Antioxidant and Antimalarial Properties of *Nigella sativa*

Jian Hung Sam[1], Yen San Chan[1,*], and Angela Siner[2]

[1]Department of Chemical and Energy Engineering, Faculty of Engineering and Science, Curtin University Malaysia, Miri, Sarawak, Malaysia

[2]Malaria Research Centre, Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Jalan Datuk Mohammad Musa, Kota Samarahan, Sarawak, Malaysia

Email: ivansjh97@gmail.com (J.H.S); chanyensan@curtin.edu.my (Y.S.C.); sangela@unimas.my (A.S.)

[*]Corresponding author

Manuscript received November 29, 2023; revised January 17, 2024; accepted March 7, 2024; published April 11, 2024

***Abstract*—Throughout the years, natural products have become a vital component contributing immensely to the healthcare industry. These natural products are highly valued for their diverse bioactive compounds which exhibit a wide range of medicinal properties and possess the potential for disease control or prevention. *Nigella sativa*, also known as black cumin, is originally native to Southern Europe, North Africa, and Southwest Asia. It is also cultivated in Malaysia and its seed serves as a culinary ingredient in the local cuisine. The seed of *Nigella sativa* has also been used by indigenous tribes around the world since long ago for traditional remedies. The objectives of this research are to evaluate the antioxidant activity and the antimalarial activity of the methanolic *Nigella sativa* extract. The DPPH assay yielded an antioxidant activity of 6.42% at 0.2 mg/ml. While, in the Mark III assay, the antimalarial activity exhibited dose-dependent results at concentrations of 1.56 µg/ml, 3.13 µg/ml, 6.25 µg/ml, 12.50 µg/ml, 25.00 µg/ml, 50.00 µg/ml and 100.00 µg/ml. In summary, the methanolic extract from *Nigella sativa* exhibits antioxidant potential which might also play a significant role in the management of malaria.**

***Keywords*—antioxidant, antimalarial, natural plants**

I. Introduction

Malaria is an infectious disease caused by the *Plasmodium* parasite from the bites of female *Anopheles* mosquitoes. The symptoms are fever, chills, fatigue, and can be fatal if not treated accordingly. In the current age, there are antimalarial drugs available for treatment such as artemisinin, chloroquine, and antibiotics. However, over the course of a decade, there has been no new class of antimalarial agents that was developed and used widely. Furthermore, antimalarial drug resistance is becoming more prominent with the passage of time [1]. Due to the lack of an effective vaccine coupled with antimalarial drug resistance, there is a need for an effective, highly available, and safe antimalarial product [1].

The indigenous community has been using traditional plants for the treatment of fever or malaria since long ago [2]. The cumulative knowledge from the indigenous community on the traditional plants is valuable and serves as a treasure trove in natural product discovery. For example, artemisinin and quinine are both derived from *Artemisia annua* and Cinchona bark which are natural products as well [3]. The abundance of antioxidants or bioactive compounds present in natural plants might help in targeting diseases.

Nigella sativa seeds are one of the natural products used traditionally for the symptoms of malaria and with great medicinal benefits [4]. It is important to identify its antimalarial properties in Malaysia. Therefore, this study is to determine the antioxidant, antimalarial and cytotoxic activity of *N. sativa* seed extract and it's potential as an antimalarial agent.

II. Literature Review

N. sativa, also widely known as black seed or *Habattus sauda* belongs to the family of *Rananculaceae*. It is native to Southern Europe, North Africa, and Southwest India, and also cultivated in various countries including Kelantan, Malaysia [5, 6]. It also serves as a culinary ingredient in various countries.

The *N. sativa* seeds were reported to possess plenty of pharmacological benefits and are called the miracle herb. Some of the benefits reported were antioxidant, antibacterial, anti-inflammatory, and more [7−9]. Today, black seed oil is widely used as a supplement and sold in many local pharmacies. Due to the overwhelming benefits, there is reason to investigate its antimalarial properties and potential to be an antimalarial agent.

III. Materials and Methods

Sample Collection and Preparation. *N. sativa* seeds were purchased from Moittry Infinity Sdn Bhd from Selangor, Malaysia. The seeds were then washed, dried and blended into powder form. The seed powders were then sealed in an airtight container at 4 °C until extraction.

Extraction of Sample. Soxhlet extract was used for the extraction of *N. sativa* seed extract with slight adaptations [10]. Firstly, 50 g of *N. sativa* seed powder were filled in the thimble and placed in the main chamber of the Soxhlet apparatus. Next, 300 mL of methanol was filled in the round bottom flask and then set up along with the rest of the Soxhlet apparatus. The extraction was carried out for 8 h. After the extraction, methanol was separated from the N. sativa extract using a rotary evaporator. The *N. sativa* seed extract was then weighted and stored in amber bottle at 4 ºC.

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging. The antioxidant activity of the extract was determined by using DPPH as a free radical with adaptation from Brand-Williams, Cuvelier, and Berset [11]. Firstly, 0.1 mL of *N. sativa* extract (0.2 mg/mL) was added to 3.9 mL of 6×10^{-5} mol/L DPPH in ethanol. The mixture was shaken vigorously and left to stand at room temperature for 30 mins in the dark. The absorbance was then recorded using UV-Vis Spectrometer at 517 nm.

$$DPPH\ activity\ (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \quad (1)$$

where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of sample.

Parasite Culture. Chloroquine-sensitive *P. falciparum*

strain 3D7 was obtained from the Malaria Research Center, UNIMAS. The parasite was cultivated and maintained according to the methods provided by the World Health Organisation (WHO) with slight modifications [12]. The parasite was retrieved and maintained in fresh *O* erythrocytes suspended in a complete RPMI-1640 medium supplemented with 25 mM HEPES, 20% D-glucose, 7.5% (w/v) sodium bicarbonate, 10 mg/mL of gentamycin, 1M hypoxanthine and 10% Albumax kept at 37 °C and 5% CO_2, 5% O_2 and N_2 balance using the candle jar method.

Antiplasmodial Mark III Assay. This assay was developed by WHO and was used to evaluate the antimalarial activity [12]. The diluted plant extract was set in a 96-well plate with erythrocytes to make 5% hematocrit. Chloroquine was also prepared as a positive control, diluted with complete media in a dose-responding manner. 50 µL of the blood mixture media (erythrocytes and complete medium) was added to each well, followed by 50 µL of the test, control, and negative samples. The 96-well plate was incubated at 37 °C and 5% CO_2, 5% O_2, and N_2 balance using the candle jar method. After 30 h, the 96-well plate was taken out of the incubator and the content of the wells was harvested to prepare thin and thick blood films. The number of schizonts with three or more nuclei per 200 parasites was noted while control and test wells were compared for the determination of inhibition percentage. All doses were done in triplicates. The inhibition of parasite growth percentage was calculated using the equation as follows.

$$Percentage\ Inhibition\ of\ Parasite\ Growth\ (\%) = \left(1 - \frac{\text{Number of schizonts in test wells}}{\text{Number of schizonts in control wells}}\right) \times 100 \quad (2)$$

In vitro Cytotoxicity Assay. The cytotoxicity of the extract was evaluated on the human dermal fibroblast cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -tetrazolium bromide) assay. A concentration of 1×10^4 cells/mL (100 µL/well) was seeded on 96-well plates. After 24 h, the cells were treated with various concentrations of the extract and then incubated for 24 h at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. After another 24 h, the media was changed and MTT solution (5 mg/mL) was applied to each well at a rate of 10 µL per well for 3–4 h at 37 °C. In each well, formazan crystals were dissolved in DMSO. A microplate reader was used to measure absorbance at 570 nm to assess the strength of purple formazan [13].

IV. Result and Discussion

A. DPPH Free Radical Scavenging Assay

Reactive Oxygen Species (ROS) and Nitrogen-Oxygen Species (NOS) are examples of free radicals formed when cells consume oxygen to produce energy. A moderate amount of these free radicals is essential to maintain physiological processes in the human body. However, when the amount of free radicals is in abundance, it can lead to cellular damage and toxicity in the human body, called oxidative stress. Oxidative stress triggers the release of free radicals which then undergoes oxidation of essential body molecules leading to diseases. Antioxidants are compounds that help maintain the equilibrium between the amount of free radicals and the condition of physiological processes in the human body.

DPPH free radical scavenging assay was selected to measure the antioxidant activity because it works in both Hydrogen Transfer (HAT) and Electron Transfer Systems (SET) which provides a complex and diverse evaluation of plant extracts through two mechanisms of action. In the presence of antioxidant compounds, electron or hydrogen atoms are transferred, converting the DPPH radical, and thus discoloring the purple DPPH solution to a pale-yellow solution [14].

The antioxidant activity evaluated by the DPPH free radical scavenging assay exhibited 6.42 ± 0.56 % at a concentration of 0.2 mg/mL from the methanolic crude extract of *N. sativa*. Besides, from another study in Morocco, *N. sativa* was extracted with methanol/water (85/15, v/v) overnight at room temperature which yielded an antioxidant activity of approximately 10% at 0.225 mg/mL [15]. Next, Soxhlet extraction of *N. sativa* in India was also conducted which exhibited an antioxidant activity of approximately 16% at 0.2 mg/mL [16]. It was important to note that the *N. sativa* seeds were soaked overnight in methanol solvent before being transferred to Soxhlet Extraction for 6 h. Lastly, another study in India also conducted a solvent extraction of the seeds in methanol for 72 h. at which yielded an antioxidant activity of approximately 35% at 0.2 mg/mL [17]. From the current study in comparison with the other studies, it was observed that the DPPH antioxidant activity varied with slightly different extraction methods, extraction time, solvent used, and geological variety.

The antioxidant activity from *N. sativa* seeds may have been attributed to the abundance of phenolic and flavonoid compounds present in the extract [17]. Thymoquinone which is the principal compound from *N. sativa* seeds and also a major phenolic compound further emphasizes its role in the antioxidant spectrum with the combination of other phytochemicals present in the extract.

B. In Vitro Antimalarial Activity of N. Sativa Seed Extract

The in vitro antimalarial activity was identified with the Mark III assay developed by WHO is dependent on the schizont maturation as an indicator for parasite growth. The schizont maturation is determined by microscopy which is considered the gold standard in malaria diagnosis. The categorization of IC_{50} values was adopted from Gathirwa which states that antimalarial activity was: high (<10 µg/mL), moderate (10−50 µg/mL), low (50−100 µg/mL) and inactive (> 100 µg/mL) [18].

From Fig. 1, the *N. sativa* seed extract inhibited the parasite growth at a dose-dependent manner from 1.5625 µg/mL to 100 µg/mL. It was observed at 50 µg/mL, the seed extract had reached over 50% of parasite inhibition. The seed extract had an IC_{50} value of 49.35 ± 9.08 µg/mL which falls on the borderline range of moderate antimalarial activity against the *Plasmodium falciparum* 3D7 strain. In another study, Omondi *et al.* [6] evaluated *N. sativa* seeds from Saudi Arabian origin against *Plasmodium falciparum* W2 and D6 strains, which exhibited an IC_{50} of 80.48 ± 12.29 µg/mL (low) and 31.93 ± 4.31 µg/mL (moderate) respectively in methanolic extract. It was observed that the *N. sativa* seed extract was more sensitive towards the D6 strain and 3D7 strain compared to the W2 strain. Geological reasons and

extraction methods might also contribute to the antimalarial activity of the seed extracts.

The phytochemical compounds present in the seed extract contributing to antioxidant activity might also be contributing to the antimalarial activity, namely, flavonoids, phenolics, terpenes, and alkaloids. Although the details regarding the mechanism of action of *N. sativa* against the plasmodium parasites remain undiscovered, the phytochemical compounds might indicate possible reasons for inhibition. One possible reason was suggested that terpenes with similar chemical structure to intermediates of isoprenoids pathway were able to inhibit dolichol biosynthesis which affects malaria pathogenesis [19]. The terpenes present in the *N. sativa* extract might cause metabolic interference to the parasite's biosynthesis of polyisoprenoids leading to parasite inhibition and death. Furthermore, alkaloids were suggested to be able to inhibit the hemozoin biocrystallization from toxic heme by the parasites created from hemoglobin feeding from erythrocyte invasion resulting to death by heme toxicity [20]. Besides, triterpenes were also suggested to be able to stop the maturation of the parasites from the ring stage to the schizont stage [1]. The antimalarial properties of *N. sativa* seed extract might have been a synergy of different bioactive compounds working in combination through a variety of mode of actions causing parasite death.

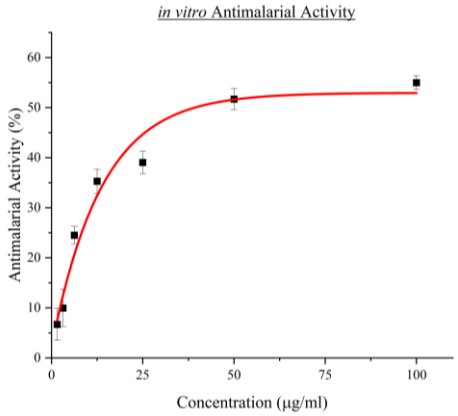

Fig. 1. *In vitro* antiplasmodial assay.

C. In Vitro Cytotoxicity Assay

Besides the evaluation of antimalarial activity, a potential antimalarial candidate should also exhibit a lack of toxicity to host cells. Therefore, cytotoxicity studies are vital in determining the lethal dose in which the extract is administered. The cytotoxicity of *N. sativa* seed extract was investigated by MTT assay for 72 hrs on human dermal fibroblast cells. The MTT assay is widely used in a variety of cell lines for viability evaluation. The chemical reaction involves a conversion of tetrazolium salt MTT to insoluble purple crystals formazan. The higher amount of viable cells, the higher the amount of purple formazan present and as such the assay is able to rapidly quantify the cell viability by means of spectrophotometric equipment.

Fig. 2 below exhibited the percentage cell viability of the extract at concentrations from 1.5625 µg/ml to 100 µg/mL. The percentage of cell viability of the extract remained constant up to 100 µg/mL of concentration which indicates lack of cytotoxicity. The results from this study demonstrated the safety of the seeds in the treatment of malaria.

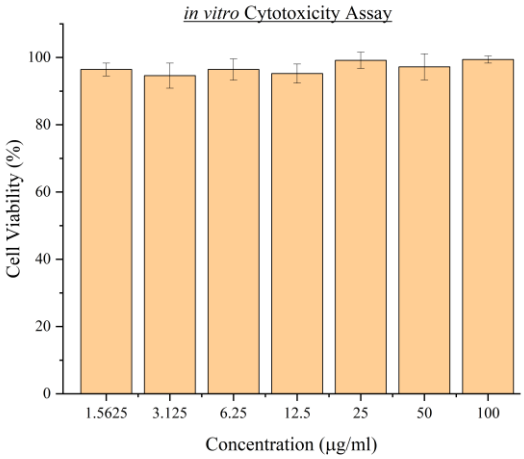

Fig. 2. *In vitro* cytotoxicity assay.

V. Conclusion

In this study, *N. sativa* seeds had been evaluated with the pLDH assay and had shown to possess capable antimalarial properties with an IC_{50} value of 49.35 ± 9.08 µg/mL. The antimalarial properties of *N. sativa* were contributed from the abundance of phytochemical compounds present in the extract, such as phenolics, flavonoids, terpenes and alkaloids. These cumulative phytochemical compounds also exhibited antioxidant activity which was evaluated to be 6.42 ± 0.56 % at a concentration of 0.2 mg/mL with DPPH assay. The antioxidant properties of *N. sativa* provided an added benefit in the management of malaria. A patient infected with malaria experiences systemic and tissue oxidative damage, therefore, having an added benefit of antioxidant activity helps reduce oxidative damage and maintain the redox balance, granting complementary therapy to antimalarial treatment. Besides, the cytotoxicity assay also exhibited safety of up to 100 µg/mL, which makes *N. sativa* a suitable candidate for future development into a potential antimalarial agent. From the results obtained, this study had contributed to the evaluation of the antimalarial, antioxidant and cytotoxicity of *N. sativa* seeds cultivated in Malaysia and indicated promising potential for further research in hopes of achieving an antimalarial agent.

However, since the scope of this study is only covering in vitro studies, which is first stage investigation in a controlled environment, future research needs to be executed *in vivo* to further grasp the effect of *N. sativa* in animal models such as cultivating *Plasmodium berghei* in mice and in-depth understanding of the mechanism of action of bioactive compounds targeting the malaria parasite. Lastly, the exploration and application of drug delivery and drug release techniques are also vital to improve the poor bioavailability of *N. sativa* seeds.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Jian Hung Sam conducted the research and wrote the paper.

Yen San Chan and Angela Siner analyzed the data and edited the paper. All authors had approved the final version.

FUNDING

This project is funded by the Sarawak Research Development Council (SRDC) with the research grant number: SRDC/RDCRG/CAT/2019/04.

ACKNOWLEDGMENT

The authors are grateful to have access to the facility at Sarawak Biovalley Pilot Plant, a facility owned by SRDC and managed by Curtin Biovalley Sdn Bhd. Besides, the authors would also like to express gratitude to Sarawak Biodiversity Centre (SBC) for the research permit approval with the permit number: SBC-2021-RDP-xx-SCYS. Lastly, we are also thankful to Curtin University Malaysia for the necessary resources that have been provided.

REFERENCES

[1] T. M. Belete, "Recent progress in the development of new antimalarial drugs with novel targets," *Drug Design, Development and Therapy*, pp. 3875−3889, 2020.

[2] R. S. Tabuti, John, and S. B. Obakiro *et al.*, "Medicinal plants used for treatment of malaria by indigenous communities of Tororo District, Eastern Uganda," *Tropical Medicine and Health*, vol. 51, 34, 2023. doi: 10.1186/s41182-023-00526-8

[3] D. G. I. Kingston, and M. B. Cassera, "Antimalarial natural products," in *Antimalarial Natural Products*, A. Kinghorn, Falk, Gibbons, Ed. Yoshinori Asakawa, Ji-Kai Liu and Verena M. Dirsch, Cham: Springer International Publishing, 2022, pp. 1−106.

[4] B. Salehi and C. Quispe *et al.*, "Nigella plants—Traditional uses, bioactive phytoconstituents, preclinical and clinical studies," *Frontiers in Pharmacology*, vol. 12, 625386, 2021.

[5] S. O. Yagoub, "Chapter four—Black cumin: morphology, physiology, growth, and agricultural yield," in *Biochemistry, Nutrition, and Therapeutics of Black Cumin Seed*, Mariod, Ed. Academic Press, 2023, pp. 19−25.

[6] J. Oyweri and A. Mohammed et al., "In vitro and in vivo antimalarial activity of Nigella sativa L. extracts," Journal of Medicinal Plants Research, vol. 13, pp. 501−508, 2019.

[7] T. Salima, O. Benayad, M. Berrabah, I. E. Mounsi, and M. Mimouni, "Phytochemical profile and antioxidant activity of Nigella sativa L. growing in Morocco," The Scientific World Journal, 6623609, 2021. doi: 10.1155/2021/6623609

[8] F. S. Shafodino, J. M. Lusilao, and L. M. Mwapagha, "Phytochemical characterization and antimicrobial activity of Nigella sativa seeds," PLOS ONE, vol. 17, no. 8, e0272457, 2022. doi: 10.1371/journal.pone.0272457

[9] R. M. Pop, and O. Sabin *et al.*, "*Nigella Sativa*'s anti-inflammatory and antioxidative effects in experimental inflammation," *Antioxidants*, vol. 9, no. 10, 921, 2020. doi: 10.3390/antiox9100921

[10] M. Alrashidi, D. Derawi, J. Salimon, and M. F. Yusoff, "An investigation of physicochemical properties of *Nigella sativa L.* Seed oil from Al-Qassim by different extraction methods," *Journal of King Saud University—Science*, vol. 32, no. 8, pp. 3337−3342, 2020. https://doi.org/10.1016/j.jksus.2020.09.019

[11] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a free radical method to evaluate antioxidant activity," *LWT—Food Science and Technology*, vol. 28, no. 1, pp. 25−30, 1995. https://doi.org/10.1016/S0023-6438(95)80008-5

[12] World Health Organization. (2001). In vitro micro-test (Mark III) for the assessment of the response of Plasmodium falciparum to chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine and artemisinin instructions for use of the in vitro micro-test kit. [Online]. Available: https://iris.who.int/handle/10665/67373

[13] S. Kamiloglu, G. Sari, T. Ozdal, and E. Capanoglu, "Guidelines for cell viability assays," *Food Frontiers*, vol. 1, no. 3, pp. 332−349, 2020. https://doi.org/10.1002/fft2.44

[14] I. Gulcin, and S. H. Alwasel, "DPPH radical scavenging assay," *Processes*, vol. 11, no. 8, 2248, 2023. doi: 10.3390/pr11082248

[15] H. Outtar, O. Zouirech, and K. Mohammed *et al.*, "In vitro study of the phytochemical composition and antioxidant, immunostimulant, and hemolytic activities of *Nigella sativa (Ranunculaceae) and lepidium sativum* seeds," *Molecules*, vol. 27, no. 18, 5946, 2022. doi: 10.3390/molecules27185946

[16] G. Barabde and A. A. S. Qureshi. (2021). Effect of extraction techniques on antioxidant activity, DNA protection potential and antimicrobial properties of *N. sativa (Kalonji)* seed extract. *The International Journal of Engineering and Science*. [Online]. vol. 7, no. 11, pp. 1−5. Available: https://www.theijes.com/papers/vol7-issue11/Version-2/A0711020105.pdf

[17] M. Dalli, S. Azizi, F. Kandsi, and N. Gseyra, "Evaluation of the in vitro antioxidant activity of different extracts of *Nigella sativa L.* seeds, and the quantification of their bioactive compounds," *Materials Today: Proceedings*, vol. 45, no. 8, pp. 7259−7263, 2021. https://doi.org/10.1016/j.matpr.2020.12.743

[18] J. Gathirwa, G. M. Rukunga, and E. N. M. Njagi, "The in vitro anti-plasmodial and in vivo anti-malarial efficacy of combinations of some medicinal plants used traditionally for treatment of malaria by the Meru community in Kenya," *Journal of Ethnopharmacology*, vol. 115, no. 2, pp. 223−231, 2008. doi: 10.1016/j.jep.2007.09.021

[19] H. B. Gabriel, R. A. C. Sussmann, E. A. Kimura, and A. Marin *et al.*, "Terpenes as potential antimalarial drugs." in *Terpenes and Terpenoids*, Shagufta and Areej, Ed. Rijeka: Intech Open, 2018. Ch. 3. doi: 10.5772/intechopen.75108

[20] F. Irabor, O. Ebohon, N. Erhunse, O. T. Okugbo, and E. S. Omoregie, "In vitro antiplasmodial activity, cytotoxicity, antioxidant action and GC-FID analysis of Allanblackia floribunda Oliv," *SN Applied Sciences*, vol. 3, 820, 2021. doi: 10.1007/s42452-021-04812-0