ASSESSING ANTIOXIDANT LEVEL IN *AZADIRACHTA INDICA* METHANOLIC EXTRACT

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Assessing Antioxidant Levels in *Azadirachta indica* Methanolic Extract

By

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Assessing Antioxidant Levels in *Azadirachta indica* Methanolic Extract

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.

Reactive oxygen species (ROS) in excess can initiate and propagate various pathophysiological conditions, including inflammation, diabetes, genotoxicity, and cancer. Managing the oxidative damage induced by ROS is essential for reducing these health issues. *Azadirachta indica,* known for its therapeutic benefits, has potential as a natural antioxidant. This study aims to evaluate the antioxidant potential of methanolic extracts of *A. indica*, exploring its effectiveness in reducing or preventing the accumulation of free radicals in biological systems. Based on the results of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity assay, methanolic extract showed lower antioxidant activity as compared to ascorbic acid. This is demonstrated by the IC_{50} values, where ascorbic acid exhibits antioxidants at 4 µg/mL, while the methanolic extract requires a significantly higher concentration of 49 µg/mL to achieve comparable activity. This suggests that *A. indica* demonstrates significant potential as an effective antioxidant for animals, capable of reducing the accumulation of harmful free radicals in their systems.

Keywords: *Azadirachta indica,* antioxidant level, methanolic extract

TAHAP ANTIOKSIDAN EKTRAK METANOLIK *Azadirachta indica*

ABSTRAK

Abstrak daripada kertas penyelidikan ini telah dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, sebagai sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

Spesies oksigen reaktif (ROS) yang berlebihan boleh mencetuskan dan mempercepatkan pelbagai keadaan patofisiologi, termasuk keradangan, diabetes, genotoksisiti, dan kanser. Menguruskan kerosakan oksidatif yang disebabkan oleh ROS adalah penting untuk mengurangkan masalah kesihatan ini. *A. indica*, yang dikenali dengan manfaat terapeutiknya, mempunyai potensi sebagai antioksidan semula jadi. Kajian ini bertujuan untuk menilai potensi antioksidan ekstrak metanolik *A. indica* dan keberkesanannya dalam mengurangkan atau mencegah pengumpulan radikal bebas dalam sistem biologi. Berdasarkan keputusan ujian aktiviti penangkapan 2,2-difenil-1-pikrilhidrazil (DPPH), ekstrak metanolik menunjukkan aktiviti antioksidan yang lebih rendah berbanding asid askorbik. Hal ini dibuktikan melalui nilai IC50, di mana asid askorbik menunjukkan aktiviti antioksidan pada kepekatan 4 μg/mL, manakala ekstrak metanolik memerlukan kepekatan yang jauh lebih tinggi iaitu 49 μg/mL untuk mencapai aktiviti yang setara. Ini mencadangkan bahawa *A. indica* mempunyai potensi yang signifikan sebagai antioksidan berkesan untuk haiwan, mampu mengurangkan pengumpulan radikal bebas yang berbahaya dalam sistem mereka.

Kata kunci: *Azadirachta indica*, tahap antioksidan, ekstrak metanolik

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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) are highly reactive molecules that play a role in cell signalling and metabolism. However, excessive ROS and RNS can cause oxidative stress, leading to damage to lipids, proteins, and DNA. This damage is associated with several diseases, including aging, cardiovascular conditions, cancer, and neurodegenerative disorders (Rahal *et al.,* 2014; Finkel, 2011). Increased ROS levels can also cause lipid peroxidation, destabilizing cell membranes and worsening oxidative damage (Sies, 2015). Maintaining a balance between ROS production and antioxidant defence is essential for cellular health, as disruption of this balance can lead to chronic oxidative stress and disease progression (Halliwell & Gutteridge, 2015).

Excessive production of reactive oxygen species (ROS), triggered by various environmental and internal stimuli, can overwhelm an organism's antioxidant defences, leading to a range of pathophysiological conditions. Chronic oxidative stress resulting from this imbalance is associated with inflammation, diabetes, genotoxicity, and cancer. ROS play a pivotal role in the inflammatory response, where they exacerbate tissue damage and promote the activation of signalling pathways that lead to the chronic inflammation observed in diseases such as arthritis and cardiovascular disorders (Rousset *et al.,* 2006).

The body possesses a formidable antioxidant defence mechanism that is essential for safeguarding cells and tissues against the detrimental effects of free radical damage. This defence system

consists of endogenous (internally generated) and exogenous (externally acquired) components. The endogenous components comprise many enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, which collaboratively neutralise reactive oxygen species (ROS) and mitigate oxidative stress. These enzymes are crucial for neutralising reactive chemicals and transforming them into less detrimental compounds (Sies, 2015; Halliwell & Gutteridge, 2015).

Dietary antioxidants, including vitamins, phytochemicals, and trace elements found in both animal and plant foods, represent exogenous components of the body's antioxidant defence system. These compounds are ingested through the diet, absorbed into biological systems, and provide significant support to the body's ability to combat oxidative stress. For example, vitamins C and E, found in fruits, vegetables, and nuts, are well-known antioxidants that help neutralise free radicals, while carotenoids and flavonoids, abundant in colourful fruits and vegetables, have also been shown to exert protective effects against oxidative damage (Blasiak *et al.,* 2015; Niki, 2014). Additionally, trace elements such as selenium and zinc play essential roles in antioxidant enzyme activity and contribute to maintaining redox balance (Valko *et al.*, 2007).

The conventional methods for extracting phenolic and antioxidant compounds from fresh plants commonly involve using organic solvents such as methanol, hexane, ethanol, and acetone, which are mixed at varying concentrations with water. These solvents are critical in accurately quantifying the antioxidants present in plant materials, as they help dissolve and extract bioactive compounds, including polyphenols, flavonoids, and other secondary metabolites, which are responsible for antioxidant activity (Alothman *et al.,* 2009). Among these solvents, methanol and ethanol are generally preferred for their ability to effectively solvate a wide range of phenolic

compounds, making them more efficient in extraction processes (Singleton *et al.*, 1999; Rufián-Henares & Cuadrado, 2009).

Azadirachta indica, commonly referred to as the neem tree or Indian lilac, is a member of the Meliaceae family and one of two species in the genus *Azadirachta*. This plant has garnered significant attention in both traditional and modern medicine due to its wide range of therapeutic properties. Neem has demonstrated notable health benefits, with research confirming its immunomodulatory, antimalarial, antioxidant, antifungal, antibacterial, anticarcinogenic, antiulcer, antimutagenic, and anti-inflammatory effects (Gupta *et al.,* 2013; Ali *et al.,* 2010). The active compounds found in neem, such as flavonoids, terpenoids, and alkaloids, contribute to these pharmacological effects, making it an essential plant in the treatment of various ailments.

Among its many properties, the antioxidant potential of *A. indica* has attracted particular interest. Studies have shown that neem exhibits strong antioxidant activity, which helps neutralise reactive oxygen species (ROS) and protects cells from oxidative stress. This property is especially relevant in the prevention and management of diseases linked to oxidative damage, including cancer, cardiovascular diseases, and neurodegenerative conditions (Rajeswari *et al.,* 2020; Sreeja & Rani, 2017). Moreover, the plant's antibacterial and antifungal properties make it a valuable resource in the fight against infections, particularly in regions where antimicrobial resistance is becoming a growing concern (Alzohairy, 2016; Goyal *et al.*, 2012).

1.2 Problem Statement

There is a need to determine antioxidant levels of methanolic extract of *A. indica* to maximise their potential therapeutic benefits. This will influence the efficiency and yield of antioxidant extraction.

1.3 Research Questions

What are antioxidant levels of methanolic extract of *A. indica* by comparing to ascorbic acid for potential therapeutic benefits in the context of veterinary medicine?

1.4 Research Hypothesis

There is a significant difference in the antioxidant level of methanolic extract of *A. indica* by comparing to ascorbic acid.

1.5 Research Objectives

The objectives of the study were to determine the antioxidant level of *A. indica* methanolic extract by comparing to ascorbic acid.

1.6 Significance of Study

Determination of antioxidant levels from plant extract can serve as valuable information and reference on reducing oxidative stress in animals due to the accumulation of free radicals using natural extracts. Other than that, the information from this study will create awareness of the importance of the antioxidant level of *A. indica* that can be added to the animal's feed to improve its quality.

CHAPTER 2

LITERATURE REVIEW

2.1 *Azadirachta indica*

A. indica or commonly known as neem, a revered and potent tree, is a precious natural resource cultivated primarily in the Indian subcontinent. Neem is a member of the mahogany family, Meliaceae. *A. indica* shows therapeutics role in health management due to the rich source of various types of constituents. The most important active constituent is azadirachtin and the others are nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin, and quercetin. Leaves contain ingredients such as nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and amino acid, 7-desacetyl-7- benzoylazadiradione, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione, and nimbiol. Quercetin and ß-sitosterol, polyphenolic flavonoids, were purified from neem fresh leaves and were known to have antibacterial and antifungal properties and seeds hold valuable constituents including gedunin and azadirachtin (Darkwah *et al.,* 2020).

Figure 2.1 *A. indica* tree plant

2.2 Assessment Method of Antioxidant Capacity

The methods used to assess plant antioxidant activities have significantly evolved in recent decades. According to Munteanu & Apetrei (2021), early techniques focused on measuring lipid oxidation to evaluate the effectiveness of antioxidants in preventing specific oxidative products. However, these methods had limitations in providing a comprehensive understanding of antioxidant mechanisms.

Recent advancements have introduced a variety of chemical tests and automated detection technologies for more precise assessments. These include techniques such as metal chelation, reducing power, and scavenging activity assays. Metal chelation assays measure an antioxidant's ability to bind metal ions, reducing oxidative reactions, while reducing power tests assess the ability to donate electrons and neutralise oxidised compounds. Scavenging activity assays evaluate how effectively antioxidants neutralise reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide.

Automated detection technologies, including high-performance liquid chromatography (HPLC) and microplate readers, have increased the efficiency and sensitivity of antioxidant testing, allowing for high-throughput screening and more accurate measurements. These advances not only improve the precision of antioxidant activity assessments but also provide deeper insights into the therapeutic potential of plant-based antioxidants. (Michalewska & Flieger, 2022)

2.2.1 DPPH Radical Scavenging Activity

The DPPH method is widely used for measuring antioxidant properties due to its simplicity, speed, and cost-effectiveness. It works by using DPPH, a stable free radical, to evaluate how well a compound can act as a hydrogen donor or free-radical scavenger. When antioxidants interact with DPPH, they reduce it to a non-radical form (DPPH-H), which results in a colour change from purple to yellow and a decrease in absorbance. This change is directly related to the antioxidant's ability to neutralise free radicals.

However, there are some limitations to the DPPH method. Certain antioxidants, such as carotenoids, have absorption spectra that overlap with DPPH, which can interfere with the accuracy of the results (Baliyan *et al.,* 2022). Additionally, the assay relies on specific organic solvents; methanol, ethanol, or acetone, which limits its ability to assess hydrophilic antioxidants. The results can also be influenced by factors such as pH, solvent type, sample concentration, and reaction time, which must be carefully controlled to ensure consistency and accuracy (Skowyra, 2014). Despite these drawbacks, the DPPH method remains a valuable tool for preliminary antioxidant screening.

2.2.2 Trolox Equivalent Antioxidant Capacity

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

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

The Ferric Reducing Antioxidant Power (FRAP) assay is a method used to assess the reducing capacity of antioxidants. According to Xu et al. (2017), the FRAP assay works under acidic conditions (pH 3.6), where antioxidants reduce a ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to a ferrous complex (Fe^{2+}-TPTZ). The ferrous complex exhibits a blue colour, and the increase in absorbance at 593 nm correlates with the antioxidant capacity of the sample. The FRAP value reflects the ability of antioxidants to donate electrons, which is vital in counteracting oxidative stress.

Benzie & Strain (1996) also note that the FRAP assay is reliable and simple, offering reproducible results. It is particularly useful for assessing the reducing power of antioxidants, which is central to their role in preventing oxidative damage. However, the FRAP method primarily measures reducing power and may not capture other antioxidant mechanisms, such as free-radical scavenging. Additionally, the presence of other reducing agents in the sample could influence the results (Benzie & Strain, 1996). Despite these limitations, the FRAP assay remains a valuable tool in antioxidant research.

2.2.4 Cupric Reducing Antioxidant Capacity

The Cupric Reducing Antioxidant Capacity (CUPRAC) assay is an effective method for measuring antioxidant activity by assessing the reduction of cupric (Cu^{2+}) to cuprous (Cu^{+}). According to Munteanu and Apetrei (2021), the CUPRAC assay was introduced in the early 2000s and has since been refined. The assay involves mixing Cu(II)-neocuproine (Nc) chelate with the antioxidant

solution, which facilitates the reduction of Cu^{2+} to Cu^{+}. After 30 minutes, the absorbance of the Cu(I)-chelate complex is measured at 450 nm, indicating the extent of antioxidant activity (Xu et al., 2017). The intensity of the colour formed is directly related to the antioxidant capacity of the sample.

In addition to its simplicity, the CUPRAC method offers advantages over other antioxidant assays. Unlike the FRAP assay, which is limited to aqueous solutions, CUPRAC can be applied to both aqueous and organic solutions, making it more versatile. Furthermore, the CUPRAC method is less influenced by interference from sample components, such as proteins or sugars, which can affect the accuracy of other assays (Apak *et al.,* 2008). However, one limitation of CUPRAC is that it primarily evaluates the reducing power of antioxidants and does not capture all antioxidant mechanisms, such as free-radical scavenging. Nevertheless, the CUPRAC assay remains a valuable tool for antioxidant evaluation, particularly in cases where other assays may not be suitable.

2.3 Antioxidants in *A. indica* and Other Plants

Plants are rich in a variety of natural antioxidants that help mitigate oxidative stress and promote health. These include vitamins, polyphenols, terpenoids, and other secondary metabolites. According to Abeyrathne et al. (2022), plant vitamins, especially vitamins C and E, are critical antioxidants. Vitamin C, a water-soluble antioxidant, helps protect cells from oxidative damage, particularly under conditions of oxidative stress. However, its antioxidant activity is less potent than that of vitamin E, which is a lipid-soluble antioxidant essential for protecting cell membranes from peroxidation. Due to their complementary antioxidant effects, both vitamins are commonly used together in food preservation and health supplements.

Polyphenols, which include flavonoids, phenolic acids, stilbenes, and lignans, are another important class of antioxidants found in plants. As noted by Naczk & Shahidi (2006), polyphenols are widely distributed in the plant kingdom and serve multiple protective roles, including shielding plants from UV radiation. Beyond this, polyphenols also contribute to the repair mechanisms of plants, aiding in the recovery from mechanical damage through oxidative polymerization by enzymes. Their antioxidant properties help in preventing the damage caused by reactive oxygen species (ROS) and provide protection against pathogens and herbivores.

Terpenes and terpenoids are among the most abundant secondary metabolites in plants and are also recognized for their antioxidant properties. These compounds are primarily non-polar, with a basic isoprene unit consisting of five carbon atoms. As Abeyrathne *et al.,* (2022) describe, terpenes polymerize into various structures and are known for their diverse health-promoting qualities. These include antioxidant, anti-aging, and anticancer effects, as well as antibacterial, anti-inflammatory, and stress-relief properties. Terpenoids are responsible for many of the characteristic odours and flavours in plants and have long been valued for their therapeutic benefits, including the alleviation of stress, depression, and migraine symptoms.

In addition to these common antioxidants, *A. indica* also known for its rich content of bioactive compounds. Neem leaves, seeds, and oil are known to possess potent antioxidant and anti-inflammatory activities, with compounds like quercetin, flavonoids, and various phenolic acids contributing to their ability to scavenge free radicals and protect against oxidative damage. The antioxidant properties of neem have been linked to its potential therapeutic benefits, including its use in traditional medicine to support immune function and combat diseases associated with oxidative stress (Ragavendran *et al.*, 2013).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample Collection and Preparation

Azadirachta indica was collected locally in Kota Bharu. The sample was then washed and rinsed using tap water to remove contaminants. It was dried by using over at 46°C for three days (Figure 3.1). The dried sample was ground into powder form using a mechanical grinder. The ground sample was kept in a sealed plastic bag with a silica gel desiccant.

Figure 3.1 Drying *A. indica* at room temperature

3.2 Sample Extraction

Approximately 100 g of the ground *A. indica* was soaked in 100 ml of hexane and extracted three times to maximise the yield of the extract obtained for 24 hours (Figure 3.2). The extract was then concentrated under reduced pressure using a rotary evaporator to achieve a paste-like consistency. The same procedure was followed for methanol. The extracts were soaked with 95% methanol for the extraction procedure to produce methanol crude extract (Figure 3.3).

Figure 3.2 Soaked sample in hexane and methanol.

Figure 3.3 Concentrating sample using a rotary evaporator.

3.3 DPPH Radical Scavenging Assay Procedure

DPPH solution (0.004% w/v) was prepared in 95% methanol. In this assay, 1 ml of different concentrations of the hexane and methanol extracts was mixed with 3 ml of DPPH solution in separate wells. The tubes were incubated for 30 minutes in the dark at room temperature. Using a plate reader, the optical density was determined at 517 nm. The absorbance of the DPPH control was also noted. Ascorbic acid was used as a reference standard. The radical scavenging activity of extracts and ascorbic acid was calculated using the formula:

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

3.4 Data Analysis

Data on the percentage of DPPH radical-scavenging was analysed using the student t-test to compare the significance of the obtained data

CHAPTER 4

RESULTS

4.1 Determination of DDPH radical scavenging assay of ascorbic acid

The absorbance of ascorbic acid at different concentrations at 517 nm is shown in Table 4.1.

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

Concentration (µg/ml)	Absorbance
1	0.810
2	0.770
3	0.688
4	0.606
5	0.541
6	0.451
7	0.321
8	0.268
9	0.218
10	0.210

The ability of ascorbic acid to neutralise free radicals was evaluated using the DPPH radical scavenging assay, with the findings presented in Table 4.2. The DPPH radical scavenging activity of ascorbic acid exhibited a dose-dependent relationship. A standard curve, shown in Figure 4.1, was constructed based on the DPPH scavenging activity of ascorbic acid at various concentrations. From the figure, inhibition concentration at 50% (IC_{50}) was determined at 4 µg/ml.

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

Concentration (µg/ml)	DDPH radical scavenging assay (%)
0	0.000
1	32.549
2	35.854
3	42.745
4	49.692
5	55.154
6	62.661
7	73.613
8	78.039
9	82.241
10	82.969

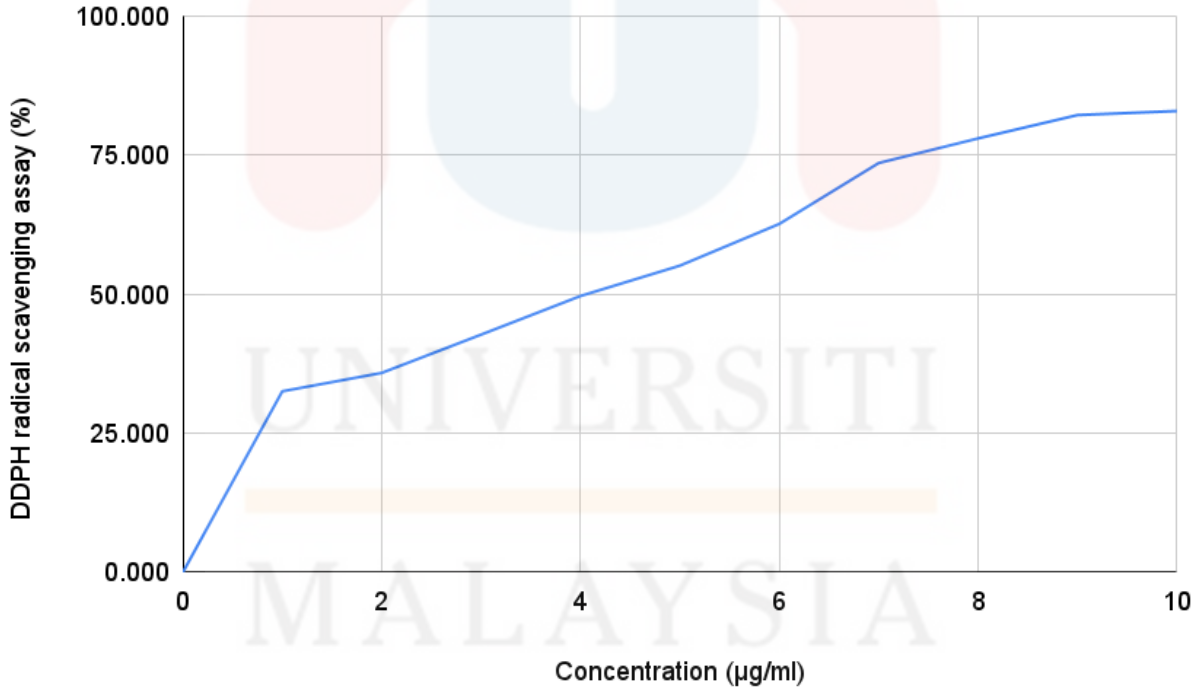

Figure 4.1 Standard curve of DPPH radical scavenging activity against concentration of ascorbic acid

4.2 Determination of DDPH radical scavenging assay of methanolic extract

The absorbance of methanol at different concentrations at 517 nm is shown in Table 4.3. As the amount of methanol rises, there is a corresponding decline in absorbance.

Table 4.3 Absorbance of methanolic extract at different concentrations at 517 nm.

Concentration (µg/ml)	Absorbance
0	1.197
20	1.153
40	0.876
60	0.271
80	0.267
100	0.165

The ability of methanol to neutralise free radicals was evaluated using the DPPH radical scavenging assay, with the findings presented in Table 4.4. The DPPH radical scavenging activity of methanolic extract exhibited a dose-dependent relationship. Figure 4.2 was constructed based on the DPPH scavenging activity of ascorbic acid at various concentrations. From the figure, IC_{50} was determined at 48 µg/ml.

Table 4.4 DPPH radical scavenging assay of methanolic extract at different concentrations.

Concentration (µg/ml)	DDPH radical scavenging assay (%)
0	0.000
20	3.669
40	26.947
60	77.815
80	78.179
100	86.695

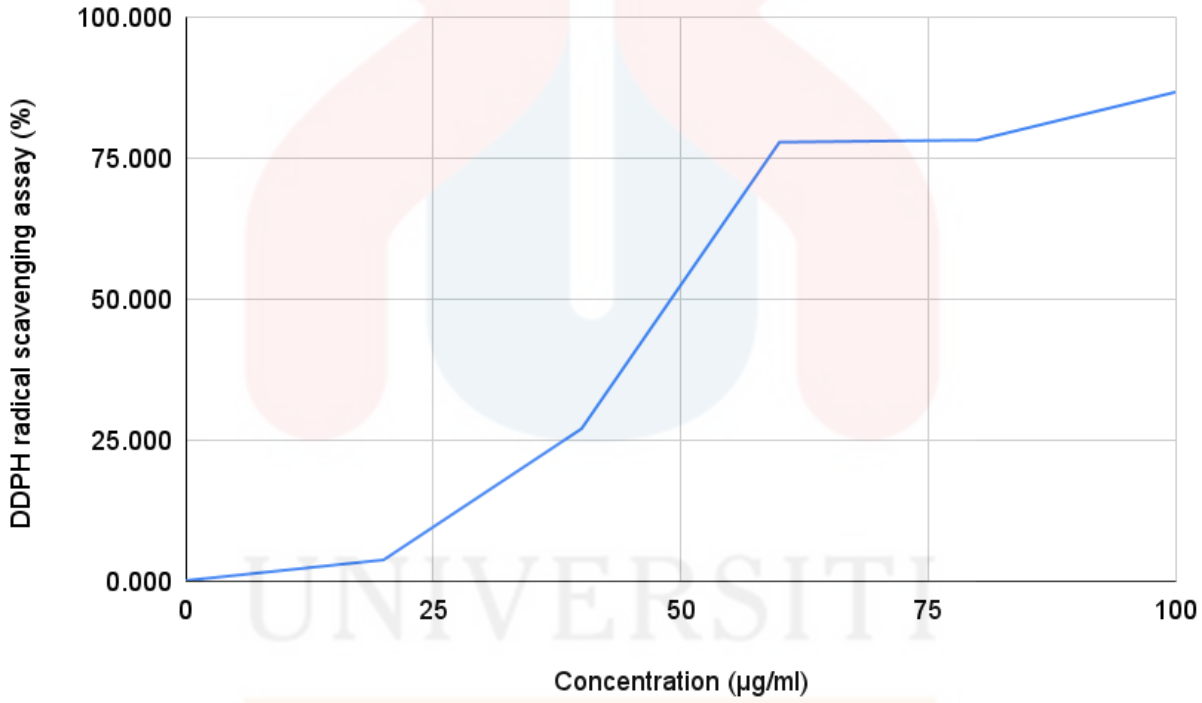

Figure 4.2 Standard curve of DPPH radical scavenging activity against concentration of methanol

CHAPTER 5

DISCUSSION

The DPPH assay, introduced in 1958, is a widely accepted method for evaluating the antioxidant capacity of substances. This assay measures the ability of compounds to scavenge free radicals, particularly the DPPH radical, which is commonly used in food and pharmaceutical research to assess antioxidant properties (Gulcin *et al.,* 2023). The DPPH radical is characterized by its purple color, which absorbs maximally at 517 nm in solvents such as ascorbic acid and methanol (Angeli *et al.,* 2021). The reduction of this color is used as an indicator of antioxidant activity, with a decrease in absorbance at 517 nm reflecting the neutralization of the radical by antioxidants.

The mechanism behind the DPPH assay involves the transformation of the DPPH radical into a stable diamagnetic molecule upon accepting an electron or hydrogen atom from an antioxidant. This process leads to the reduction of DPPH's characteristic violet color, turning it from purple to yellow. The degree of this color change is proportional to the antioxidant capacity of the tested compound, with a stronger color change indicating greater antioxidant activity. This makes the DPPH assay an essential tool for evaluating the potential of various substances to neutralize harmful free radicals, which can cause oxidative stress and contribute to diseases such as cancer, and cardiovascular disease (Ioneta *et al.,* 2014).

In this study, the antioxidant potential of *Azadirachta indica* leaf extracts was assessed using the DPPH assay, with ascorbic acid used as a reference antioxidant. Ascorbic acid, also known as vitamin C, is a well-established and potent antioxidant, frequently used in research as a benchmark due to its high efficacy in scavenging free radicals. The use of ascorbic acid as a reference allows

for a standardized comparison, ensuring the validity and reliability of the results. Methanol was chosen as the solvent for extraction because it is effective in dissolving bioactive compounds such as flavonoids, alkaloids, and phenolic compounds, which are known for their antioxidant properties (Ouerfelli *et al.,* 2022)

The results of this study showed that ascorbic acid exhibited significantly higher radical scavenging activity compared to the methanolic extract of *A. indica*. This finding aligns with a wealth of research demonstrating that ascorbic acid is highly effective at neutralizing free radicals due to its ability to donate electrons. The potent antioxidant capabilities of ascorbic acid are well-documented, and its role in protecting cells from oxidative damage has been widely studied (Arshad *et al.,* 2018). The methanolic extract of *A. indica*, while showing some antioxidant potential, demonstrated lower radical scavenging activity. This is consistent with previous studies indicating that methanol extracts, although rich in bioactive compounds, tend to have lower antioxidant potency compared to pure compounds or extracts obtained using other solvents like ethanol (Sahu *et al.,* 2016).

Flavonoids and phenolic compounds present in *A. indica* are known to exhibit antioxidant activity. These compounds act as electron donors, neutralizing free radicals and preventing oxidative damage. However, the relative effectiveness of these compounds can vary depending on the extraction method and solvent used. Methanol, although commonly employed for extracting antioxidant compounds, may not always be the most efficient solvent for extracting all types of bioactive molecules from plant material. Studies have shown that other solvents, such as ethanol, may yield higher amounts of polyphenolic compounds, which have been shown to possess stronger antioxidant properties (Sahu *et al.,* 2016). This variability underscores the importance of solvent selection in determining the antioxidant potential of plant extracts.

The comparison between the ascorbic acid standard and the *A. indica* extract highlights the differences in antioxidant activity based on solvent choice and the types of compounds extracted. Pure compounds like ascorbic acid are often more potent in antioxidant assays due to their higher concentrations of active compounds. However, this does not diminish the potential of *A.indica* as a source of antioxidants. The plant contains a variety of phytochemicals, including flavonoids, alkaloids, and phenolic compounds, which contribute to its overall antioxidant potential. While the methanol extract in this study showed lower scavenging activity than ascorbic acid, the plant still holds promise as a natural antioxidant source, particularly when more efficient extraction methods are employed (Darkwah *et al.,* 2020)

These findings have important implications for future research and the potential applications of *A. indica* in the pharmaceutical industries. Given the variability in antioxidant activity based on extraction methods, further studies could focus on optimizing extraction conditions, such as solvent choice, extraction time, and temperature, to maximize the yield of bioactive compounds. Additionally, investigating the specific phytochemical constituents of *A. indica* responsible for its antioxidant activity could provide valuable insights into its therapeutic potential (Sultana *et al.,* 2009)

The DPPH assay effectively demonstrated the antioxidant activity of *A. indica* leaf extracts, with ascorbic acid serving as a potent benchmark. While the methanol extract showed lower radical scavenging potential than ascorbic acid, it still exhibited antioxidant activity, highlighting the plant's potential as a source of natural antioxidants (Ahmed *et al.,* 2023).

CHAPTER 6

CONCLUSION

This study highlights the significant antioxidant potential of *Azadirachta indica*, with ascorbic acid demonstrating a higher DPPH radical scavenging activity (IC_{50} value at concentration 4µg/mL) compared to methanolic extract (IC_{50} value at concentration 48 µg/mL). This suggests that *A. indica* contains compounds capable of donating hydrogen and scavenging free radicals, underscoring its therapeutic potential. The findings also emphasise the critical role of solvent selection in optimising antioxidant extraction, which is vital in phytochemical research. These results provide valuable insights into the antioxidant capacity of *A. indica,* reinforcing its potential as a natural antioxidant source for applications in veterinary medicine.

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