



## Formulation and Evaluation of Therapeutic Potential of Nanoemulsion of a Blend of Antimicrobial Oils

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### ARTICLE INFO

#### Article history:

Received 30 December 2017

Revised 02 February 2018

Accepted 05 February 2018

Published online 07 February 2018

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### ABSTRACT

This work is aimed at producing an effective self-emulsifying nanoformulation of a combination of 9 antimicrobial edible plant oils against bacterial and fungal infections. The oils are olive, eucalyptus, dill, castor, peppermint, garlic, ginger, sunflower and lemongrass oils. Oil Formulation (OF) was produced by mixing a simple Oil Blend (OB) of the oils with polysorbate 80 at the ratio of 1:4. Particle size distribution, polydispersity index and zeta potential of OF and Nanoemulsion (NE) were determined. The antimicrobial properties of OB and OF were determined using selected bacteria and fungi. The most sensitive bacteria and fungi were then selected for *in vivo* assay of OB and OF using albino Wistar rats (n = 5). Oral treatment began 4 days post-infection and lasted 5 days. Ciprofloxacin and Ketoconazole were used as positive controls. Safety screenings (hematological, liver function tests (LFT's) and histological) were then conducted. The results showed that the optimal Oil/Polysorbate 80 ratio was 1:4. NE had mean particle size (151.12 ± 6.21) nm, polydispersity index (0.12) and zeta potential (-13.70 ± 4.40) mV, which after 90 days became (202.50 ± 4.11) nm, 0.09, and (35.00 ± 6.15) mV respectively. OF had mean particle size (158.00 ± 3.08) nm, polydispersity index (0.07) and zeta potential (-15.50 ± 3.01) mV, which after 90 days became (162.90 ± 6.01) nm, 0.08, and (-19.00 ± 0.00) mV respectively. Nanoemulsion formulation of the oil blend significantly (p<0.05) improved antimicrobial activity in infections of *Staphylococcus aureus* and *Aspergillus niger* and it was not toxic to the rats.

**Keywords:** Nanoemulsion, Formulation, Antimicrobial oils, Therapeutic potential, Safety.

### Introduction

There is a growing need to combat antibiotic resistance, or at least enhance antimicrobial activity against resistant organisms through formulation.<sup>1</sup> Possible solutions may lie in the use of herbs or their products.<sup>2</sup> There have been reports of synergism among herbal products<sup>3</sup> and some natural oils derived from plants possess antimicrobial properties. The oils of *Mentha piperita* (peppermint),<sup>4</sup> *Cymbopogon citratus* (lemon grass)<sup>5,6</sup> and *Eucalyptus*<sup>7,8</sup> have been reported to possess antibacterial, antiviral and antifungal properties. Even though the chemical composition of the essential oil of *Cymbopogon citratus* (lemon grass) varies according to the geographical origin,<sup>9</sup> some species have demonstrated activity against these organisms. *Eucalyptus* oil has been reported to show activity against *Herpes simplex viruses*, some strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *Candida albicans*, and *Aspergillus flavus*.<sup>10,11</sup> Similarly, castor oil is a known source of ricinoleic acid, possess antibacterial and antifungal activity.<sup>12</sup>

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**Citation:** Osonwa UE, Okechukwu SA, Ihekwereme CP, Chukwu KI, Eluu SC, Azevedo RB. Formulation and Evaluation of Therapeutic Potential of Nanoemulsion of a Blend of Antimicrobial Oils. Trop J Nat Prod Res. 2018; 2(2):67-73. [doi.org/10.26538/tjnpr/v2i2.3](https://doi.org/10.26538/tjnpr/v2i2.3)

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The oil of *Anethum graveolens* (dill oil) is also reported to possess antibacterial and antifungal activity.<sup>13-15</sup> *Allium sativum* (garlic) possesses antifungal and antibacterial properties and is associated with allicin, ajoene, thiosulfonates and a wide range of other organophosphate compounds.<sup>16</sup> Its antibacterial spectrum covers *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus epidermidis* and *Klebsella pneumonia*.<sup>17,18</sup> *Zingiber officinale* (ginger) has activity against some Gram-positive and Gram-negative bacteria including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Haemophilus influenzae*.<sup>19,20</sup> Gingerols and gingerdiol from ginger are identified as being active against 13 human pathogens.<sup>21</sup> *Helianthus annuus* (Sunflower) seed oil has antifungal and antibacterial properties.<sup>23</sup> Oleic acid, phenolic constituents, and squalene are the major active components of the fruit of *Olea europaea* (olive oil).<sup>24</sup> Olive oil has demonstrated activity against a variety of organisms including intestinal and respiratory pathogens.<sup>25</sup> A combination of these oils will potentially have a broad spectrum of activity, and may also be an important agent against resistant organism due to their multicomponent nature. These oils are miscible and multicomponent in nature and they may have the capacity to withstand microbial resistance by acting synergistically against organisms that are normally resistant to single molecules. More so, these oils are edible and generally regarded as safe. The present work is therefore aimed at formulating the selected oils into a nanoemulsion system for treatment in cases of bacterial and fungal infections possibly occurring together.

## Materials and Methods

### Reagents

The following reagents were purchased from a local supplier: ketoconazole 200 mg tablets (Hovid Bhd, Malaysia), ciprofloxacin 500 mg tablets (Fidson Healthcare, Limited, Nigeria). They were standardized in terms of drug content and release. Olive, eucalyptus, dill, castor and peppermint oils were purchased from a local supplier (Anselem Chemicals, Nigeria). Garlic, ginger, sunflower and lemongrass oils were donated by the Nanobiotechnology Laboratory, Department of Genetics and Morphology, University of Brasilia, Brazil.

Other reagents were immersion oil (Panzonar Laboratory Supplies, Canada), alanine aminotransferase and aspartate aminotransferase (Randox Laboratory, UK), chloroform (Sigma-Aldrich, USA), Drabkin's solution (Organo Biotech. Laboratories, India), EDTA (Fushun Shunnu Chemical, China), paraffin wax (Green Mountain, China), xylene (Shivan Industries, India), haematoxylin (Abbey Colour, Philadelphia), eosine (Abbey Colour, Philadelphia), RBC diluting fluid (Organo Biotech. Laboratories, India), WBC diluting fluid (Alpha Chemicals, India), formol saline (Vet Way Industries, UK), phosphate buffer (Vet Way Industries, UK) and Polysorbate 80 (Sigma-Aldrich, Brazil).

### Experimental Animals

Forty-five (45) Wistar rats of both sexes weighing 108-188 g and twelve albino mice of both sexes weighing 18-22 g were acquired from the Animal House of the Department of Veterinary Medicine, University of Nigeria, Nsukka. They were housed in groups of three in an environment with controlled conditions of humidity and 12 h light and darkness cycles. They were given rodent feed *ad libitum* and had unrestricted access to water during the adaptation period of 7 days.

### Microbial culture

Clinical isolates of Gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhi*) bacteria and fungi (*Candida albicans* and *Aspergillus niger*) were previously isolated from clinical samples and maintained in the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka. These microorganisms were used in this study.

### Formulation and Characterization of the Nanoemulsion System

A blend of the 9 oils with antimicrobial properties (olive oil, eucalyptus oil, dill oil, castor oil, peppermint oil, garlic oil, ginger oil, sunflower oil and lemongrass oil) was produced by introducing eight milliliters (8 mL) of each in a 200-mL beaker and gently blending for 10 min, using a magnetic stirrer (model RH IKA Labortechnik, Germany). This was designated as Oil Blend (OB). Using a predetermined ratio, calculated quantities of polysorbate 80 and water were introduced and blended together for another 10 min. A total of twelve (12) different formulations were made. The products were stored and observed for one week. The ratio of oil to polysorbate 80 that forms stable, freely miscible oil-in-water nanoemulsion was selected as desired one, and designated NE, while the corresponding oil-surfactant blend was selected as the desired Oil Formulation (OF). One preparation each of the OB, OF and NE was made and assessed for stability before using for further studies.

The particle size, polydispersity index (pdi) and zeta potential of NE and OF were measured using Zetasizer (Malvern, USA) after five-fold serial dilutions with distilled water. The mean of 21 readings was used in each instance. The readings were taken on days 0 to 90. The readings initially were taken daily for one week, and then weekly over the period. Because the NE was not stable on long-term storage, further assays were only done with OB and OF which will nanoemulsify *in situ*.

### In vitro Antimicrobial Assay of the Nanoemulsion

The media (Nutrient agar (Marine Chemicals Limited India) and Sabouraud Dextrose Agar (SDA) (Titan Biotech Limited, India) were prepared as specified by the manufacturers. Briefly, a specified quantity of the powder (nutrient agar 28 g/L, SDA 65 g/L) was dispersed in distilled water, homogenized and sterilized in the autoclave at 121°C for 15 min. The microorganisms (100 cfu) were incubated in agar culture containing 1.5625 - 100 µL/mL of OB and 0.3125-20 mg/mL of OF using agar dilution method. The bacteria were incubated at 37°C for 24 h while the fungi were incubated at 25°C for 48 h.

A 1% polysorbate 80 dispersion was used as negative control while ciprofloxacin (1.5625-50 µg/mL) and ketoconazole (0.625-2 µg/mL) were used as positive controls for antibacterial and antifungal activities respectively. The fungal spores and the bacterial colonies were counted physically using a counting chamber.

### Determination of Acute Toxicity of Oil Blend (OB)

The oral median lethal dose (LD<sub>50</sub>) of OB was determined by the method of Lorke<sup>26</sup> with the white albino mice and observed.

### In vivo Antimicrobial Assay

In this experiment, only 24 h-cultures of *Staphylococcus aureus* and *Aspergillus niger* were used because of their highest sensitivity to OB and OF in the *in vitro* antimicrobial assay. Nine (9) groups of five animals (n = 5) each were randomized into three categories of four groups for antibacterial studies, another four groups for antifungal studies. One group served as a reference for both studies.

Except for the reference group, the animals were infected with the pathogenic microorganisms (0.1ml of 10<sup>6</sup> CFU), according to McFarland's dilution standards, using sterile hypodermic 1 mL syringe. The reference category was neither infected nor treated. Out of the four groups used for the antifungal studies category, one group was infected but not treated, serving as the negative control. Another group that was infected but treated for 5 days with ketoconazole (2.86 mg/kg/day) served as a positive control. The remaining two groups in this category were infected and treated with either OB or OF. In antibacterial study category, the negative control group was infected with *S. aureus* but not treated while the positive control group was infected with *S. aureus* and treated with a daily ciprofloxacin dose (7.14 mg/kg) for 5 days. The remaining two groups also received either OB or OF as treatment post infection. The doses of the OB and OF administered were 0.37 mL/kg/day OB (1/50th of the LD<sub>50</sub>) and 1.85 mL/kg OF (equivalent doses based on density calculations). Treatments were started 4 days post-infection. Samples of whole blood (0.5 ml) were taken from the animals on days 1, 3 and 5 post-treatment from the medial cantus vein of the eyes. The microbial load of the organisms (*S. aureus*, *A. niger*) in blood were assessed by culturing a 10-fold dilution of the blood samples. The colonies of bacteria and spores of fungi were physically counted using tally counter after 24 h (*S. aureus*) and 48 h (*A. niger*).

### Hematological Parameters Assessment

Three days post-treatment; blood samples were collected from the medial cantus of each rat using a capillary tube. The blood samples were used to determine the PCV, hemoglobin, total red blood cell (RBC), and total white blood cell (WBC) of the rats using standard method.<sup>27</sup>

### Assessment of Biochemical Parameters

At the end of the test period, the rats were sacrificed. Blood samples were collected from each experimental rat by cardiac puncture. The blood samples were centrifuged to obtain sera which were stored at -4°C until further assay. The stored sera were used for assessment of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) after first warming to room temperature. AST and ALT activities were determined by the methods of Reitman and Frankel<sup>29</sup> as outlined in Randox kit (Randox Laboratories, UK). Activities were expressed as IU/L.

### Histopathological assessment

Histopathological changes of the liver and kidney of each rat in the respective categories and groups were assessed after treatment. Tissue sections of the liver and kidney were fixed in 10% formal saline and dehydrated in 80% ethanol overnight and then in 100% ethanol for one hour. Thereafter, the tissues were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The paraffin blocks were later mounted and sectioned with a rotary microtome (HM-325) at a 5-6 microns thickness. Sections were deparaffinized in xylene and subsequently stained with Haematoxylin and Eosin (H and E) for light microscopy.<sup>30</sup>

### Statistical Analyses

Means and standard deviations of all readings obtained during the characterization of OF and OB, were calculated using Microsoft (MS) Excel. ANOVA was done using SPSS version 16.0 at 5% level of significance.

## Results and Discussion

### Formulation Characteristics

The mean particle size, polydispersity index (pdi) and zeta potential of the freshly formulated NE were  $151.12 \pm 6.21$  nm, 0.12 and  $-13.70 \pm 4.40$  mV, respectively and after 90 days became  $202.50 \pm 4.11$  nm, 0.09, and  $-35.00 \pm 6.15$  mV, respectively. The readings for the fresh OB were  $158.00 \pm 3.08$  nm, 0.07, and  $-15.50 \pm 3.01$  mV, respectively and after 90 days became  $162.90 \pm 6.01$  nm, 0.08, and  $19.00 \pm 0.00$  mV, respectively. Hence, NE was not used for further assays since it is not stable on prolonged storage. These results are shown in Figures. 1-3. Gentle agitation of OF with water forms a stable uniform phase.

The size range achieved has been reported to enhance the activity of antimicrobial agents.<sup>31,32</sup> Nanostructures are achieved when they are less than 500 nm. The more the number of structures with larger sizes, the more unstable the formulation becomes. The result of the size analysis shows it conforms to the size range of nanoemulsions. The pdi is an indication of the width of the particle size distribution. The pdi value that reflects the quality of the dispersion usually ranges from 0 to 0.5. Pdi values  $\leq 0.1$  indicate the highest quality of dispersion. Although, most researchers rate pdi values  $\leq 0.3$  as optimal, values  $\leq 0.5$  may be acceptable.<sup>33</sup> The pdi values of the nanoemulsion were within the acceptable range.

Aggregation of suspended particles results in formation of larger particles which quickly results in unstable formulation. As a result, the degree of repulsion between similarly charged particles in a formulation during storage shows how stable a suspension is. This repulsive force, measured through the zeta potential is indicative of physical stability of a formulation.<sup>34</sup> A minimum zeta potential of about  $-60$  mV yields a formulation with excellent physical stability, while a zeta potential of approximately  $-30$  mV yields a formulation with fairly good physical stability.<sup>35</sup> The range of values of our formulation suggests a product with good physical stability.

### In vitro Antimicrobial Assay

The values of MIC of OB against *S. aureus*, *S. typhi*, *C. albicans* and *A. niger* were  $\leq 1.25$   $\mu\text{L/mL}$ . *E. coli*, *E. faecalis* and *B. subtilis*, had values between 2.5  $\mu\text{L/mL}$  - 10  $\mu\text{L/mL}$  (Table 3). *P. aeruginosa* and *K. pneumonia* had values  $\geq 20.0$   $\mu\text{L/mL}$ . The most sensitive organisms (*C. albicans*, *A. niger*) recorded 0.63  $\mu\text{L/mL}$  while *P. aeruginosa* was resistant at the highest concentration used. For the OF, *S. aureus* (3.125  $\mu\text{L/mL}$ ) was the most sensitive. Some of the organisms (*S. typhi*, *E. faecalis*, *C. albicans*, *A. niger*) recorded an MIC between 12.5  $\mu\text{L/mL}$  - 25  $\mu\text{L/mL}$  while *E. coli*, *B. subtilis* and *K. pneumonia* had a value range 50 - 100  $\mu\text{L/mL}$ . Though *B. subtilis* (3.12  $\mu\text{g/mL}$ ) was the most sensitive to the positive antibacterial control (ciprofloxacin), all the sample bacteria recorded sensitivity within the range of 3.12  $\mu\text{g/mL}$  and 25  $\mu\text{g/mL}$ . *P. aeruginosa* (25  $\mu\text{g/mL}$ ) and *S. typhi* (25  $\mu\text{g/mL}$ ) were the least sensitive. Intermediate values were obtained at 6.25  $\mu\text{g/mL}$  (*S. aureus*, *E. faecalis*) and 12.5  $\mu\text{g/mL}$  (*E. coli*, *K. pneumonia*). In comparison, OB had lower MIC values than their corresponding values for OF (Table 1). The lowest

MIC recorded for OB and OF were 0.625  $\mu\text{L/mL}$  (*A. niger* and *C. albicans*) and 3.125  $\mu\text{L/mL}$  (*S. aureus*), respectively. All the organisms except *P. aeruginosa* were sensitive at the maximum concentration used. When OB and OF are compared based on equivalent oil content, OB recorded a lower MIC than OF for all the organisms except for *K. pneumonia* (Figure 1). Ciprofloxacin had its lowest MIC (3.125  $\mu\text{g/mL}$ ) on *Bacillus subtilis* while OB and OF recorded MICs of 5  $\mu\text{L/mL}$  and 20  $\mu\text{L/mL}$ , respectively on the same organism.

With the fungus, *Candida albicans*, ketoconazole recorded the lowest MIC (0.25  $\mu\text{g/mL}$ ), while OB and OF recorded MICs of 0.625  $\mu\text{L/mL}$  and 5  $\mu\text{L/mL}$ , respectively. Results of the *in vitro* MIC assay show that OB has a lower MIC for most of the organisms than OF. The higher MIC value of OF was probably due to the poor dispersion of OF in the incubation medium. Poor dispersion possibly resulted from the absence of water, which could have been an agent for nanoemulsification. Another source of poor dispersion could be the excipients in OF. Presence of formulation excipients may affect the dispersion profile of an active drug principle in a medium. However, the results may not be representative of what happens inside the body.

### In Vivo Studies

#### Efficacy Studies

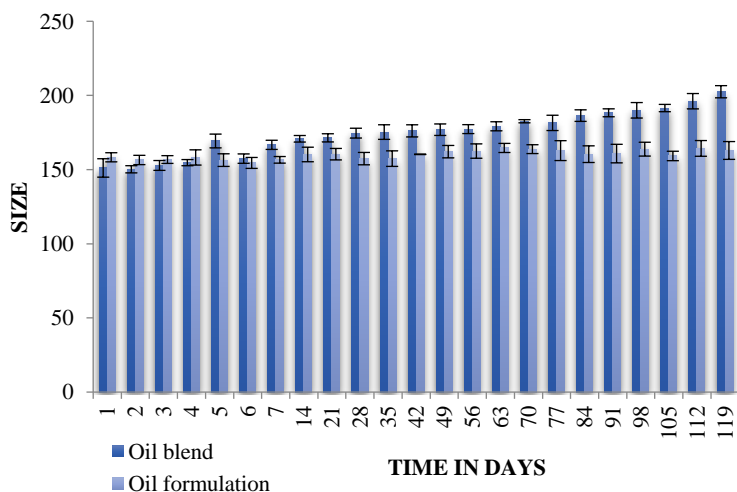
The antifungal count showed that the results for the positive control (ketoconazole treated group), OB and OF were similar and there was no significant difference ( $p > 0.05$ ) between the ketoconazole group and OF. After 5 days, the fungal load was reduced to 55%, 71% and 60% with ketoconazole, OB and OF, respectively (Figure 4).

Ciprofloxacin performed slightly better than OF in antibacterial activity (Figure 5). The order of activity at the test doses used was ciprofloxacin  $>$  OF  $>$  OB. After 5 days, the bacterial load was reduced to  $17 \pm 1.7\%$ ,  $67 \pm 4.0\%$  and  $28 \pm 3.6\%$  for ciprofloxacin, OB and OF, respectively. Overall, results of the *in vivo* experiments for both bacteria and fungi show that OF performed better than OB in microbial elimination. Consequently, the studies imply that nanoemulsification improved microbial clearance since the equivalent dose of oil was used both in OB and OF. The improved performance in *in vivo* studies may be due to *in situ* nanoemulsification, which is facilitated by agitation that accompanies both swallowing, and peristaltic movement of the gastrointestinal tract, thus enhancing absorption.

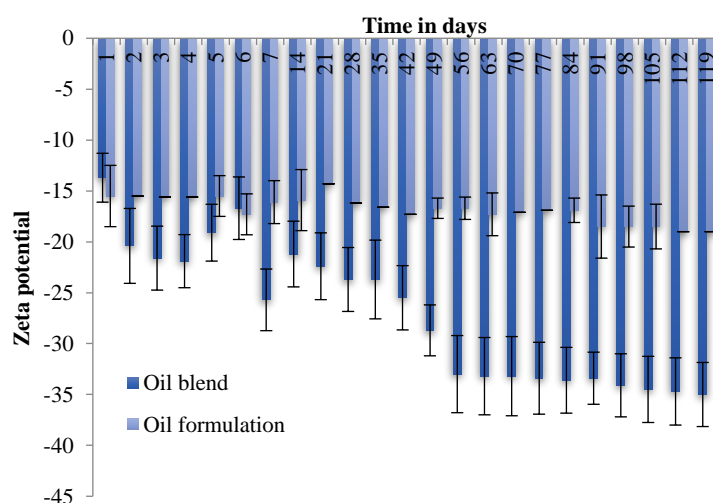
#### Safety Studies

##### LD<sub>50</sub>

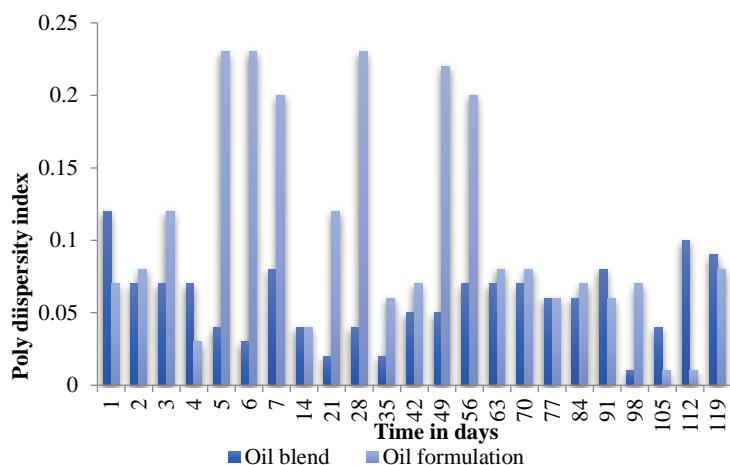
The LD<sub>50</sub> of the formulation was calculated to be 18.5 mL/kg equivalent to 15,910 mg/kg based on a calculated relative density of 0.86 mg/mL. The result of the acute toxicity test shows that the formula is safe.



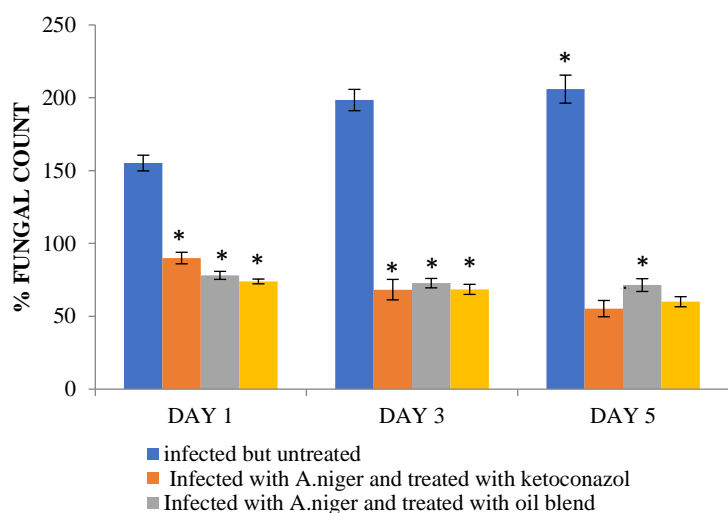
**Figure 1:** Stability results of the preparations showing changes in particle size



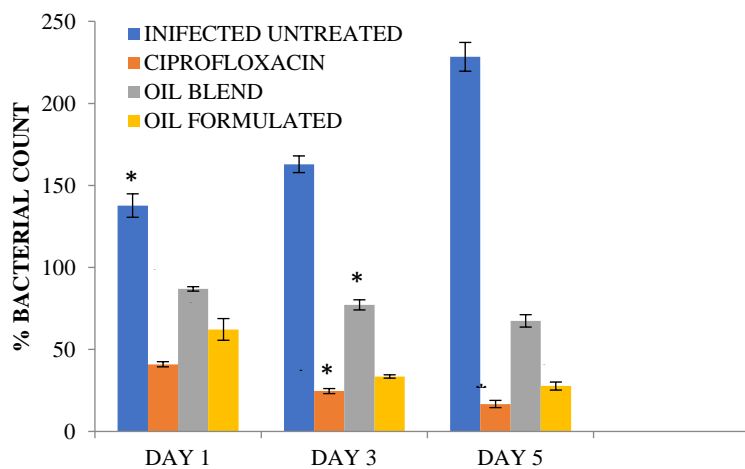
**Figure 2:** Stability results of the preparations showing changes in Zeta potential



**Figure 3:** Stability results of the preparations showing changes in poly dispersity index.



**Figure 4:** Effect of Treatment on Blood Fungal Count.  
\* Significant at  $p < 0.05$  when compared to infected untreated group.



**Figure 5:** Effect of treatment on blood bacterial count.  
\* Significant difference at  $p < 0.05$  when compared to infected untreated group.

#### Effect of treatment on hematopoietic parameters

The order of improvement in PCV (Figure 6) was ciprofloxacin = OF > OB for the bacteria-infected group. There were no significant differences in PCV for the fungus-infected animals (Figure 7). The test samples showed an improvement in RBC count in both infections (bacterial and fungal).

All other treated animals performed better than the ketoconazole treated group. All the groups infected but treated showed improvement in hemoglobin content in both infections (bacteria and fungi) (Figure 8). The effect on hemoglobin content for all the animals treated with the test sample was comparable to that of the uninfected animals. The results suggest that OF and OB do not cause erythropoenia, unlike ketoconazole treatment, which showed some adverse effects.

WBC results (Figure 9) show that infection increases WBC count ( $p < 0.05$ ). All the interventions ameliorated the effect of infection on WBC count (Figure 9). Values for OB and ciprofloxacin were similar in bacteria-infected groups. Ketoconazole treated group had less WBC count increase than OB, OF or ciprofloxacin-treated groups.

Hematological indices are reliable parameter for assessment of health status of animals.<sup>36</sup> The severity of hematopoietic changes depends on the species, physiological state of the host, and acuteness or chronicity of the infection. Since there is no significant difference ( $p < 0.05$ ) in RBC or PCV of pre-infected animal groups, it is taken that all the groups were of similar health status before treatment. The post-treatment values of PCV and RBC count indicates the level of correlation with microbial clearance. More adverse effects were observed with the bacteria-infected groups, than with the fungus *A. niger*. Immune-mediated hemolysis in infection may result from the production of hemolytic antibodies. Hemolysis may also be initiated by chemicals or metabolites that result in erythrocyte membrane damage. The damaged RBCs are subsequently recognized by splenic macrophages and destroyed.

Increase in WBC count usually arises as an immune defense mechanism against invading microorganisms or foreign particles. The reduction in WBC count for the treated groups is associated with microbial reduction arising from treatment. With OB and OF, like ketoconazole and ciprofloxacin WBC proliferation was reduced.

#### Effect of Treatment on Liver

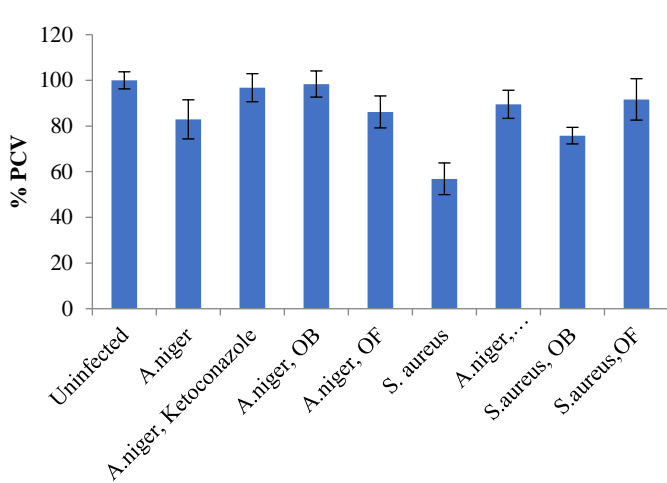
The liver function tests AST, ALP and ALT give a good indication of the condition of the liver. Increase in plasma level of the hepatic enzyme may be due to injury or altered liver cell integrity caused by infection. The amount of these enzymes in the blood is used as a measure of the extent of damage sustained. The interventions mitigated the increases in ALP in both infections (bacterial and fungal) (Figure 10). The effect of treatment on ALP for all the animals treated with the test samples showed slight increases ( $\leq 15\%$ ) in values when compared with the uninfected group. All interventions ameliorated the effect of infection on AST (Figure 11) for all the animals. Values for OB, OF, and ketoconazole were similar ( $p > 0.05$ ) in fungal infection. On the other hand, ciprofloxacin showed less increase with OB and OF.

Figure 12 shows photomicrographs of liver sections of the rats. Varying degrees of periportal hepatitis (mainly mononuclear cell infiltration of the portal area-P) are associated with the controls (negative and positive) of both the fungal and bacteria-infected groups. OB showed varying degrees of periportal mononuclear cell infiltration of cells both in the bacterial and fungal group. In addition, there was vascular congestion observed with negative control (fungal), ketoconazole and OB. There was no observable histopathological change associated with OF in both the fungal and bacterial groups. The results from the liver studies show that OF is safe on the liver.

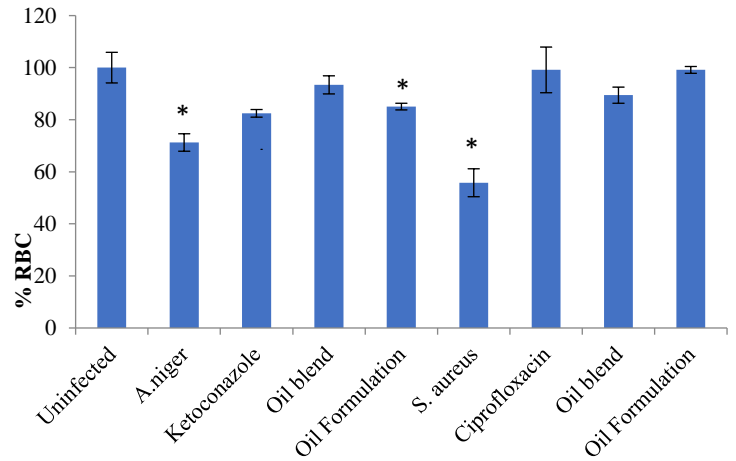
**Table 1:** Results of *In vitro* MIC Screening.

Conc (µg/mL) →	OB	OF	OB	OF	OB	OF	OB	OF	OB	OF	OB	OF	OB	OF
	20	100	10	50	2	25	2.5	12.5	1.25	6.3	0.63	3.1	0.31	1.6
<i>S. Aureus</i>	-	-	-	-	-	-	-	-	MIC	-	+	MIC	+	+
<i>E. coli</i>	-	MIC	MIC	+	+	+	+	+	+	+	+	+	+	+
<i>P. aeruginosa</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. subtilis</i>	-	+	-	+	MIC	+	+	+	+	+	+	+	+	+
<i>S. typhi</i>	-	-	-	-	-	MIC	-	+	MIC	+	+	+	+	+
<i>K.pneumoniae</i>	MIC	-	+	MIC	+	+	+	+	+	+	+	+	+	+
<i>E. faecalis</i>	-	-	-	-	-	MIC	MIC	+	+	+	+	+	+	+
<i>C. albicans</i>	-	-	-	-	-	MIC	-	+	-	+	MIC	+	+	+
<i>A. niger</i>	-	-	-	-	-	-	-	MIC	-	+	MIC	+	+	+

- = NO GROWTH; + = GROWTH; MIC = Minimum Inhibitory Concentration.

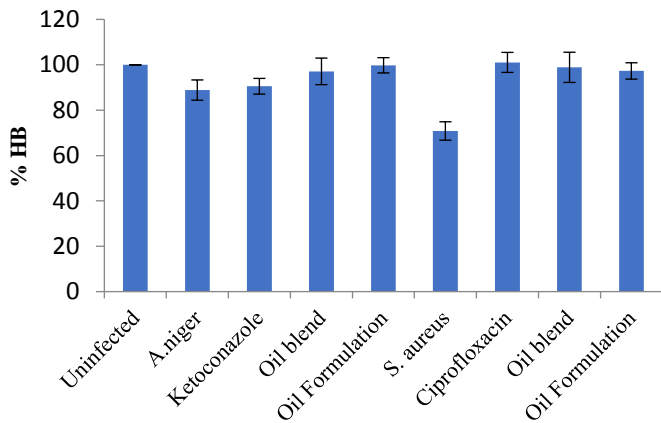


**Figure 6:** Effect of treatment on packed cell volume.



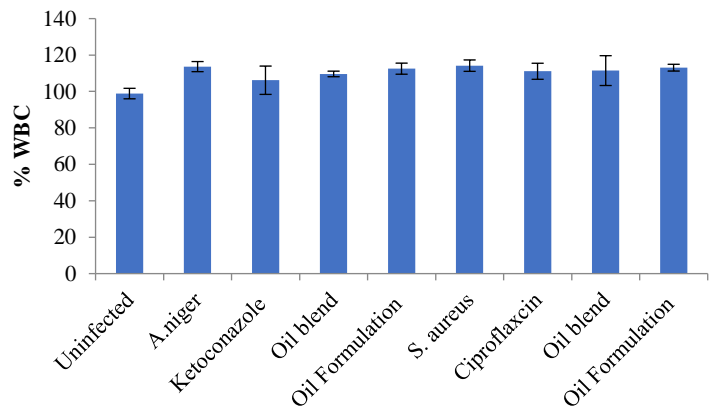
**Figure 7:** Effect of treatment on RBC.

\* Significant difference at p<0.05 when compared to uninfected group.

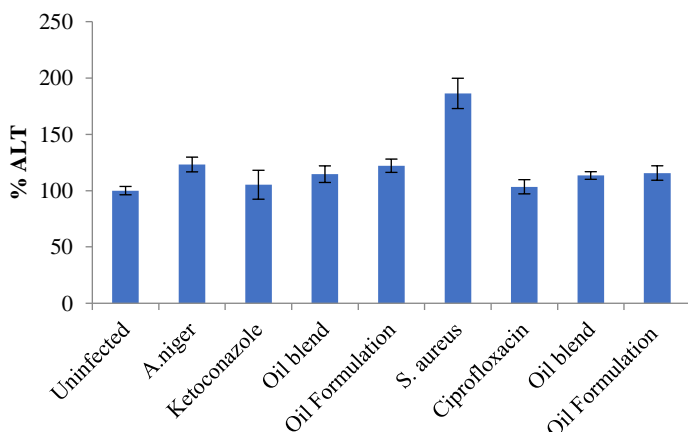


**Figure 8:** Effect of treatment on hemoglobin (Hb).

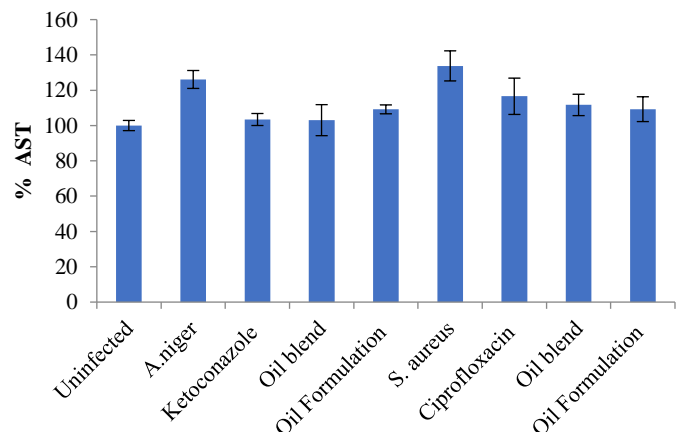
\* Significant difference at P < 0.05 when compared to uninfected group.



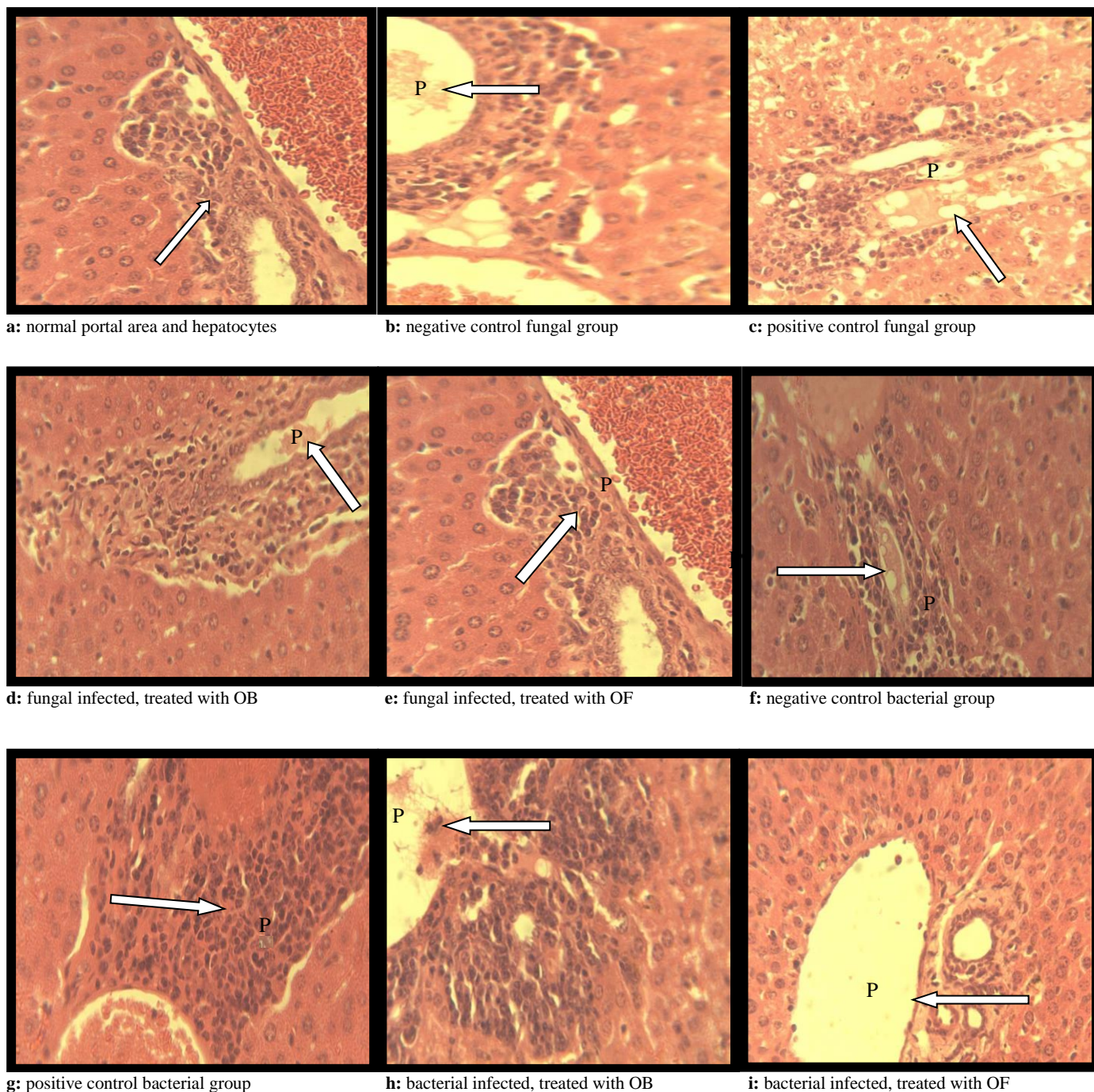
**Figure 9:** Effect of treatment on White blood cells (WBC).



**Figure 10:** Effect of treatment on ALT.



**Figure 11:** Effect of treatment on AST.



**Figure 12 (a-i):** Photomicrograph of liver section of rats (H and E x 400). Note the infiltrating cells (arrow) and the mononuclear cell infiltration of the portal area-P.

### Conclusion

In conclusion, this pilot study has demonstrated that the formulation of a blend of antimicrobial oils into nanoemulsion resulted in significant microbial clearance *in vivo*, which, possibly is due to improved bioavailability. Acute toxicological, hematological and hepatological studies show that the nanoemulsion is safe for consumption. Further studies will be needed to improve and comprehensively characterize the stability of the product over time.

### Conflict of interest

The authors declare no conflict of interest.

### Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

### Acknowledgements

We thank the staff of Department of Pharmaceutics and Pharmaceutical Technology, Nnamdi Azikiwe University-Awka, Anambra State, Nigeria, and Department of Genetics and Morphology, Institute of Biological Sciences, University of Brasilia, Brazil, for their material and technical supports to this research. We wish to acknowledge also the INCT, Brazil, that funded the preliminary part of this work.

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