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Formulation and *in vitro* trypanocidal evaluation of garlic oil nanoemulsions

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ABSTRACT: Human African Trypanosomiasis (HAT) is a neglected tropical disease with complex clinical manifestations and is difficult to treat. Most treatment options currently available are old, expensive, not too effective, and usually associated with adverse drug reactions and resistance. To combat this challenge, garlic oil nanoemulsions were formulated and evaluated in this study. Six batches of the garlic oil nanoemulsions were formulated using the low-energy method. The formulations were characterized using droplet size analysis, pH, and viscosity determination. Gas chromatography of the pure garlic oil used was carried out. *In-vitro* antitrypanosomal activity test was carried out on three selected batches (3, 4 and 6) of the formulations using *Trypanosome brucei brucei* infected Wistar rats. The size distribution of the nanoemulsion droplets was unimodal with batches 2, 4 and 6 showing a droplet size of 154.6, 145.5 and 241.6 nm respectively and polydispersity indices (PDI) of 0.149, 0.195 and 0.434 respectively. There were slight alterations in the pH and viscosity of the six batches after the 3-month observation period. Gas chromatography of pure garlic oil showed major sulfur compounds which include: Sufralem, disulfide, allicin, and trisulfide. Anti-trypanosomal *in vitro* assay showed positive activity for all batches tested with batch 6 having a similar activity with the positive control at all the concentration ranges used. The study shows that nanoemulsion is a viable dosage form in delivering garlic oil for the treatment of trypanosomiasis at low concentrations of the oil.

KEYWORDS: Trypanosomiasis; Garlic oil; Nanoemulsion; Gas chromatography; Poly dispersity index.

1. INTRODUCTION

Human African trypanosomiasis (HAT) also referred to as sleeping sickness is a disease caused by the protozoan parasite *Trypanosoma brucei* spp and transmitted by the vector tsetse fly (Genus Glossina) [1]. HAT is a neglected tropical disease with a complex clinical presentation that occurs in low-income populations in Africa [2, 3]. Two forms of HAT exist and they are caused by different subspecies. *Trypanosoma brucei gambiense* is the prevalent species in Central and West Africa and this gives rise to a chronic form of the disease, which takes about 36 months to progress from the haemolymphatic stage (First stage) to death. *Trypanosoma brucei rhodesiense* is the prevalent species in Eastern and Southern Africa that gives rise to an acute form of the disease, which takes only weeks to progress from infection point to death [4, 5]. The use of vaccines is not an effective measure to deal with HAT because they possess the ability to change their surface glycoproteins [6] and available medications that are being used in the treatment of trypanosomiasis are no longer ideal because they are toxic, lack efficiency, associated with severe adverse reactions and are expensive [7, 8]. These drawbacks have facilitated the search for newer and more effective alternatives.

In this study garlic oil (*Alluim sativum*) was used. It belongs to Alliaceae family and has several medicinal properties such as lowering blood cholesterol, anti-inflammatory and anticancer activity and antibacterial, antifungal, antiviral and antioxidant properties [9, 10]. It was formulated into a nanoemulsion, which is a

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thermodynamically and kinetically stable dispersion of two immiscible liquids (oil and water) and is stabilized using an appropriate surfactant to form a single-phase [11]. The utilization of garlic oil on its own is limited due to its high volatility, strong odour, water insolubility, and low physiological stability [10]. The formulations were characterized using pH, viscosity, globule size analysis, and polydispersity index. In vitro evaluation of selected formulations was carried out on Trypanosoma brucei brucei infected Wister rats.

2. RESULTS AND DISCUSSIONS

1.1 Characterization of formulated garlic oil nanoemulsion

1.1.1 Droplet size and poly dispersity index

The results are presented in Table 1. Three batches of the formulated nanoemulsion (batch 2, 4 and 6) were analyzed for droplet size and polydispersity index.

Table 1. Droplet size and poly dispersity index

Batch no	Zeta Size (d.nm)	Poly Dispersity Index
2	154.6	0.149
4	145.5	0.195
6	241.6	0.434

The nature and concentration of surfactant determine the droplet size. Upon emulsification, the emulsion gave nanosized droplets, which play a pivotal role in effective drug release, *in vivo* absorption, and stability [12]. Droplet size is thought to affect drug absorption. The smaller the droplet size, the larger the interfacial surface area available for drug absorption. Besides, larger sizes may be predisposed to early drug precipitation before absorption. Polydispersity is the ratio of standard deviation to the mean droplet size and is inversely proportional to droplet size uniformity; the higher the polydispersity, the lower the uniformity of droplet size [13–15]. The polydispersity index describes the degree of uniformity in droplet size within a formulation. An increased droplet size was observed in batches where surfactant concentration was high; batch 6, prepared using both tween 80 (7 %) and span 80 (3 %) and batch 4 in which only tween 80 (10 %) was used, and in batch 2 where tween 80 (5 %) was used. By increasing surfactant concentration, further molecules of surfactant formed micelles in a continuous phase on particle surface resulting in increasing aggregation and increase in particle size [16]. On combination of two surfactants that is; tween 80 and span 80 in batch six, there was an observed increase in both the droplet size (241.6 nm) and polydispersity index (0.434) compared with batch 2 (droplet size; 154 nm and PDI of 0.149) and 4(droplet size; 145.5 nm and PDI of 0.195) in which only one surfactant was used. Batch 2 gave a better result as a smaller droplet size will allow for a larger surface absorption.

1.1.2. pH determination

The result for pH determination of batches of formulated garlic oil nanoemulsion is presented in Table 2.

Table 2. pH determination of garlic oil nanoemulsion

Batch	**Day 1 (X ± SD)**	**Day 2 (X ± SD)**	**Day 30 (X ± SD)**	**Day 90 (X ± SD)**
1	4.20 ± 0.00	4.20 ± 0.00	3.50 ± 0.00	3.00 ± 0.00
2	4.20 ± 0.00	4.20 ± 0.00	3.50 ± 0.00	3.13 ± 0.06
3	4.33 ± 0.06	4.40 ± 0.00	3.70 ± 0.00	3.20 ± 0.00
4	4.60 ± 0.00	4.63 ± 0.06	3.90 ± 0.00	3.40 ± 0.00
5	4.83 ± 0.06	4.80 ± 0.00	4.13 ± 0.06	3.87 ± 0.06
6	4.83 ± 0.06	4.80 ± 0.00	4.07 ± 0.06	3.77 ± 0.06

Key: X = mean; SD = standard deviation

Monitoring the pH value is important for determining the emulsion's stability [17,18]. From the result obtained for pH determination for all formulated batches it was observed that for the initial pH on day 1 of the garlic oil nanoemulsion ranges from 4.20-4.83 (± 0.00 - 0.06) were observed while after three months assessment, ranges of 3.00 - 3.77 (± 0.00 - 0.06) were observed. This result shows a statistical variation in the pH of formulated garlic oil nanoemulsion over storage. The decrease in pH could be due to temperature fluctuations which could cause a chemical reaction and affects the stability of the formulated garlic oil nanoemulsion as temperature is a parameter that affects the pH stability of the nanoemulsion [17,18].

1.1.3 Viscosity determination

The result for the viscosity determination of batches of formulated garlic oil nanoemulsion is presented in Table 3.

Table 3. Viscosity determination of the formulated garlic oil nanoemulsion

Batch	Day 1 X ± SD (N/m^2)	Day 7 X ± SD (N/m^2)	Day 30 X ± SD (N/m^2)	Day 90 X ± SD (N/m^2)
1	16.03 ± 0.01	16.01 ± 0.00	15.01 ± 0.01	13.01 ± 0.01
2	18.03 ± 0.01	18.01 ± 0.01	17.01 ± 0.01	15.01 ± 0.01
3	19.13 ± 0.01	19.01 ± 0.01	18.02 ± 0.01	16.02 ± 0.01
4	21.05 ± 0.06	21.01 ± 0.01	20.02 ± 0.02	18.01 ± 0.01
5	23.01 ± 0.01	23.00 ± 0.01	21.35 ± 1.16	20.01 ± 0.01
6	24.02 ± 0.01	24.01 ± 0.01	23.01 ± 0.01	21.03 ± 0.01

Key: X-mean; SD-standard deviation

Viscosity is an important criterion for nanoemulsions as it measures their physical stability[19]. From the result obtained for all formulated batches, it was observed that the initial viscosity on day one of the garlic oil nanoemulsion ranged from 16.03-24.02 N/m^2 and after three months of assessment was observed in the range of 13.01-21.03 N/m^2. The viscosity of the nanoemulsion slightly decreased during the three months period. This slight decrease may be due to temperature fluctuations as the period progressed, since the preparations were stored at room temperature.

1.2 Gas chromatography

The major components obtained from the gas chromatography of the garlic oil are presented in Table 4.

Table 4. Major compounds found in garlic oil obtained from gas chromatography

Serial No	Peak number	Retention Time	Compound ID	Compound Common Name	Abundance (%)
1	1	7.03	3H-1,2-dithiole	Sufralem	95
2	4	11.14	Diallydisulfide	Garlicin	72
3	10	12.59	4-methyl-1,2,3-trithiolane	Silane	90
4	14	13.9	3-vinyl-1,2-dithiacyclohex-4-ene	Disulfide	98
5	16	14.41	2-vinyl-4H-1,3-dithine	Allicin	96
6	19	17.69	Trisulfide, di-2 –propenyl	Allitin	97
7	36	23.55	Tetrasulfidedi-2-propenyl	Sulfane	90
8	38	24.62	6-methyl-4,5,8-trithia1,10-undecadiene	Trisulfide	92
9	41	26.62	1,2-dithiolane	Thiolane	81
10	47	29.41	1-ally-3-(2-(allythio)propyl)trisulfane	Allitridine	87

Gas chromatography- mass spectroscopy of garlic oil shows different peaks with the composition of compounds. The compounds found in high proportion include: 3H-1,2-dithiole (Sufralem) (95 %) ,3-vinyl-1,2-dithiacyclohex-4-ene(disulfide) (98 %), 2-vinyl-4H-1,3-dithine(allicin) (96 %) and trisulfide, di-2 –propenyl(allitin) (97 %). Previous reports show garlic oil to predominantly contain diallydisulfide, dially trisulfide, allypropyldisulfide, disulfide and diallypolysulfide [20, 21]. This shows that the garlic oil used is a pure garlic oil with similar compounds to some of the previously reported compounds.

Anti-trypanosomal *in vitro* assay

Results for antitrypanosomal in vitro assay of some batches of the formulated garlic oil nanoemulsion are presented in Table 5.

Table 5. Antitrypanosomal in vitro assay of garlic oil nanoemulsion

Concentration % v/v	Batch 3	Batch 4	Batch 6	GO	PC	NC
50	++	++	++	++	++	-
25	++	++	++	++	++	-
12.5	++	++	++	++	++	-
6.25	++	++	++	++	++	-
3.125	++	++	++	++	++	-
1.56	+	-	++	+	++	-

Key: (++) total clearance; (+) active but no total clearance; (-) inactive; GO-Garlic oil

PC-Positive control (Media and DIMINAVETO®); NC-Negative control (Media and trypanosomes); GON-Garlic oil nanoemulsion

Antitypanosomal *in vitro* assay of formulated garlic oil nanoemulsion was performed against *Trypanosoma brucei brucei.* Batch samples three, four and six plus pure garlic oil were analyzed for activity.

The choice of three of the batches was based on the stability of prepared nanoemulsions. The three batches chosen were selected to evaluate the effect of oil concentration (Batches 3 and 4) and choice of surfactant (batches 4 and 6) on the antitrypanosomal action of the nanoemulsions.

At different dilution concentrations (from 50 – 1.56 % v/v). A cessation or drop in parasite motility in test groups compared with that of parasite loaded blood without formulations (control group) was taken as a measure of trypanocidal activity [22]. The three batches tested showed antitrypanosomal activity at 50-3.125 %v/v concentration. At a concentration of 1.56 % v/v there was little or no activity, signifying that activity is dependent on the concentration of the garlic oil nanoemulsion. The activity of garlic oil nanoemulsion can be attributed to the presence of the following compounds: 3H-1,2-dithiole, diallydisulphide,4-methyl-1,2,3-trithiolane, trisulfide di-2-propenyl,1,2dithiolane, 1-allyl-3-(2-(allythio) propyl) trisulfane which correlates with the work of El-sayed et al., [20]. The antitrypanosomal *in vitro* activity of evaluated formulated batches of garlic oil nanoemulsion was observed to give similar results to that of the pure garlic oil and positive control (diminazene diaceturate (Diminaveto®)), with batch six showing a better activity as there was total clearance even at 1.56 % v/v concentration. The negative control (media) showed no activity against the organisms. The action of the garlic oil nanoemulsion involves the interaction of garlic oil compounds with the trypanosome causing conformational changes in the parasite membrane structure leading to a loss in membrane stability and disruption of key enzymes in the parasite glycolytic pathway [23].

3. CONCLUSION

Summarily, different batches of garlic oil nanoemulsions were formulated and characterized. The viscosity of the formulations was stable throughout the 90-day storage period. The formulations (batches 3, 4 and 6) showed antitrypanosomal activity against *Trypanosoma brucei brucei* with batch six giving the best antitrypanosomal activity. The activity of the nanoemulsions thus presents them as a viable dosage form for delivering garlic oil in the treatment of trypanosomiasis. They are advantageous as a lower dosage will be used in comparison with the raw oil, thereby leading to fewer adverse effects.

4. MATERIALS AND METHODS

4.1 Materials

Garlic oil (China), Span 80 (Lobechem, India), Tween 80 (Lobechem, India), Benzoic acid (Griflin and George, India), Adult Wister rat (NITR animal house), *Trypanosoma brucei brucei,* RPMI 1640 media (Sigma-Aldrich, Germany), Diminazine diaceturate (Nozomil; kepro B.V., Holland).

4.2 Nanoemulsion preparation

Nanoemulsion was developed using a low energy method by Sundararajan et al., [24] with slight modification. Using different component compositions of garlic oil, Tween 80, Span 80, and benzoic acid as a preservative. Benzoic acid (0.1 g) was weighed and dissolved in 90 ml of distilled water in a beaker. The distilled water was transferred into a burette attached to a retort stand with a magnetic stirrer fixed beneath it. A quantity of 5 ml garlic oil was measured using a measuring cylinder and transferred into a beaker and 5 ml of tween 80 was measured and transferred into the beaker containing garlic oil. A magnetic rod was inserted into the beaker and the beaker was placed on a magnetic stirrer (79-1, China). The content in the beaker was stirred at 800 rpm using the magnetic stirrer for 30 min. Then, water was added drop wise at a flow rate of 3.5 ml/min. The mixture was stirred at 800 rpm for 60 min [24]. A total of six batches were prepared according to the formula in Table 6. The prepared nanoemulsions were stored at room temperature (25 ± 2°C) and stability was assessed after 1, 7, 30, and 90 days post-preparation.

Table 6. Batch composition of nanoemulsion

	Batch					
Ingredients	**1**	**2**	**3**	**4**	**5**	**6**
Oil	5 ml	10 ml	5ml	10 ml	5ml	10 ml
Tween 80	5 ml	5 ml	10 ml	10 ml	7 ml	7 ml
Span 80	-	-	-	-	3 ml	3 ml
Benzoic acid	0.1 g	0.1 g	0.1 g	0.1g	0.1g	0.1 g
Water	to 100 ml	to 100 ml	to 100 ml	to 100 ml	to 100 ml	to 100 ml

4.3 Characterization of formulated garlic oil nanoemulsion

4.3.1 Droplet size analysis

The droplet size of the formulated nanoemulsion was determined using the Zeta Potential analyzer (Zetasizer ZS, Malvern, UK) according to the method described by Sundararajan et al., [24].

4.3.2 pH determination

The pH of formulated garlic oil nanoemulsions was determined using a pH meter (Hanna instrument, Romania) according to the method described by Bernardi et al., [25]. Measurement was carried out in triplicate and the average taken for each result. Determinations were carried out at intervals of 1, 7, 30, and 90 days.

4.3.3 Viscosity determination

The viscosity of nanoemulsions was determined using a viscometer (NDJ-5S rotary viscometer, China) according to the method described by Laxmi et al., [19]. Spindle number one was used, and the speed of the spindle was adjusted to 100 rpm. The viscosity was determined in all six formulated garlic oil nanoemulsion batch samples, measurement was carried out in triplicate for each sample and the average taken. The determinations were carried out at intervals of 1, 7, 30 and 90 days.

4.4 Gas chromatography analysis (GC analysis)

The identification of the secondary metabolite constituents of the garlic essential oils was performed by Gas-Chromatography according to the method described by Sundararajan et al., [24]. The JOEL GC model was used in the analysis equipped with secondary electron multiplier (Agilent Technologies 6890N Network GC system for gas chromatography).

4.5 Determination of parasitaemia

The protocols for the care and use of animals by the research institute were followed in concordance with the guide for the care and use of laboratory animals by the European union (EU directive 2010/63/EU). Two adult Wister rats obtained from the Nigerian Institute for Trypanosomiasis Research (NITR) Kaduna State were infected with the *Trypanosoma*

brucei brucei via the subcutaneous route. Parasitaemia was monitored in blood obtained from the tail of the infected adult Wister rats pre-sterilized with methylated spirit using the wet blood film. Here, the droplet of blood (about 2 µL) was placed on a clean microscope slide and covered with a coverslip (22 mm × 22 mm). The blood was examined microscopically at × 400 total magnification with phase-contrast microscopy (Olympus, USA). Approximately 30 fields were examined. Trypanosomes were recognized by their movement

among the red blood cells. The number of parasites was determined using the rapid matching of Herbert and Lumsden. The method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with RPMI 1640 media[(22].

4.6 In vitro antitrypanosomal activity

In vitro antitrypanosomal activity was performed according to the method described by Atawodi et al., [26] with slight modification. *In vitro* trypanocidal activity was performed in duplicates in 96 well micro titer plates (Flow laboratories Inc., McLean, Virginia 22101, USA). The garlic oil nanoemulsions were constituted in concentrations ranging from 100 to 1.56 %(v/v) in a twofold serial dilution using a glucose phosphate media. Afterwards, 30 uL of blood suspension in media was added to each well of the titre plate. The trypanosome counts after adding the blood were about 20-25 trypanosomes per microscopic field at the commencement of the assay. Control wells containing only media and trypanosomes (negative control); media and diminazene diaceturate (Diminaveto®)-a commercial trypanocidal drug (positive control); and another well containing media and garlic oil alone were also included. After an hour of incubation, wet smears of blood-garlic oil nanoemulsion mixture were prepared and examined under a light microscope and trypanosomes were observed for their rate of motility relative to those in the control well. Cessation or drop in motility of the parasites in extract-treated blood, compared to that of parasite-loaded control blood without the garlic oil nanoemulsion were taken as a measure of trypanocidal activity.

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