is more significant when compared to the inhibition of other types of cancer cells, such as human colon carcinoma (HT29), human cervical carcinoma (HeLa), and human liver carcinoma (HepG2).[61] Tannin compounds have been isolated from red maple (*Acer rubrum*) species, which produces Mapplexin C. It has anticancer activity against MCF-7 cells with an IC_{50} of 73.7 ppm. It is known that these compounds belong to the class of gallotannins.[62] Previous studies have never isolated tannins as an anticancer in water hyacinth, so it needs to be proven by separating the extract to produce purified tannin fractions to determine the mechanism of action of tannins as anticancer in water hyacinth plants.

A literature search regarding the biological activity of the tannin fraction in water hyacinth was reported by Al-Azawi *et al*.[63] only limited activity against bacteria and fungi. Tannins in several parts of the water hyacinth plant can inhibit the growth of *Klebsiella pneumoniae*, *Shigella*, *S. aureus*, and *S. epidermidis*.

Relationship between Antioxidant and Cytotoxic Activity

The IC_{50} value in Table 2 shows the ability of cytotoxicity against MCF-7 cells, which has promising results on the leaves. The antioxidant activity of the leaf extract may be related to the cytotoxicity of MCF-7. This study shows that the best antioxidant activity of water hyacinth is in the leaves with the highest TTC. Likewise, the best cytotoxic activity against cancer cells was tested on the leaves. This fact is supported by previous research on the ethanol extract of *Ficus sycomorus*. Their study found that the highest antioxidant activity was shown in the leaves with an IC_{50} of 18.443 µg/ml. The best anticancer activity was also obtained from the leaves against MCF-7 breast cancer cells with an IC_{50} of 219 µg/ml. The tannin content in the leaves had the highest levels compared to other plant parts, namely 11.11 ± 0.23 g/100 g.[64]

Elevated ROS levels play an essential role in carcinogenesis, tumor metastasis, and maintenance of the phenotypic characteristics of cancer cells. By increasing antioxidant intake, increasing cellular antioxidants, and targeting sources of ROS, oxidative stress can be eliminated, further causing growth inhibition and increased susceptibility to cell death in cancer cells.[65] Antioxidant compounds are able to attack and neutralize ROS and RNS. Antioxidants mainly act as chemical electron scavengers and stop or reduce free radical chain reactions. Antioxidant effectiveness is related to many factors, including activation energy, rate constant, oxidation-reduction potential, and solubility properties. Antioxidant efficiency increases with decreasing phenolic oxygen-hydrogen bond strength. Therefore, modulation of intracellular ROS/RNS levels by antioxidants could be used to target oxidative stress-mediated cancer initiation, promotion, and progression.[66]

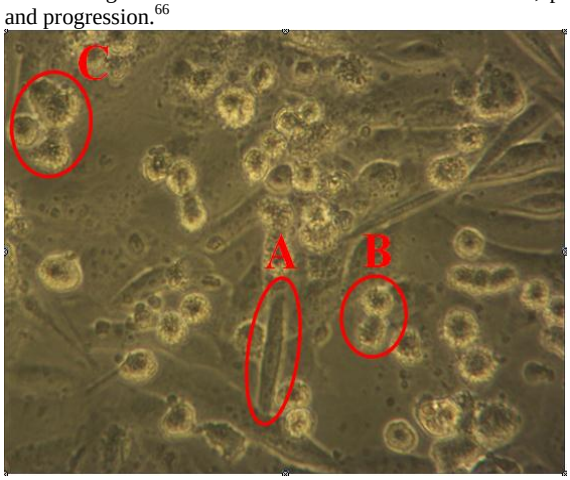

Figure 2: Morphology of MCF-7 cell after the application of leaves extract (25 µg/mL). (A) Normal MCF-7 Cell (B) Cell shrinkage (C) Membrane blebbing.

Conclusion

This study proved that tannins have the highest levels in the parts of water hyacinth leaves that are relevant to their antioxidant and anticancer activities.

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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