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Comparative Studies on the Antimalarial and Antioxidant Activities of Extracts and Silver Nanoparticles from *Spermacoce verticillata*

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Abstract

Malaria stands as a pervasive infectious disease, posing a substantial global public health challenge with 3.3 billion individuals residing in endemic regions across 100 countries. This study investigated and compared the anti-malarial and antioxidant properties of ethanol and aqueous extracts with those of nano-particles derived from *Spermacoce verticillata*, a plant, commonly employed in our local community for treating bacterial skin infections and fever. The phytochemical analysis, conducted using established methods, involved both quantitative and qualitative assessments. Antimalarial potency was evaluated through an *in-vitro* assay measuring *β*-hematin formation inhibition, while antioxidant activity was gauged using the DPPH radical scavenging model. The results highlighted the presence of key phytochemicals such as saponins, tannins, alkaloids, phenols, and flavonoids in both ethanol and aqueous extracts in substantial amounts. Notably, the antimalarial assay demonstrated that silver nanoparticles exhibited the highest percentage inhibition at 77.96 ± 6.52, a statistically significant difference from ethanol (47.20 ± 3.01) and aqueous (48.64 ± 2.48) extracts, as well as the standard drug chloroquine (63.88 ± 4.56) at ($p < 0.05$). While all extracts demonstrated antioxidant activity, the nanoparticles with IC_{50} 16.5 µg/mL; surpassed those of the Aqueous (51.0 µg/mL), Ethanol (36.0 µg/mL) extracts and were comparable to that of the standard vitamin C (19.5 µg/mL) ($P < 0.05$) using one-way ANOVA. This study underscores the potential of nanotechnology in enhancing the therapeutic properties of herbal remedies against malaria, opening avenues for further exploration and application in global health initiatives.

Keywords: *Antimalarial, Antioxidant, Hermatin, Nanoparticle, Plasmodium, Spermacoce verticillata*

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1. Introduction

According to WHO (2022), malaria is an acute febrile illness caused by *Plasmodium* parasites, transmitted through the bites of infected female Anopheles mosquitoes. There are five *Plasmodium* parasite species (namely: *P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi*) responsible for malaria in humans, with *P. falciparum and P. vivax* posing the greatest threat globally. As of the latest World Malaria Report (2020), the disease remains a significant public health challenge, particularly in sub-Saharan Africa, where it is responsible for a high number of cases and deaths.

The African Region continues to bear a disproportionately high share of the global malaria burden, with 95% of all malaria cases and 96% of deaths reported in 2020 (World Malaria Report, 2022). Malaria deaths are especially prevalent among children under 5 years of age, accounting for approximately 80% of all malaria-related fatalities in the region. The disease's impact is further emphasized by four African countries—Nigeria, the Democratic Republic of the Congo, the United Republic of Tanzania, and Mozambique—contributing to over half of all malaria deaths worldwide.

Despite progress in malaria control, challenges such as insecticide resistance among Anopheles mosquitoes threaten global efforts. The World Malaria Report (20202) notes that seventy-eight countries reported mosquito resistance to at least one of the four commonly-used insecticide classes between 2010 and 2019. Additionally, twenty-nine countries reported resistance to all main insecticide classes.

Early diagnosis and treatment remain crucial in reducing disease burden and preventing deaths. WHO recommends confirming all suspected cases of malaria through parasite-based diagnostic testing (World Malaria Report, 2022). The best available treatment, especially for *P. falciparum* malaria, is artemisinin-based combination therapy (ACT), aiming for the rapid and complete elimination of *Plasmodium* parasites.

Malaria is characterized by inflammatory and oxidative stress, contributing to severe complications (World Malaria Report, 2020). The recent understanding of free radicals and reactive oxygen species (ROS) in malaria has led to the exploration of therapeutics that maintain oxidative balance in patients (Bagchi and Puri, 1998; Lobo *et al*., 2010). This aligns with the fact that malaria is highly inflammatory and oxidative, with host phagocytes producing reactive oxygen species as a crucial response to *Plasmodium* infection.

In recent years, antimalarial drug resistance has emerged as a threat, prompting exploration of new solutions from natural sources, particularly plants. This research also focused on the anti-oxidant activity of the plant understudy.

Antioxidants play a crucial role in protecting biological systems from oxidative damage. They are classified based on their environment and functions. Antioxidants are substances that can delay or prevent the oxidation of molecules, even at low concentrations. They neutralize free radicals and minimize oxidative damage, maintaining the balance between oxidants and antioxidants in metabolism. Antioxidants are effective at different stages of the oxidative process and depend on factors such as concentration, structure, and localization. Synthetic antioxidants like Butylated hydroxytoluene (BHT), propyl gallate (PG), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) have been widely used in the food and pharmaceutical industries but raise concerns due to their potential side effects, including enzyme inhibition. As a result, there's a growing preference for natural antioxidants that offer lower toxicity, high biodegradability, and safer action (Gulcin and Alwasel, 2023). This is one reason we investigated *S. verticillata* for another green and natural source of antioxidants. Various methods, including the use of DPPH, are employed to assess antioxidant efficacy (Gulcin *et al*., 2010).

Nanoparticles, particularly silver nanoparticles (AgNPs), are products of nanotechnology and are synthesized from plant extracts. These nanoparticles have gained attention for their unique properties and diverse applications in various fields (Smith *et al*., 2006; Wei *et al*., 2005). The study aims to contribute to the understanding of the antimalarial and antioxidant potential of *Spermacoce verticillata*, considering both traditional leaf extracts and nanoparticles. Malaria remains a significant global health challenge, particularly in sub-Saharan Africa, and efforts to combat the disease face challenges such as insecticide resistance and treatment failures. The study focused on *Spermacoce verticillata*, a medicinal plant known for traditional use in treating various ailments, including fever (Conserva and Ferreira, 2012). *Spermacoce verticillata* is considered

due to its potential antimalarial properties, and the research explored both leaf extracts and nanoparticles synthesized from aqueous extracts, aimed to assess their antimalarial and antioxidant activities. The study on *Spermacoce verticillata*'s antimalarial and antioxidant properties provides valuable insights into potential alternative therapies. The study aims to contribute to the understanding of the antimalarial and antioxidant potential of Spermacoce verticillata (Figure 1) considering both traditional leaf extracts and nanoparticles.

Figure 1: Spermacoce Verticillata Plant

Source: https://assessment.ifas.ufl.edu/assessments/spermacoce-verticillata/

2. Materials and Methods

2.1. Materials

2.1.1. Equipment

UV/spectrophotometer UV-2500. Labomed Inc., USA. Weighing balance (Shimadzu, Japan).

2.1.2. Glass Wares and Other Material

Graduated cylinder (10 ml, 50 ml and 100 ml), Beakers 50 ml, Glass rod, Stirrer, Filter paper (Whatman filter paper No. 1).

2.1.3. Reagents

Hydrochloric acid, potassium hexacyano ferric cyanide, Olive oil, Sodium hydroxide, Folin-Crocalteu's reagent, sodium carbonate, Hematin porcine, chloroquine diphosphate, Oleic acid, sodium dodecyl sulfate (SDS), sodium acetate, magnesium sulfate, sodium hydrogen phosphate, sodium chloride, potassium chloride and sodium hydroxide. All reagents and chemicals were purchased from Aldrich Chemical Company while Hydrochloric acid was purchased from Merck.

2.2. Methods

2.2.1. Collection Identification and Extraction of the Plant Material

The fresh and healthy leaves of *spermacoce verticillata* were collected from the Faculty of Pharmacy Garden, University of Uyo, Akwa Ibom State, Nigeria. All the plants were identified by Dr Imoh I. Johnny of the Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo and assigned voucher number in the Herbarium as follows: *Spermacoce verticillata (UUPH 67(a).*The leaves and stem bark were air dried at room temperature. The dried plant materials were then pulverized using a laboratory miller. The pulverized plant part was subjected to cold maceration using ethanol and distilled water to obtain ethanol and aqueous extract respectively.

2.2.2. Quantitative-Phytochemical Analysis (Taiwo and Olaoluwa, 2020).

2.2.2.1. Determination of the Total Phenols

Folin-Crocalteu's reagent (1 ml) was added to 1 ml of the plant extract (dissolved with distilled water) after which 2 ml of 7% Na_2CO_3 was added. 1ml of distilled water was added after 5 minutes and mixed thoroughly. The mixture was incubated for 90 minutes at 25 °C. The absorbance was taken at 750 nm. The total phenol content was determined from standard curve of garlic acid solution and expressed as mg of Gallic acid equivalent (GAE) of dried sample.

2.2.2.2. Determination of Total Flavonoids

Methanol (3.4 ml, 30%) was added to 0.3 ml of the extract after which $NaNO_2$ (0.5 M, 0.15%) and $AlCl_3.6H_2O$ (0.5 ml, 0.3 M) were added and mixed. NaOH (1 ml, 1 M) was then added after 5 minutes and kept at room temperature for 30 minutes. The absorbance was taken at 586 nm using uv/visible spectrometer. The determination was done in triplicate. The flavonoid content was calculated using standard graph of quercetin and the result expressed as quercetin equivalent (QUE) in mg/ml.

2.2.2.3. Determination of Total Saponins

The extract (0.5 g) was dissolved with distilled water, shaken and allowed to stand for 1 hour. Formation of a stable foaming was observed. Olive oil (1 mL) was added to 1 mL of the mixture and shaken to obtain a cloudy appearance. The absorbance was measured at 620 nm using spectrophotometer. Done in triplicate. Calculate using standard graph of saponin.

2.2.2.4. Determination of Total Tannin

Small quantity of extract was dissolved in distilled water and filtered. $FeCl_3$ (1 ml, 0.1 M) was added to the filtrate (2.5 ml), after which $K_4Fe(CN)_6.3H_2O$ (0.3 ml 0.0088 M) was added. Absorbance was measured at 395 nm. Result was expressed in terms of tannic acid (mg/ml) using standard tannic acid graph.

2.2.2.5. Determination of Total Alkaloids

HCl (5 ml, 0.1 N) was added to the extract's supernatant (5 mL) in a flask and shook thoroughly for 3 minutes and allowed to stand. 5 mL of the lower layer was titrated with NaOH (0.1 N) till the colour changed from red to yellow. Total alkaloid was calculated using the relationship:

1 mL 0.1 N HCl = 0.0612 g of alkaloid.

2.2.2.6. Synthesis of Silver Nano-Particles of the Extract (Jackson *et al.*, 2018)

Silver nitrate solution was prepared by dissolving 1 g of silver nitrate in 1 litre of solution using distilled water to obtain a solution of concentration of 1 mg/mL. Further dilutions were carried out to obtain solutions of concentrations 10, 20, 40, 80 and 100 µg/mL. To 5 mL of each concentration of silver nanoparticles (AgNPs), 5 ml of the aqueous extract was added in drops with constant stirring using a magnetic stirrer assembly for 5 min, to obtain [Ag]+ dispersion. The resultant suspension of silver nanoparticles were lyophilized using Virtis 2KBTXL-75 Bechtop SLC Freeze Dryer.

2.2.3. Determination of Anti-Malarial Activity

2.2.3.1. Study Sample

The samples tested were Ethanol extract, Aqueous extract, and silver nanoparticles. The samples were prepared in alcohol, which also acted as vehicle control. The standard drug used was Chloroquine diphosphate (500 µg), a known anti-malarial drug. The given samples were also evaluated in various concentrations.

2.2.3.2. *In vitro* β-Hematin Formation Assay (Afshar *et al.*, 2011; Joshi *et al.*, 2017)

The samples were subjected to incubation with 3 mM of hematin, 10 mM oleic acid, and 1 M HCl. The total volume was adjusted to 1 mL using a sodium acetate buffer with a pH of 5. Chloroquine diphosphate was employed a positive control. The reaction mixtures were left to incubate overnight at 37 °C with continuous

gentle agitation. After incubation, the samples were centrifuged at 14,000 rpm for 10 minutes at 25 °C. The hemozoin pellet formed was subjected to repeated washing during the incubation every 15 minutes at 37 °C by shaking in a 2.5% (w/v) SDS solution in phosphate buffered saline, followed by a final wash in 0.1 M sodium bicarbonate until the supernatant became clear (this required 7 washing cycles). After the last wash, the supernatant was decanted, and the pellets were dissolved in 1 mL of 0.1 M NaOH. The hemozoin content was then determined by measuring the absorbance at 400 nm. The results were expressed as the percentage inhibition (I%) of heme crystallization in comparison to vehicle controls, using the following equation:

$$I\% = \frac{AbsC - AbsS}{AbsC} x\ 100$$

Where AbsC: Absorbance of the control; and AbsS: Absorbance of the sample.

2.2.3.3. DPPH Free Radical Scavenging Activity (Shekhar and Anju, 2014; Johnson *et al.*, 2017)

The antioxidant activity of the extract is based on its ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. A dilution series was prepared for the plant extracts. The reaction mixture contains 0.5 mL of the extract and 1 mL of DPPH solution (0.4 mM) in methanol. The mixture was incubated in a dark room for 30 min at room temperature. Ascorbic acid was used as a standard. The reduction in the DPPH-free radical was measured using a spectrophotometer at 517 nm. The percentage inhibition (I%) of the radicals was estimated using the following formula:

The percent DPPH scavenging effect was calculated by using following equation:

$$I\% = \frac{AbsC - AbsS}{(AbsC} x\ 100$$

Where AbsC: Absorbance of the control; and AbsS: Absorbance of the sample.

The IC_{50} were determined using calibration graph of vitamin C standard solution. Reference standard compound being used was ascorbic acid and experiment was done in triplicate. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. Lower absorbance of the reaction mixture indicated higher free radical activity.

2.3. Statistical Analysis

All results were expressed as mean ± SEM and were analyzed by two-way ANOVA using MS excel 2019. P <0.05 was taken as significant.

3. Results and Discussion

3.1. Phytochemical Analysis

The phytochemical investigation of the aqueous extract of *S. verticillata* revealed important levels of saponins, phenolics, flavonoids, alkaloids and tannins (Table 1). Each of these phytochemicals was found to be present in good amount. The quantity of each of these compounds were comparable in each of the extracts; total saponins having the highest amounts of 9.03±1.05 and 10.75±0.53 mg/g in ethanol and aqueous extracts respectively. Several studies have indicated that the polyphenols are mainly accountable for the antioxidant potential and anti-inflammatory properties of multiple medicinal plants (Fawole *et al.*, 2009; and Costa *et al.*, 2012). In addition to phenolics, flavonoids and tannins are widespread and important bioactive molecules in plants. Tannins have a potent antioxidant and antibacterial activities (Soldado *et al.*, 2021, and Yuan *et al.*, 2022).

The formation of nano-particles was indicated by the different UV absorption peaks on the spectrum before and after freeze drying.

3.2. Antimalarial Activity Test

In the study of Malaria, it has been reported that the digestion of hemoglobin within the food vacuole of the

Table 1: Quantitative Estimation of Phytochemicals in of *S. verticillata*

Sample	Total Phenolics GAE mg/g)	Total Flavonoids (QUE mg/g)	Total Saponins (SA mg/g)	Total Tannins (TA mg/g)	Total Alkaloids (g)
Ethanol extract	2.74±0.53	0.123±0.07	9.03±1.05	0.973±0.44	0.138±0.06
Aqueous extract	2.44±0.03	0.125±0.03	10.75±0.53	0.895±0.23	0.113±0.01

Note: All values represent Mean ± SD, n = 3.

malaria parasite leads to the generation of substantial quantities of redox-active toxic free heme. Plasmodium species employ a unique process known as hemozoin (beta-hematin) formation to detoxify this free heme. Inhibitors of hemozoin formation have the potential to serve as antimalarial drugs and represent a promising target for the development of new anti-malarial agents. The inhibition of hemozoin formation is crucial in combating malaria, and drugs capable of achieving this can offer effective antimalarial activity (Alam *et al.*, 2009). Consequently, many antimalarial drugs, including 4-aminoquinolines like quinine, mefloquine, and chloroquine, rely on inhibiting hemozoin formation as their mode of action. This inhibition process is regarded as a favorable target for drug screening initiatives (Joshi *et al.*, 2017). In this research study, all the three samples namely: the ethanol extract the aqueous extract and the silver nanoparticles exhibited inhibition of hemozoin but the inhibition of the nanoparticles was greater than those of both extracts and the positive control Chloroquine diphosphate used in the study (Table 2). That is, the prepared nanoparticle showed a significantly higher ($p<0.05$) anti-malarial activity than both aqueous and ethanol extracts of *S. verticillata.*

Table 2: Inhibition of Hemozoin Formation by the Extracts and Nano-Particles

S/No.	Sample	% Inhibition
1	Aqueous extract	48.64 ± 2.48
2	Ethanol extract	47.20 ± 3.01
3	AgNPs	77.96 ± 6.52*
4	Chloroquine diphosphate (500 µg)	63.88 ± 4.56

Note: All values represent Mean ± SD, n = 3; *$p<0.05$; as compared to the extracts; (ANOVA followed by post-hoc tests).

3.3 Antioxidant Test

In the antioxidant studies our findings revealed that all extracts of the plant had an antioxidant activity (Table 3 and Figures 2-6). Antioxidants are substances that can neutralize or reduce damage caused by free radicals (Raj Narayana *et al.*, 2001). The nanoparticles as expected showed greater activity than the extracts and was not significantly different from that of the standard vitamin C ($P<0.05$). IC_{50} of the samples in DPPH radical-removing ability were also determined (Figures 2-4). IC_{50} is a quantitative measure that indicates how much of a particular inhibitory substance is needed to inhibit, *in vitro*, a given biological process or biological component by 50% (Stewart and Watson, 1983; Shekhar and Anju, 2014). The lower the IC_{50} values, the higher the DPPH radical-removing ability of the antioxidants. In this context, the IC_{50} value of the AgNPs was the lowest showing that it has the highest scavenging capacity (Gulcin and Alwasel, 2023) compared to others and even the standard - Ascorbic acid (Figure 5). This activity could be due to the presence of phenolics and flavonoids in the extracts (Djeridane *et al.*, 2006). All the extracts contained these compounds. The phenolic compounds especially flavonoids are endowed of the antioxidant activity (Nair and Gupta, 1996; Costa *et al.*, 2012). Other studies have indicated that alkaloids, flavonoids, phenols and tannins are responsible for the antioxidant potential of medicinal plants (Allagui *et al.*, 2023). These plants' chemicals have been found in substantial quantities in *S. verticulatta* in our experiments in this research work. Hence the antioxidant activity observed here may be directly attributed to them. The nanoparticle of the plant extract indicated higher activity because of its high solubility and the strong reducing ability.

Table 3: Percentage Inhibition of DPPH Radicals by the Extracts and Nano-Particles of *S. verticillata* and the IC_{50} of the Samples

Conc. g/mL	Aqueous Extract		Ethanol Extract		AgNPs		Ascorbic Acid	
	Abs	(%I)	Abs	(%I)	Abs	(%I)	Abs	(%I)
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.296 ±0.04	40.20	0.292±0.10	41.01	1.045±0.16	58.79	0.2356±0.11	52.40
20	0.260±0.03	47.47	0.230±0.11	53.53	0.570±0.05	77.52	0.1394±0.16	71.84
40	0.235±0.01	52.52	0.221±0.07	55.35	0.430±0.12	83.04	0.0749±0.12	84.87
80	0.210±0.07	57.57	0.205±0.04	58.58	0.351±0.22	86.16	0.0564±0.02	88.60
100	0.201±0.02	59.19	0.196±0.01	60.60	0.321±0.05	87.34	0.0391±0.01	92.10
IC_{50}	51.0 µg/mL		36.0 µg/mL		16.5 µg/mL		19.5 µg/mL	

Note: Values represent Mean ± SD, n = 3, Blank absorbance (517 nm) = 0.495 for aqueous, ethanol extract and ascorbic acid; 2.536 for nanoparticles (517 nm).

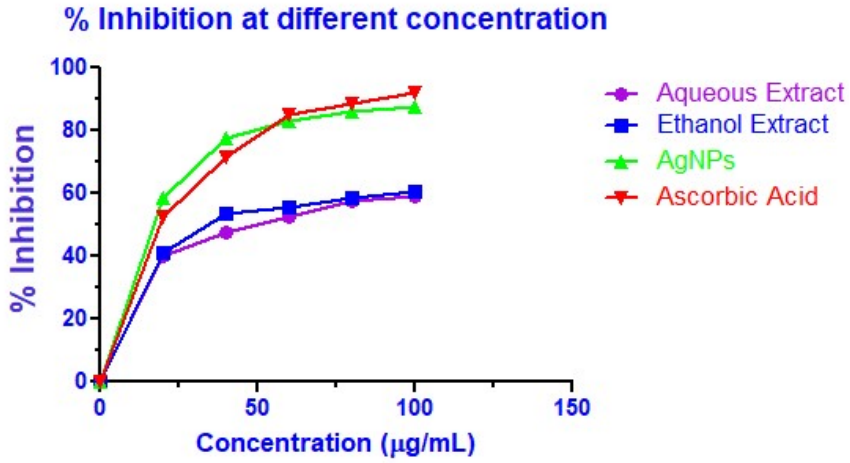

Figure 2: Percentage Inhibition of DPPH Radicals by the Extracts and Nano-Particles of *S. verticillata*

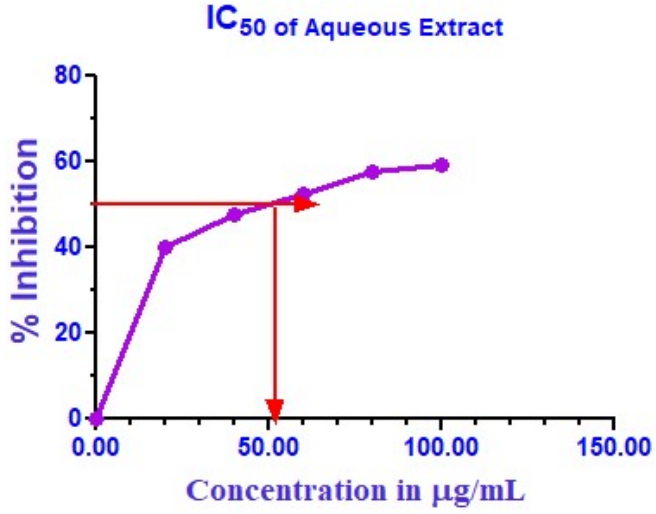

Figure 3: IC_{50} of the Aqueous Extract of *S. verticillata*

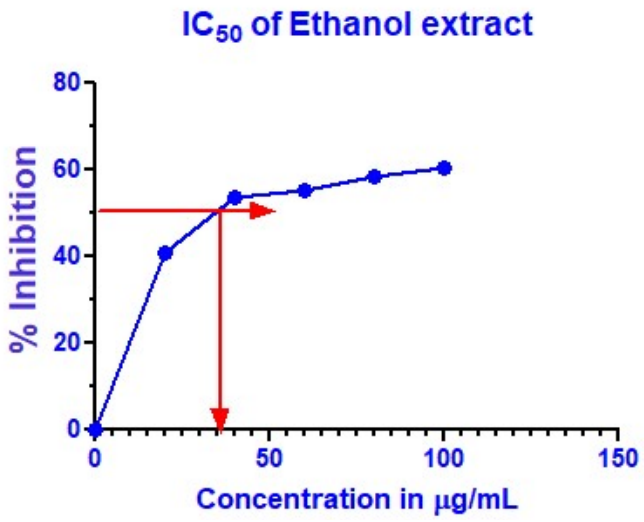

Figure 4: IC_{50} of the Ethanol Extract of *S. verticillata*

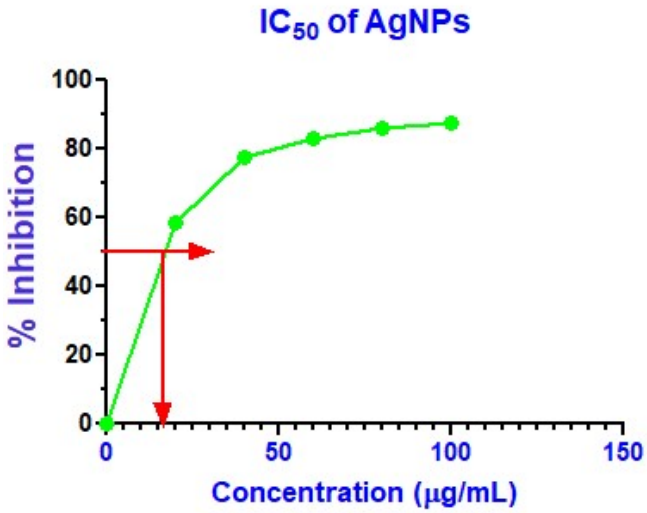

Figure 5: IC_{50} of the Silver Nano-Particles of the Extract of *S. verticillata*

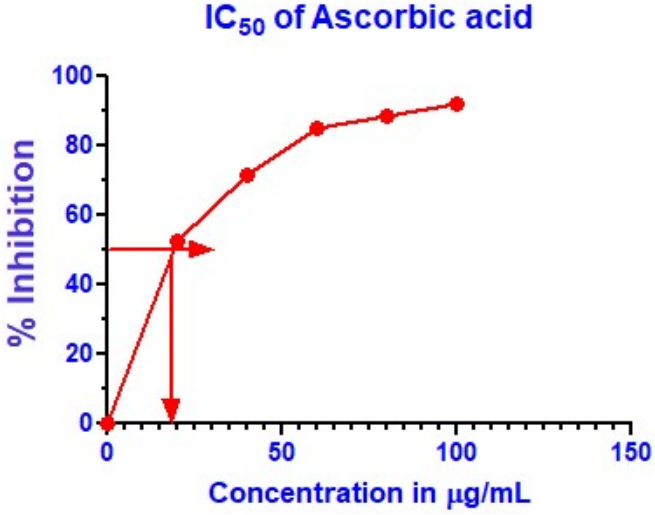

Figure 6: IC_{50} of Ascorbic Acid

3.4. Significance of the Research Work

This research has shown that formulation of nano-particles of the extracts of medicinal plants will improve their pharmacological activities. This is useful in drug manufacturing and drug delivery systems.

4. Conclusion

This research showed that both the crude ethanol and aqueous extract of *S. verticillata* as well as its silver nanoparticles possessed anti-malarial and antioxidant activities. This research also showed that the silver nano-particles of the extracts possessed higher anti-malarial and antioxidant activity than the crude aqueous extract of the plant and potentially more promising source of good leads.

Conflicts of Interest

The authors declare no conflict of interest.

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