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Eucalyptus Oil Nanoemulsion: Evaluation of *In vitro* Activity against *Trypanosoma brucei*

Emmanuel Maduabuchi Uronnachi[1], Patience Sunday Ngbo[2], Somtochukwu Richard Ewuzie[1*], Chidalu Benedicta Ikeotuonye[1], Anthony Amaechi Attama[3]

[1]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.
[2]Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Kaduna State University, Kaduna State, Nigeria.
[3]Department of Pharmaceutics, University of Nigeria, Nsukka, Enugu State, Nigeria.

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ABSTRACT

Background: Natural products and their secondary metabolites are a good source of pharmacologically active constituents and have been widely developed into modern medicines. The research aims to use an essential oil from eucalyptus and then formulate a nanoemulsion. It would be characterized and evaluated for its *in vitro* anti-trypanosomal activity against *Trypanosoma brucei* (a parasite endemic in developing and most African countries).

Material and Methods: Several batches of the eucalyptus oil nanoemulsion were produced using Tween 80 and/or Span 80 as surfactants, in different proportions. The batches were characterized for *in vitro* anti-trypanosomal activity against *Trypanosoma brucei* using contaminated blood smears from Wistar rats. The nanoemulsions were also analyzed for particle size, polydispersity index, pH, and viscosity. The eucalyptus oil used for the formulation was also evaluated using gas chromatography-mass spectroscopy (GC-MS).

Results: The nanoemulsion possessed a concentration-dependent anti-trypanosomal activity and had a similar result to the positive control (commercially available standard). The eucalyptus oil analysis revealed the presence of aromandendrene, alloaromadendrene, methyl palmitate, 7-octadecenoic acid methyl ester, eucalyptol, and other organic compounds.

Conclusions: The eucalyptus oil nanoemulsion demonstrated strong trypanocidal efficacy against *Trypanosoma brucei* and was comparable to the positive control.

1. Introduction

Human African trypanosomiasis (HAT) is a parasite-induced disease, also known as sleeping sickness; and can lead to death unless it is properly treated [1]. *Trypanosoma brucei (T. bb)* is the microorganism mostly implicated in HAT and Animal African trypanosomiasis (AAT) disease [2]. Although HAT caused devastating epidemics in the 20th century, the number of reported cases has decreased to a historically low level in recent years due to consistent and well-coordinated efforts [3].

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The World Health Organization (WHO) aims to completely eradicate the disease, with less than 3000 cases reported worldwide in 2015. The disease is still endemic in several regions of sub-Saharan Africa where it significantly burdens rural people despite these recent advancements [1]. HAT should also be taken into account in the differential diagnosis for visitors, tourists, migrants, and expatriates who have been to or resided in endemic areas because patients have also been documented from non-endemic nations [1]. Financial resources have been mobilized for more fundamental and practical research due to the recent outbreaks and have increased the opportunities for translational science for sustainable HAT control. This has produced new insights into the physiology, genetics, and genomes of parasites and tsetse vectors [4]. There is no vaccine available for the disease thus disease control depends solely on vector control, case detection, and treatment. The medications already on the market are also suboptimal thus the ongoing research and clinical trials should offer hope for safer and more straightforward therapies [1]. This increases the need for supplementary active agents like essential oils from plants with proven pharmacological actions.

Essential oils (EOs) are also known as essences or volatile oils. They are complex natural combinations of volatile and odorous compounds found in 'fragrant' plants [5]. They possess a wide range of pharmacological effects including anti-parasitic effects. A study evaluated the essential oils from eighteen Myrtaceae species for *in vitro* activity against *Trypanosoma cruzi* (*T. cruzi)* and demonstrated their essential oils as valuable sources of bioactive compounds against *T. cruzi* [6]. EOs antibacterial, antiviral, and anti-inflammatory qualities suited them early on for both the causative and symptomatic treatment of a variety of ailments, as well as prevention. EOs constitute a classic example of a multicomponent mixture with up to several hundreds of different compounds, which in a complicated composition make up the attribute of a specific complete EO [7]. Eucalyptus oil is a typical example of an essential oil.

Eucalyptus oil is extracted from the leaf of the eucalyptus tree which is a native of Australia and is known to possess many medicinal values [8]. Several other studies have been published on the medical benefits of eucalyptus oil. Essential oils like eucalyptus oil when used without dilution, can irritate mucosa and would not be palatable for oral use. To overcome this side effect, nanoemulsions can be formulated. Eucalyptus oil nanoemulsion formulation should involve the incorporation of lower amounts of the oil in a dosage form that also contains an aqueous layer that would prevent these side effects while improving the bioavailability of the active oil constituent. It has been confirmed that eucalyptus oil-based nanoemulsions can possess good physical stability over many days or weeks and can be used as key drug carriers in the pharmaceutical, food, and cosmetic industries [9]. Another study also revealed the greater potential of eucalyptus oil nanoemulsion against synthetic pesticides while concluding that an optimized form of it can be achieved with a 6% EO concentration [10]. This research aims to formulate a eucalyptus oil nanoemulsion and evaluate its *in vitro* anti-trypanosomal activity against the parasite – *Trypanosoma brucei* (*T. bb*). There is also no documented literature work as at this date focusing only on HAT implicated by *T. bb and* using eucalyptus oil nanoemulsion.

2. Material and methods

2.1. Materials

Eucalyptus oil (Bells, England), Benzoic acid (Griffin and George, India), RPMI 1640 medium (Sigma-Aldrich, Germany), diminazine diaceturate (Nozomil, Holland), Span 80 (Lobechem, India), and Tween 80 (Lobechem, India). Other reagents and materials used were of analytical grade.

2.2. Methods

2.2.1. Test Organism Source

The *Trypanosoma brucei* parasite was utilized and obtained from stables kept at the parasitology laboratory in the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

2.2.2. Nanoemulsion preparation

Eucalyptus oil nanoemulsion batches were developed using a low-energy emulsification method as described but slightly modified [11]. For each batch, 0.1 g of benzoic acid was weighed with an analytical weighing

scale and dissolved in a beaker in 90 ml of distilled water. The resulting solution of benzoic acid was moved into a burette that was fastened to a retort stand. Additionally, a magnetic stirrer (Malvern, England, 1043), was fixed underneath it. The formula for the nanoemulsion batches **[Table 1]** shows that the required varying volumes of eucalyptus oil, the tween 80 and span 80 were measured and transferred into a beaker placed under the burette containing the earlier produced benzoic solution. The magnetic stirrer was used to agitate the beaker's contents for 30 minutes at 800 rpm. Each batch was then filled to 100 ml with the benzoic acid solution, which was added drop by drop at a rate of 3.5 ml/min while being stirred with a magnetic stirrer. Following the full addition of the necessary benzoic acid solution, the magnetic stirrer was used to mix for an additional 60 minutes at 800 rpm [11]. The prepared nanoemulsions were then stored at room temperature (30 ± 2 °C).

Table 1: Formula for the batches of the eucalyptus oil nanoemulsion

Excipients	Batches					
	1	**2**	**3**	**4**	**5**	**6**
Eucalyptus Oil (ml)	5	10	5	10	5	10
Tween 80 (ml)	5	5	10	10	7	7
Span 80 (ml)	0	0	0	0	3	3
Benzoic acid (g)	0.1	0.1	0.1	0.1	0.1	0.1
Water (ml)	to 100	to 100	to 100	to 100	to 100	to 100

2.2.3. PH determination

The pH of all the batches of the formulated eucalyptus oil nanoemulsions was determined with a slight modification of a described method [13], using a pH meter (Hanna instrument, Romania) and measurements were taken in triplicates at 25 °C at intervals of 1, 7, 30, and 90 days. Calibration was done using pH 4, 7, and 10 standards before each day's use.

2.2.4. Viscosity measurement

Using a rotary viscometer (NDJ-5S, China), the viscosity of each batch of the prepared eucalyptus oil nanoemulsions was measured using a described method [14]. Spindle number one was utilized, and its speed was set to 100 rpm. Measurements were made in triplicate at 1, 7, 30, and 90-day intervals for each of the six batches of prepared eucalyptus oil nanoemulsion samples.

2.2.5. Evaluation of the prepared eucalyptus oil nanoemulsion

Analysis of droplet size and the polydispersity index (PDI)

Based on an initial physical stability result after a day post-production of all the batches; batches 3, 4, and 6 were selected for the droplet size and PDI analysis (as batches 1,2 and 5 didn't form properly). Zeta size analyzer (Zetasizer NS Malvern 1162 England) was used to measure the droplet size of the prepared nanoemulsion [12].

Analysis of eucalyptus oil using gas chromatography-mass spectroscopy (GC–MS)

Gas chromatography coupled with mass spectrometry (GC-MS) was used to identify the secondary metabolite components of the eucalyptus essential oil. Equipped with a secondary electron multiplier, the JOEL GC model (Agilent Technologies 6890N, USA) was employed in the analysis [15].

2.2.6. Determination of parasitemia

The study followed the recommendations made in the "National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978)". The protocols for the care and use of animals in the facility of the National Institute for Trypanosomiasis Research, Kaduna, Kaduna State, Nigeria were also followed.

Three adult Wistar rats were used and were infected with *Trypanosoma brucei* with the aid of a syringe and needle through the subcutaneous route.

Using a wet blood film, parasitemia was tracked in blood drawn from the tail of an adult Wister rat that had been infected and had been previously sterilized with methylated spirit. Here, a coverslip (22 mm × 22 mm) was placed over a drop of blood (about 2 µL) on a sanitized microscope slide. Phase-contrast microscopy was used to examine the blood under a microscope at a total magnification of ×400. About 30 fields were looked at. The migration of trypanosomes among red blood cells allowed for their identification and the microscopic count of parasites per field in pure blood was conducted [16].

2.2.7. *In vitro* test of antitrypanosomal activity

The test for *in vitro* antitrypanosomal activity was performed according to a slightly modified method [16] . Ninety-six-well microtitre plates were used to monitor *in vitro* activity (in duplicates). The prepared eucalyptus oil nanoemulsion was tested *in vitro* against T. bb.

Batch 3 eucalyptus oil nanoemulsion (earlier used for droplet size test and confirmed as a nanoemulsion because of its PDI value) and the eucalyptus oil sample were used. Through the use of a two-fold serial dilution procedure, the nanoemulsion and the eucalyptus oil were reconstituted from 100% to 1.56% (v/v). Each well of the titer plate was then filled with 30 uL of blood suspension in a parasite medium. At the start of the assay, there were roughly 20–25 trypanosomes per microscopic field following the addition of the blood.

The negative control consisted of control wells with just media and trypanosomes; the positive control consisted of media and Diminaveto® (a commercial trypanocidal medication that contained 1.05 g diminazene diaceturate, 1.31 g antipyrine, and 1 mg vitamin B12); and finally wells containing the media and eucalyptus oil alone were also included. Following an hour of incubation, the blood-eucalyptus oil nanoemulsion mixture was formed into wet smears, which were then seen under a light microscope.

The motility rate of trypanosomes was measured in comparison to those in the control well.

As a gauge of anti-trypanosomal activity, the emulsion-treated blood's ability to stop or reduce parasite movement was compared to that of the parasite-loaded control blood that did not include the eucalyptus oil nanoemulsion.

3. Results

3.1. Droplet size analysis and polydispersity index (PDI) of formulated eucalyptus oil nanoemulsion

The results obtained showed that batches 3 and 4 were monodispersed with a PDI of 0.29±0.00 and 0.44±0.02 respectively while batch 6 was polydispersed and non-homogenous with a PDI of 0.76±0.03. **[Table 2]**. The particle size plots for batches 3, 4, and 6 are also shown below **[Figures 1 - 3]**.

Table 2: Droplet size analysis and polydispersity index of batches of formulated eucalyptus oil nanoemulsion

Batch Number	Z-average (d.nm)	PDI
3	201.10±0.00	0.29±0.00
4	199.10±0.00	0.44±0.02
6	63.13±0.00	0.76±0.03

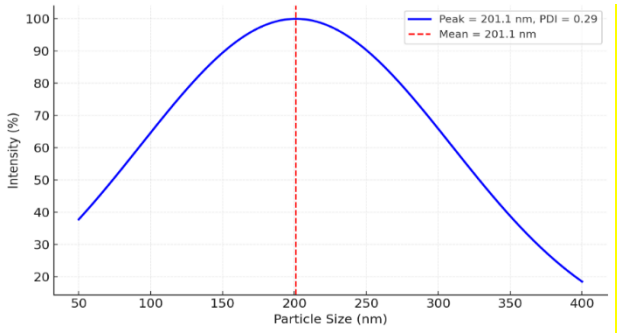

Figure 1: Particle size distribution plot for batch 3 nanoemulsion used for *in vitro* analysis

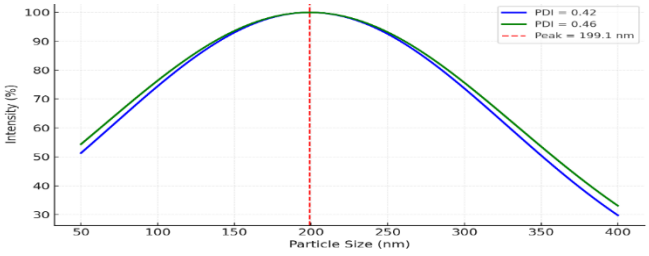

Figure 2: Particle size distribution plot for batch 4 nanoemulsion

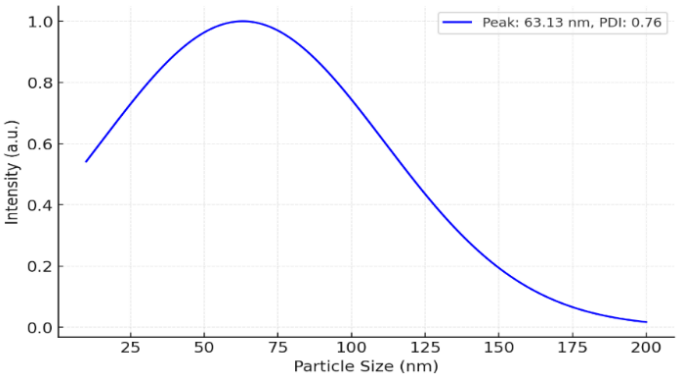

Figure 3: Particle size distribution plot for batch 6 emulsion

3.2. Determination of pH of formulated eucalyptus oil nanoemulsion

Batches 1 & 2 gave ranges between 3.20±0.00 to 4.50±0.00 while batches 5 & 6 gave ranges between 4.13±0.05 to 5.11±0.00 **[Table 3].**

3.3. Determination of viscosity of formulated eucalyptus oil nanoemulsion

The eucalyptus oil nanoemulsions generally showed low viscosity values. Batches 1 and 2 with a single surfactant (tween 80) exhibited lower viscosity compared to batches 5 and 6 with surfactant combinations (tween 80 and span 80) **[Table 4]**.

Table 3: PH values of batches of formulated eucalyptus oil emulsions

Batch	Days			
	1	7	30	90
1	4.50±0.00	4.20±0.00	3.80±0.00	3.20±0.00
2	4.50±0.00	4.17±0.06	3.90±0.00	3.37±0.05
3	4.80±0.00	4.71±0.06	4.60±0.00	3.38±0.12
4	4.63±0.06	4.63±0.05	4.40±0.00	3.37±0.05
5	5.00±0.00	4.80±0.00	4.50±0.00	4.13±0.05
6	5.11±0.00	5.00±0.00	4.40±0.00	4.03±0.05

Table 4: Viscosity Result (in N/m^2) of batches of formulated eucalyptus oil nanoemulsion

Batch	Days			
	1	7	30	90
1	15.80±0.01	15.51±0.01	14.51±0.01	13.50±0.01
2	17.04±0.01	17.01±0.01	16.41±0.01	14.51±0.01
3	18.14±0.01	18.01±0.02	17.03±0.01	15.02±0.01
4	18.15±0.01	18.11±0.01	17.08±0.01	15.12±0.01
5	19.16±0.01	19.12±0.01	18.12±0.01	16.12±0.15
6	20.04±0.01	20.01±0.09	19.00±0.01	17.02±0.02

3.4. Gas chromatography-mass spectroscopy (GCMS) analysis of eucalyptus oil

GCMS of eucalyptus oil showed different peaks with compound compositions. The most significant ones above 70 percent composition are enumerated **[Table 5]**. Notably, aromandendrene, alloaromadendrene, methyl palmitate, and 7-octadecenoic acid methyl ester had up to 99% abundance while, 1, 8-cineole, and alpha-pinene had 98% abundance, and alloocimene had 97% abundance; amongst others.

3.5. Anti-trypanosomal *in vitro* activity of formulated eucalyptus oil nanoemulsion

The trypanocidal activity witnessed was dependent on nanoemulsion concentration; with higher concentrations yielding higher cidal effects **[Table 6, Figure 4]**.

Table 5: Major compounds found in eucalyptus oil

Peak Number	RT	Constituents	Qual (%)
1	6.26	Alpha pinene	98
2	9.52	Eucalyptol(1,8-cineole)	98
3	13.02	Alloocimene	97
4	14.51	Alpha-Terpineol	96
5	18.31	Camphene	90
6	20.65	Aromandendrene	99
7	21.18	Alloaromadendrene	99
8	22.03	Alpha-Gurjunene	94
9	27.51	Myristic acid	72
10	30.61	Methyl palmitate	99
11	32.13	7-Octadecenoic acid	99

Table 6: Antitrypanosomal *in vitro* assay result of eucalyptus oil nanoemulsion, eucalyptus oil, and the controls

Concentration (%v/v)	EON	EO	PC	NC
50	++	++	++	-
25	++	++	++	-
12.5	++	++	++	-
6.25	++	++	++	-
3.125	++	++	++	-
1.56	+	+	++	-

Dash (-) means inactive; ++: total clearance; +: active but no total clearance; EO: Eucalyptus oil; PC: Positive control (1.05 g diminazene diaceturate + 1.31 g antipyrine + 1 mg vitamin B_{12} and Media); NC: Negative control (Media and rypanosome); EON: Eucalyptus oil nanoemulsion

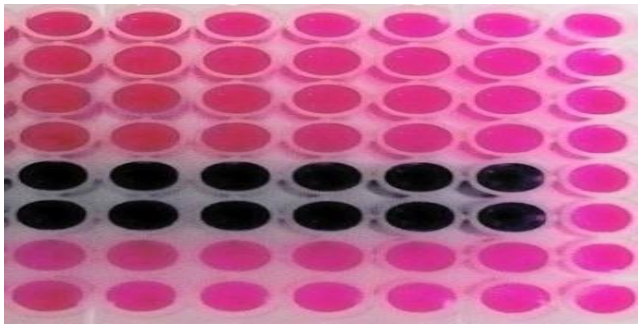

Figure 4: Microtitre plates showing inactivity/parasite motility (dark colour) of negative control; activity (red colour) for the eucalyptus oil nanoemulsion and eucalyptus oil at 1.56% concentration only; and total clearance (pink colour).

3.6 Statistical Analysis

Data analysis was done using stochastic statistics as well as chi-square where necessary. Data sets are mean and standard deviation. **S**tatistical significance of $P< 0.05$.

4. Discussion

The PDI has been described as the degree of consistency in droplet size within a formulation [17]. It is also reported that particle sizes are inversely proportional to the PDIs as higher PDI shows smaller droplet sizes [18]; thus, from the results, the monodispersed batch 3 showed higher droplet sizes. It has also been suggested that low PDI values indicate a nano-sized emulsion system [13,19], thus batch 3 is a nanoemulsion and only the batch 3 nanoemulsion (with the least PDI) was further used for the *in vitro* anti-trypanosomal study. The stability and physicochemical characteristics of emulsions can be impacted by emulsifying parameters, including concentration, homogenization parameters, and emulsifier type and concentration [18]. A decrease in droplet size was observed in batch 6, possibly due to the surfactant combination (tween 80 and span 80) used. Drug absorption is believed to be influenced by droplet size because a smaller droplet size results in a higher interfacial surface area available for drug absorption [20]. Batches 3 and 4 in which only tween 80 was used had higher droplet sizes and lower PDIs. This recommends the need for further pharmacokinetic study with the chosen batch 3 nanoemulsion drug.

For the batch 3 nanoemulsion used, there was only a slight reduction in pH value from 4.80±0.00 to 4.30±0.00 over 30 days. Properties of emulsions like the pH can be easily destabilized under various conditions during preparation and storage and can compromise the quality of the product as reported [21]. From the results obtained, it can be deduced that all the batches had a good result though batches 5 and 6 which had surfactant combinations of tween 80 and span 80 had a lesser variation in the pH compared to batches 1, 2, 3, and 4 which had single surfactant (tween 80). PH measurements are also employed to determine the occurrence of phase inversion processes, especially with multiphase systems like emulsions.

Viscosity assessment is an important parameter for the physicochemical characterization of nanoemulsions. It has been observed that less concentrated emulsion formulations generally exhibit lower viscosity than highly concentrated ones because the droplets are evenly distributed and there is little flocculation [22]. This suggests that the rate of flocculation in this research is notably low and that individual emulsion droplets are evenly distributed, particularly for batches 1, 2, and 3. Viscosities also reveal the nature of emulsions i.e. whether it is an oil-in-water (o/w) or water-in-oil (w/o) emulsion. High-viscosity systems typically suggest a water-in-oil (w/o) type system, while low-viscosity systems invariably tend to imply an oil-in-water (o/w) type system as suggested in some literature [23,24]. It can thus be inferred that the systems produced were o/w nanoemulsions.

GC-MS results revealed eleven major constituents of the eucalyptus oil as shown in Table 5. 1,8-cineole (also known as eucalyptol) is mostly extracted from the essential oils of plants and is the main constituent reportedly implicated in its pharmacological actions. In a study, it showed extensive pharmacological properties including anti-inflammatory and anti-oxidation mainly via the regulation of some markers. It was used to treat respiratory and cardiovascular diseases [25]. Another work also revealed that elettaria cardamomum L. essential oil also has 1,8-cineole as its main compound and exhibited potent protoscolicidal activity both *in vitro* and *ex vivo* and it was suggested that more research be done on its effectiveness and toxicity in clinical situations as well as on animal models [26]. Another study also confirmed the anti-microbial properties and its use for arthritis, respiratory ailments, and skin disorders [26]. The other constituents of the eucalyptus in 99% abundance - alpha-pinene and alloaromandendrene have been reported to have antimicrobial qualities [27,28]; methyl palmitate in 99% abundance has also been described more widely in the literature as a naturally occurring cardioprotective and a nematicidal agent and for its anti-apoptotic role [29-31]. Despite all these reports, the anti-parasitic properties of eucalyptus or its constituents were rarely revealed in the available literature.

This work has confirmed its anti-trypanosomal or anti-parasitic activity. These studies have all shown that eucalyptus oil or at least its components have multiple pharmacological actions and modes of action and the

quick concentration-dependent decrease in parasite number in the *in vitro* test results with this research indicates that the eucalyptus oil just as the eucalyptus oil nanoemulsion is killing the parasites by an unknown mechanism. It can suffice to say that the activity of the nanoemulsion is due to one or more of its eucalyptus oil constituents and thus calls for more research and investigation into the exact mode(s) of action for the trypanocidal effect seen. Parasite motility is a key factor for pathogenicity and as such the motility in the nanoemulsion-treated blood was measured and compared to parasite-loaded control blood that did not include either the oil or the nanoemulsion. This was taken as a measure of anti-trypanosomal activity [32]. The eucalyptus oil nanoemulsion exhibited total clearance (no motility) from the 50 %v/v to the 3.125%v/v concentrations and was very similar in result to the eucalyptus oil alone. The activities decreased with a decrease in concentration. The negative control with just the media and the trypanosomes (without the eucalyptus oil) exhibited no *in vitro* activity during the examination while the positive control exhibited total clearance of the organism with all the concentrations used. This research showed a trypanocidal activity as a complete cessation of motility with the parasites is taken as a complete trypanocidal effect [32].

The result from this study has also shown that the formulated nanoemulsion is comparable to the pure oil based on *in vitro* capabilities against *T. bb*. They can also be preferable to the positive control media used being that they are natural derivatives that can also offer comparably lesser adverse or side effects when they are considered to be used in humans and animals. However, more research is still needed to guarantee its safety and bioavailability as it comes in contact with gastric and intestinal fluid during absorption.

5. Conclusion

The eucalyptus oil nanoemulsion demonstrated strong trypanocidal efficacy against *Trypanosoma brucei* and was comparable to the eucalyptus oil and the positive control. The formulation had good physical properties and exhibited its anti-parasitic effect through an unknown mechanism but possibly due to one or more of its constituents.

More studies on the mechanism of action, safety, and pharmacokinetics of the eucalyptus oil nanoemulsion are recommended especially if it is to be used in animals or humans.

Declarations

Funding
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Ethical Approval
No ethical approval was required because none of the animals were killed and they were further examined by the institute after the research duration.

Competing Interests
The authors declare no conflict of interest (financial or personal ties, rivalries, religious convictions, etc) with this work.

Data Availability
Any data needed from the research will be made available at any time to the readers, upon request.

Authors' Contribution
Emmanuel Uronnachi, Somtochukwu Ewuzie – Conceptualization, Data Curation, Investigation, and Methodology; **Patience Ngbo** – Data Curation, Writing Original Draft, Methodology, and Validation; **Chidalu Ikeotuonye** – Investigation and Methodology; **Anthony Attama** – Conceptualization, Project Administration, Supervision, and Validation.
All the listed authors have also approved the final article sent for publication.

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