ANTIOXIDANT LEVEL OF *Azolla* spp. WITH DIFFERENT SOLVENT EXTRACTION

MUHAMMAD SHAMMEEM PUTRA BIN SHAMSUL @ KAMAR

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Antioxidant Level of *Azolla* spp. with Different Solvent Extraction

By

Muhammad Shammeem Putra Bin Shamsul @ Kamar

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ABSTRACT

An abstract of the research paper was presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 55204 – Research Project.

Excessive reactive oxygen species (ROS) can lead to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity, and cancer. The efficacy of *Azolla* spp., an aquatic fern as an antioxidant in animals must be explored in order to reduce or prevent the accumulation of free radicals. This study investigated the antioxidant potential of methanol and hexane extracts of *Azolla* spp. Based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity results, the methanol extract has higher antioxidant level as compared to hexane extract, by comparing to ascorbic acid reference. The antioxidant level of methanol was at 26.36%. This indicates that the *Azolla* spp. exhibits significant potential as an effective antioxidant for animals that can reduce the building up of free radicals in animals.

Keywords: *Azolla* spp., antioxidant level, methanol, hexane

TAHAP ANTIOKSIDAN *Azolla* spp. DENGAN MENGGUNAKAN BAHAN PELARUT YANG BERBEZA

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

Spesies oksigen reaktif (ROS) yang berlebihan boleh membawa kepada pelbagai proses patofisiologi seperti keradangan, diabetes, genotoksisiti dan kanser. Keberkesanan *Azolla* spp., paku pakis air sebagai antioksidan dalam haiwan harus dikaji untuk mengurangkan atau mencegah pengumpulan radikal bebas. Kajian ini menyiasat potensi antioksidan ekstrak metanol dan ekstrak heksana *Azolla* spp. Berdasarkan keputusan aktiviti pelenyapan 2,2-diphenyl-1-picrylhydrazyl (DPPH), ekstrak metanol mempunyai tahap antioksidan yang lebih tinggi berbanding ekstrak heksana, dibandingkan dengan rujukan asid askorbik. Tahap antioksidan metanol adalah pada 26.36%. Ini menunjukkan bahawa *Azolla* spp. mempamerkan potensi yang besar sebagai antioksidan yang berkesan untuk haiwan dengan mengurangkan pembentukan radikal bebas dalam haiwan

Kata kunci: *Azolla* spp., level antioksida, metanol, heksana

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LIST OF ABBREVIATIONS

ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
CUPRAC	Cupric Reducing Antioxidant Capacity
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH-H	2,2-diphenyl-1-picrylhydrazine
TPTZ	2,4,6-tripyridyl-s-triazine
FRAP	Ferric Reducing Antioxidant Power
FRS	Free-radical scavengers
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TEAC	Trolox Equivalent Antioxidant Capacity
UV	Ultraviolet

LIST OF SYMBOLS

%	Percent
cm	Centimeter
Cu	Copper
Cu^{+}	Cuprous
Cu^{2+}	Cupric
H_2O_2	Hydrogen peroxide
IC_{50}	Half-maximal inhibitory concentration
ml	Milliliter
nm	Nanometer
µg	Microgram

CHAPTER 1

INTRODUCTION

1.1 Research Background

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), originating from oxygen and nitrogen, respectively, are the predominant types of free radicals found in biological systems. The physiological processes of the organism require only a small amount of ROS, but an excess will lead to oxidative damage in several molecules, adversely affecting deoxyribonucleic acid (DNA) and proteins of the cells as well as leading to the lipid peroxidation of cellular membranes (Rahal et al., 2014). Besides, a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity, and cancer can be caused by the excessive production of ROS which is induced by various stimuli that exceed the antioxidant capacity of the organism (Kourounakis et al., 1999; Gulcin et al., 2002; Gulcin et al., 2003). Fortunately, a strong defence mechanism known as the antioxidant defence system effectively guards tissues and cells against free radical damage. Exogenous and endogenous parts make up this defence system.

Dietary antioxidants found in animal and plant foods, such as vitamins, phytochemicals, and trace elements, may be categorized as exogenous components because they are consumed as food (feed) and enter biological systems through the processes of digestion and absorption. Endogenous antioxidants include enzymes like catalase, glutathione peroxidase, superoxide dismutase, and glutathione reductase as well as non-enzyme components like nutrient-based antioxidants like tocopherols and tocotrienols, carotenoids, and lipoic acid and metal-binding proteins like albumin, ferritin, ceruloplasmin, and lactoferrin (Ponnampalam et al., 2022).

Azolla spp. or commonly known as mosquito fern, duckweed fern, fairy moss, water fern belongs to the family Salviniaceae. It is a small pteridophyte (aquatic fern) and indigenous to the tropics, subtropics region and warm temperate regions of Africa, Asia and the Americas. *Azolla* spp. has been used by farmers as a substitute for other protein sources because of its nutritious value and affordable price (Samad et al, 2020). The nutrients that are contained in *Azolla* spp. are minerals like iron, calcium, and magnesium as well as amino acids like leucine and alanine (Bhaskaran & Kannapan, 2015). In the present study, the methanol and hexane extracts of the *Azolla* spp. were screened for antioxidants using the standard method. The finding from this work may add to the overall value of the medicinal potential of the plant.

1.2 Problem Statement

Highly reactive molecules from the metabolism of oxygen can cause extensive damage to cells and lead to various types of diseases in different animals. To combat this issue, synthetic or natural antioxidants should be added to the feed ingredients. The need to determine the most effective solvent for extracting antioxidants from *Azolla* spp. to maximize their antioxidant levels and potential therapeutic benefits. The choice of solvent significantly influences the efficiency and yield of antioxidant extraction, and it is essential to identify the optimal solvent for veterinary applications.

1.3 Research Questions

What is the most effective solvent for extracting antioxidants from *Azolla* spp. to maximize their antioxidant levels and potential therapeutic benefits in the context of veterinary medicine?

1.4 Research Hypothesis

There is a significant difference in the antioxidant level of *Azolla* spp. using different solvents.

1.5 Research Objectives

The objectives of the study were to analyse the antioxidant level of *Azolla* spp. using methane and hexane extracts.

1.6 Significance of the Study

This study is being conducted due to limited awareness of this plant among the general populace. Furthermore, this research can contribute significantly to the scientific community by addressing critical gaps in our understanding of the antioxidant potential of *Azolla* spp. The exploration of various solvents and their impact on antioxidant extraction provides valuable insights, enabling researchers to employ more accurate and reliable methods in future studies.

CHAPTER 2

LITERATURE REVIEW

2.1 *Azolla* spp.

In 1783, Lamarck was the first to use the term Azolla (Roy et al., 2016). The genus *Azolla* belongs division Pteridophyta, class Polypodiopsida and order Salviniales. It is a member of the Salviniaceae family and consists of two subgenera and six living species (Lumpkin & Plucknett, 1980). *Azolla* spp. thrives in freshwater in tropical, subtropical, and warm-temperate climates across the world (Roy et al., 2016). The macrophyte of *Azolla* spp. is known as a frond, and it can grow to a length of 15 cm or more in the largest species, such as *A. nilotica*, and between 1 cm and 2.5 cm in species like *A. pinnata* (Raja et al. 2012). *Azolla* spp. is widely used as a feed supplement in various livestock such as ruminants, pigs, rabbits, poultry and fish (Roy et al., 2016). In a study conducted by Nor Nawaz et al. (2014), *Azolla* spp. has been shown to have significant antioxidant activity and it could be utilized to create agents which are effective against oxidative stress.

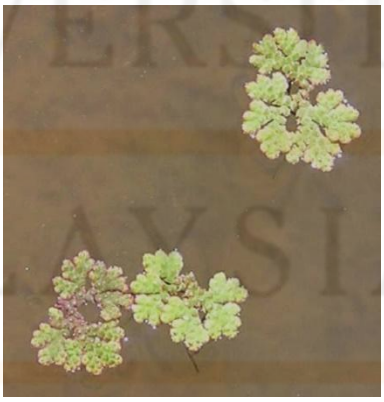

Figure 2.1. Single plants of *Azolla* spp.

Source: Hussner, (2010)

2.2 Assessment Method of Antioxidant Capacity

The procedure and equipment used to analyze the antioxidant activities in plants have made astounding advancements over the previous few decades. Munteanu & Apetrei (2021) stated that early techniques rely on measuring lipid oxidation to determine how effective antioxidants are at preventing the production of specific oxidation product species. Until now, different chemical tests in conjunction with extremely sensitive and automated detection technologies have been used to assess antioxidant activity through specific techniques, such as metal chelation, reducing power, and scavenging activity against various forms of reactive oxygen species.

2.2.1 DPPH Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is a popular, quick, simple, and inexpensive method for measuring antioxidant characteristics, which incorporates the use of free radicals to assess the ability of compounds to serve as hydrogen providers or free-radical scavengers (FRS). The DPPH test is based on the removal of DPPH, a stabilised free radical. DPPH is a dark-coloured crystalline substance composed of stable free-radical particles. The DPPH free radical reacts with an odd electron to create a maximum absorption wavelength of 517 nm (purple colour). Antioxidants react with DPPH and the presence of a hydrogen source (for example, a free-radical scavenging antioxidant), resulting in the reduction of DPPH to 2,2-diphenyl-1-picrylhydrazine (DPPH-H) and a decrease in DPPH absorbency. In contrast to the DPPH-H form, decolourization (a yellow hue) happens as the number of electrons gathered increases (Baliyan et al., 2022).

According to Skowyra (2014), there are a few disadvantages of the DPPH radical scavenging activity assay. Firstly, some antioxidants, such as carotenoids, exhibit spectra that overlap with DPPH at 515 nm, interfering with the results. The only

organic solvents that can dissolve the DPPH radical are methanol, ethanol, and acetone, which poses a constraint for analysing the function of hydrophilic antioxidants. Other than that, pH, type of solvents, sample concentration and reaction time may affect the assay.

2.2.2 Trolox Equivalent Antioxidant Capacity

According to Xu et. al (2017), to assess an antioxidant's capacity to scavenge the ABTS radical, the Trolox equivalent antioxidant capacity (TEAC) assay is frequently used. This assay is available in two variations, depending on the type of oxidation agent used. The first one is metmyoglobin-H_2O_2 oxidizes ABTS to produce the coloured ABTS form; the green colour is subsequently lost upon the presence of antioxidants. Secondly, potassium persulfate oxidizes ABTS to produce the coloured ABTS form; the green hue is subsequently lost in the presence of antioxidants.

2.2.3 Ferric Reducing Antioxidant Power

According to Xu et. al (2017), ferric-reducing antioxidant power (FRAP) assay directly assesses antioxidant reducing capacity. Under pH 3.6 conditions, antioxidants can reduce a ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to a ferrous complex (Fe^{2+}-TPTZ) in parallel with a blank sample in a ferric-reducing antioxidant reactive system. The ferrous complex (Fe^{2+}-TPTZ) is a blue ferrous form with a maximal UV-vis absorption at 593 nm. The ability of antioxidants in samples (FRAP value) is positively connected to absorbance rise.

2.2.4 Cupric Reducing Antioxidant Capacity

Munteanu & Apetrei (2021) discussed that to measure the antioxidant activity based on the reduction of cupric (Cu^{2+}) to cuprous (Cu^{+}), the CUPRAC assay was

developed in the early 2000s. However, it has already undergone modifications. CUPRAC is performed by combining Cu(II)-neocuproine (Nc) chelate with an antioxidant solution. After 30 minutes, the absorbance of the coloured Cu(I)-chelate as a result of redox reaction is measured at 450 nm (Xu et al., 2017).

2.3 Antioxidants in *Azolla* spp. and Other Plants

Plants contain a variety of natural antioxidant compounds. These compounds are classified as vitamins (vitamins C and E), polyphenols (flavonoids, phenolic acids, stilbenes, and lignans), and terpenoid groups. According to Abeyrathne et al. (2022), plant vitamins are the primary antioxidants. Vitamin C offers protection from oxidative stress-related cellular damage, whereas vitamin E serves as a crucial lipid-soluble antioxidant. Vitamin C has a negligible antioxidant effect compared to vitamin E, which is why both are used in food.

According to Naczk & Shahidi (2006), polyphenols became ubiquitous in the plant kingdom for the protection of plants against UV radiation. Polyphenols further provide a repair mechanism for plants through oxidative polymerization by enzymes following mechanical damage. These compounds play an important role in plant growth and reproduction, providing efficient protection against pathogens and predators, besides contributing to the colour, sensory characteristics and nutritional properties of fruits and vegetables.

Other than that, Abeyrathne et al. (2022) mentioned that terpenes and terpenoids are good plant-derived antioxidants. They are the most abundant secondary metabolites in plants and contain. Isoprene, a hydrocarbon skeleton with five carbons that is present in both terpenes and terpenoids, polymerizes into different terpenes when two or more molecules are present. For the most part, they are non-polar chemicals. They contribute odour and flavour, as well as antioxidant and antibacterial capabilities, antiaging and

anticancer characteristics, and other health-promoting qualities like lowering stress, depression and migraines.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample Collection and Preparation

Azolla spp. was acquired from a local farm in Kota Bharu. Then, the sample was washed and rinsed using tap water to remove contaminants. The sample was dried at room temperature (27°C) for seven days. The dried sample was ground into powder form using a mechanical grinder. The ground sample was kept in a sealed plastic bag with silica gel desiccant.

Figure 3.1 Drying *Azolla* spp. at room temperature.

Figure 3.2. Ground dried *Azolla* spp.

3.2 Sample Extraction

Approximately 100 g of ground *Azolla* spp. was soaked in 1000 ml of 99.6% methanol for 24 hours. The extract was passed through Whatman No. 1 filter paper to obtain particle free extract. Then, the extract was concentrated under reduced pressure using a rotary evaporator and a paste-like consistency extract was acquired. The same procedure was followed for hexane.

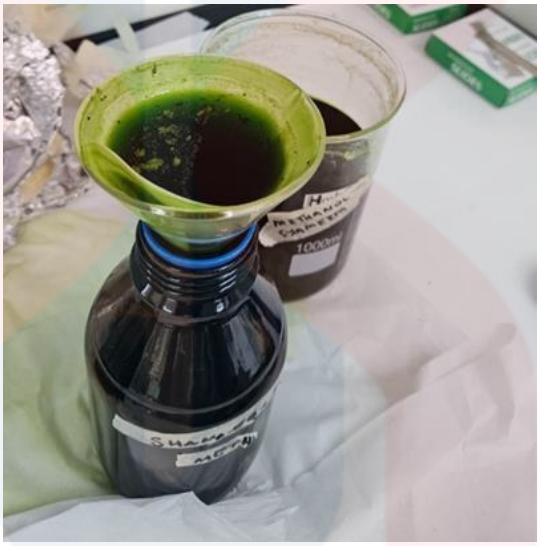

Figure 3.3. Filtering soaked sample.

Figure 3.4. Concentrating sample using a rotary evaporator.

3.3 DPPH Radical Scavenging Assay Procedure

DPPH radical scavenging assay was used to determine the radical scavenging potential of hexane and methanol extract of the *Azolla* spp. following the procedure of Hamid et al. (2010) with slight modifications. DPPH solution was prepared in 95% methanol. In this assay, 20 µl of hexane and methanol extracts were mixed with 80 µl of DPPH solution in separate wells. Using a plate reader, the optical density will be determined at 517 nm. The DPPH control's absorbance will also be noted. As a reference standard, different concentrations of ascorbic acid was employed with concentration ranging from 1-10 µg/ml. The procedures were repeated three times for each extract and concentration of the ascorbic acid. The radical scavenging activity of extracts and ascorbic acid will be calculated using the formula:

% DPPH radical-scavenging = [(Absorbance of control - Absorbance of test sample)/ (Absorbance of control)] x 100

CHAPTER 4

RESULTS

4.1 Standard Curve of Ascorbic Acid

The absorbance of ascorbic acid at different concentrations at 517 nm is shown in Table 4.1. As the amount of ascorbic acid rises, there is a corresponding decline in absorbance.

Table 4.1 Absorbance of ascorbic acid at different concentration at 517 nm.

Concentration (μg/ml)	Absorbance
1	0.140
2	0.143
3	0.133
4	0.124
5	0.117
6	0.116
9	0.107
10	0.087

The efficacy of ascorbic acid to scavenge free radical was assessed by DDPH radical scavenging assay and the result is shown in Table 4.2. The DPPH radical scavenging activity of the ascorbic acid was dose dependent. A standard curve was generated using the DPPH radical scavenging assay value of ascorbic acid against different concentrations, as shown in Figure 4.1.

Table 4.2. DPPH radical scavenging assay of ascorbic acid at different concentrations.

Concentration (µg/ml)	DDPH radical scavenging assay (%)
1	15.49
2	16.70
3	19.71
4	25.35
5	29.57
6	29.98
9	35.41
10	47.48

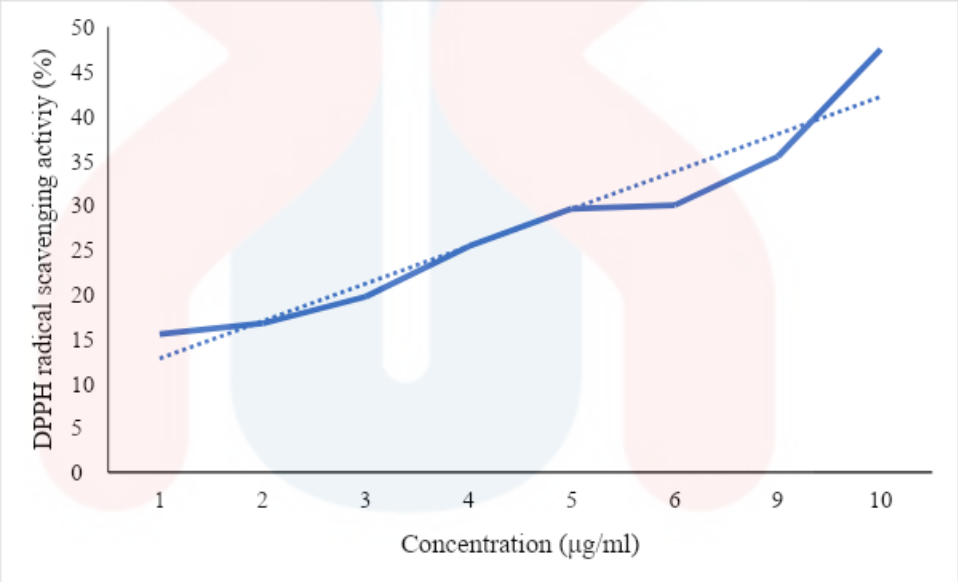

Figure 4.1. Standard curve (y = 4.1966x + 8.5801) of DPPH radical scavenging activity against concentration of ascorbic acid (R^2 = 0.9264).

4.2 Antioxidant Level of Different Solvents

Table 4.3 shows the average percentage of DPPH radical scavenging activity of methanol and hexane. Among the sample extracts, methanol displayed stronger scavenging potential (26.36%) as compared to hexane (0%).

Table 4.3. DPPH radical scavenging activity of methanol and hexane.

Samples	DPPH radical scavenging activity (%)
Methanol	26.36
Hexane	0

CHAPTER 5

DISCUSSION

The DPPH method introduced almost half a century ago, is extensively employed to assess the capacity of compounds to function as scavengers of free radicals or donors of hydrogen. It is a widely accepted approach for evaluating antioxidant capabilities (Proestos et al, 2013). DPPH, an enduring nitrogen-centered organic free radical, exhibits an absorption peak ranging from 515 to 528 nm (with a specific value of 517 nm) when dissolved in alcohol (Nor Nawaz et al., 2014). The author also stated that DPPH transforms into a stable diamagnetic molecule upon acquiring an electron or hydrogen atom. The amount of discolouration in the DPPH solution signifies the plant extracts' antioxidant activity, which is determined by their capacity to donate hydrogen (Onoja, et al., 2014). The total reaction depends on the antioxidants' capacity to donate hydrogen. The antioxidants convert the purple-coloured DPPH radical to the yellow-coloured molecule diphenylpicrylhydrazine (Nor Nawaz et al., 2014).

In this study, the absorption of DPPH solution of *Azolla* spp. extracts was measured at 517 nm using different methanol and hexane solvents. We employed both methanol and hexane as solvents, chosen for their differing polarities. Solvent selection is a critical aspect of phytochemical extraction, influencing the spectrum of compounds obtained from plant matrices (Thouri et al., 2017). It was observed that the radical scavenging activities of methanol extract is higher (26.26%) compared to the reference standard (25.35%) and hexane (0%) extract. Hence, clearly demonstrated the potential to donate hydrogen, indicating that they may act as main antioxidants by scavenging free radicals (Nor Nawaz et al., 2014). This is consistent with the results of research by Dai et al. (2012) which demonstrated that the *Azolla* spp. is capable of scavenging free radicals. At

19.08 µg/ml, the anthocyanins of *A. imbricata* showed dose-dependent DPPH free radical scavenging.

The observed higher DPPH radical scavenging activity of the methanol extract aligns with research conducted by Thouri et al. (2017) and Ghasemzadeh et al. (2011) which revealed that the polar solvent contained the greatest number of bioactive chemicals such as flavonoids and polyphenols.

CHAPTER 6

CONCLUSION

The findings of this study revealed significant antioxidant potential in *Azolla* spp., with methanol extraction exhibiting higher DPPH radical scavenging activity (26.36%) compared to hexane, suggesting that *Azolla* spp. possesses compounds capable of donating hydrogen and scavenging free radicals, emphasizing its potential therapeutic benefits. The choice of solvent appeared to influence the efficiency of antioxidant extraction, underscoring the importance of solvent selection in phytochemical studies. The study provides valuable insights into the antioxidant capacity of *Azolla* spp., reinforcing its potential application as a natural antioxidant source in veterinary medicine.

For future investigations, it is imperative to incorporate biological replication in the extraction process to enhance statistical robustness and reliability. The addition of statistical analysis, such as variance assessments, can provide a more nuanced understanding of the antioxidant levels within Azolla spp. and contribute to the overall scientific rigor of similar studies. Furthermore, exploring various statistical techniques, such as analysis of variance (ANOVA) or t-tests, can help discern significant differences in antioxidant levels between extraction methods and solvents. This comprehensive approach will not only strengthen the validity of the results but also facilitate a more informed optimization of antioxidant extraction methods from Azolla spp. for potential applications in veterinary care.

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