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Original Research Article

A study on the composition and antimicrobial activities of stored and freshly extracted leaf essential oils of *Ocimum gratissimum* L.

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ABSTRACT

There have been concerns expressed on the biological activities of certain essential oils following long-term storage. The aim of this study was therefore to determine the composition and antimicrobial activities of samples of stored and freshly extracted essential oils of *Ocimum gratissimum* Linneo (Lamiaceae) in order to provide an exhaustive comparison of these properties in the studied oils. The essential oils were obtained using steam distillation from fresh leaves of *O. gratissimum* L. collected in Ile-Ife (Nigeria) in the early morning hours in April 2005 and March 2013. These essential oils were then stored in amber coloured bottles in a refrigerator at 4 °C. GC-MS analyses of the stored and fresh essential oils led to the identification of twelve and five constituents, respectively. The proportions in the stored and fresh essential oils were thymol (46.2 and 76.0%), linalool (14.0 and 7.0%), neral (8.4 and 6.7%), eucalyptol (3.9 and 5.3%), terpinen-4-ol (1.8 and 2.4%), geranial (11.7 and 0%), α -terpineol (2.1 and 0%) and nerol (1.8 and 0%), respectively. The antibacterial activities of the two essential oils were comparable, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of which were from 0.25% v/v to 4.0% v/v. On the other hand, the stored essential oils showed higher antifungal effects with MIC and minimum fungicidal concentration (MFC) of < 0.0625%v/v against *Candida* spp. Both essential oils completely killed 1.0×10^8 CFU/mL bacterial inoculum within three hours. The differences in the antimicrobial activities obtained for the fresh and stored essential oil of *O. gratissimum* L. were presumably due to different proportion of constituents.

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1. Introduction

Man has used plants for various purposes since ancient times. Plants are being used as medicines, foods, flavours, ornamentals, fumigants, spices, insect repellents and cosmetics. Of the plants used for medicinal purposes, those that are used for treating microbial infections constitute the biggest group. Essential oils constitute major extractives from these plants used as antimicrobial agents (Mohammadhosseini et al., 2016; Mohammadhosseini, 2017).

A large number of studies have clearly shown that essential oils possess a wide variety of pharmacological properties including antiplasmodial, acaricidal, antioxidant, antimicrobial, antiviral, antimutagenic, anticancer, anti-inflammatory, and immunomodulatory activities (Camilo

et al., 2017; Mohammadhosseini, 2017). The various biological effects of essential oils have been attributed to their various phytochemicals (Mohammadhosseini, 2017).

Ocimum Linneo, is a versatile aromatic genus (family Lamiaceae) well-known for its medicinal properties and also for economically important essential oils. The *Ocimum* genus comprises annual, perennial herbs and shrubs native to the tropical and subtropical regions of Asia, Africa, and South America (Kalita and Khan, 2013).

Ocimum gratissimum L. is one of the major species of this genus. It is widely distributed in tropical and warm temperature regions (Sulistiarini et al., 1999; Prabhu et al., 2009; Joshi, 2017). The therapeutic use of its essential oil is extremely broad and varies according to the countries (Prabhu et al., 2009; Chen et al., 2015;

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Paula-Freire et al., 2016).

In Nigeria, *O. gratissimum* L. has many uses. It is used for culinary purposes. The fresh leaves are squeezed to release extracts to stop nose-bleeding. Decoctions of the leaves are also drunk as antidiarrhoeal. As a fresh herb, it is used as a vegetable in soup and also to flavor foods such as other vegetables, poultry, fish, jelly, honey, tea, and liquor. The essential oils have been shown to have antibacterial, antifungal, antiviral, hypoglycaemic, antipyretic, anti-nociceptive, antioxidant, anti-inflammatory, anthelmintic, anticarcinogenic, free radical scavenging, radio protective, antidermatophytic activities along with numerous other pharmacological uses (Orafidiya et al., 2007; Prabhu et al., 2009; Saliu et al., 2011; Olugbade et al., 2017).

Moreover, the antimicrobial activity of the essential oil of *O. gratissimum* L. has been recognized worldwide (Orafidiya et al., 2006; Silva et al., 2016). It has been established that the constituents of the essential oil are responsible for its reported antimicrobial actions (Sainsbury and Sofowora, 1971; Silva et al., 2016).

Several chemotypes, reflecting the most abundant constituents of the essential oil, have been identified. Two main chemotypes, thymol and eugenol, were the first to be reported in 1948 (Guenther, 1948). Most early investigations and recent studies from Nigeria are in agreement with this classification (Sofowora, 1970; Sainsbury and Sofowora, 1971; Orafidiya et al., 2006; Saliu et al., 2011; Olugbade et al., 2017). Since 1948, however, many other chemotypes have been reported in literature around the world. Examples include geraniol (Charles and Simon, 1992), methyl cinnamate (Fun and Baerheim Svendsen, 1990), ethyl cinnamate (Ali and Shamsuzzaman, 1968), eugenol/methyl eugenol (Matasyoh et al., 2007; Unnithan and Sushen, 2017), citral (Hegnauer, 1967), linalool/methyl chavicol (Lawrence, 1992), eugenol/l,8-cineole/sesquiterpenes (Sulistiarini et al., 1999), γ -terpinene (Saliu et al., 2011) and eugenol- α -bulnesene- β -caryophyllene (Joshi, 2017). Recent chemotypes found in Nigeria include γ -terpinene/ α -pinene chemotypes (Owokotomo et al., 2012). The variety of chemical constituents of the plant essential oil may serve to explain its differential medicinal uses by people of different climes around the world.

It has been reported that the components of essential oils easily convert into one another through various interactions such as oxidation, isomerisation, polymerization, disproportionation, cyclization or dehydrogenation reactions which may be triggered either enzymatically or chemically (Börje et al., 2004). Aside from natural conversion that has been reported to possibly take place in the plant, shown by the constituents obtained from plants harvested at different age or stage of maturity, as well as different times and seasons (Burfield, 2004; Figueiredo et al., 2008), this is also known to occur during storage of essential oils (Wabner et al., 2006; Oladimeji and Orafidiya, 2007;

Bråred-Christensson et al., 2009; Akinkunmi et al., 2015). As a result of this occurrence, there have been concerns expressed on the biological activities of certain essential oils following long term storage (Oladimeji and Orafidiya, 2007; Akinkunmi et al., 2015). However, reports on the antimicrobial effects of stored essential oil of *O. gratissimum* L. are not found in literature.

It was therefore the objective of this study to determine the composition and antimicrobial activities of stored essential oil of *O. gratissimum* L. in comparison with freshly extracted oil. For the purpose of the study, essential oil of *O. gratissimum* L. stored in amber coloured well closed glass bottles in the refrigerator over a period of eight years and freshly extracted oil collected in similar containers were used for the analysis.

2. Experimental

2.1. Collection of plant and extraction of oil

The fresh oil sample was extracted from the leaves of *O. gratissimum* L. (Fig. 1) collected at Ile-Ife, Nigeria (Fig. 2) in March, 2013 at about 10.00 am and used immediately for the experiments. The plant, as authenticated by the botanist in charge of the Herbarium of the Department of Botany, OAU, Ile-Ife, Nigeria, was identical with the Voucher Specimen (IFE 14849) of the plant from which the stored oil was extracted in 2005. The British Pharmacopoeia method was used for the extraction of the two oils (British Pharmacopoeia, 1988). The old oil was stored in the refrigerator at 4 °C in tightly closed amber colored bottles.



Fig. 1. *Ocimum gratissimum* L. plant photographs of leaves, flowers and twigs.

2.2. GC-MS Analysis

For GC-MS analysis, the samples were injected into a gas chromatograph (GC) coupled with mass spectrometry detector (MSD) equipped with an Agilent

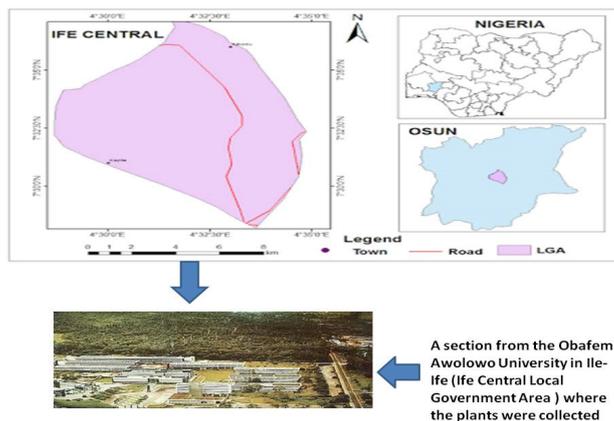


Fig. 2. Geographical map of the sampling area (Modified from <http://www.google.com.ng/mapofile-ifeosunstatenigeria>).

19091J413HP-5MS capillary column (30 m x 320 μm x 0.25 μm) consisting of 5% phenyl methyl siloxane. The injector was set at 250 $^{\circ}\text{C}$ and the detector at 250 $^{\circ}\text{C}$. The stepped temperature program of the column oven was as follows: 50 $^{\circ}\text{C}$ for 5 min, then increased to 250 $^{\circ}\text{C}$ at the rate of 5 $^{\circ}\text{C}/\text{min}$, and held for 10 min. The GC-MS interface temperature was at 325 $^{\circ}\text{C}$. The injection volume was 1 μL . The solvent delay was 3 min and injected in a split ratio of 50:1. The mass range used was over the m/z 30-550 range.

Compounds were identified by the calculation of their Kovats retention indices using C_8 - C_{32} alkane standards (Adams, 2017) and also by comparing their mass spectral data with those in the National Institute of Standards and Technology library (NIST11.L) and those reported in literature (Olugbade et al., 2017; Unnithan and Sushen, 2017).

2.3. Test organisms

The organisms used were type and clinical strains obtained from the stock collection in our laboratory and included *Bacillus cereus* (NCIB 6349), *B. subtilis* (NCIB 3610), *Clostridium sporogenes* (NCIB 532), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (NCIB 950), *P. fluorescence* (NCIB 3756), *Staphylococcus aureus* (ATCC 29213), *Klebsiella pneumoniae* (clinical strain), *Proteus mirabilis* (clinical strain), *Candida albicans* (ATCC 24433), *C. pseudotropicalis* (NCYC 6). The organisms were stored in Nutrient and Sabouraud Dextrose Agar Slope at room temperature and subcultured in the appropriate broth when needed.

2.4. Disk diffusion assay

The agar disk diffusion method was employed for the screening of antimicrobial activities of the oils following the protocol described earlier (Aiyelabola et al., 2012). Tobramycin (15 $\mu\text{g}/\text{disc}$) and acriflavin (6.3 mg/mL/disc) were used as positive controls for the bacteria and fungi strains, respectively.

2.5. Dispersion of the oil samples

Each of the fresh and old oil sample was dispersed in 50% v/v aqueous methanol separately to produce a stock solution each of 32% v/v of the oil as earlier described (Orafidiya et al., 2006). The dispersed solution was mixed homogenously using the rotamixer.

2.6. Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The stock preparation was used in the determination of MIC, MBC and MFC using the microbroth dilution method and following the protocol described earlier (Ajileye et al., 2015). The range of concentration used for the microbroth dilution assay was 8% to 0.0625% v/v. Streptomycin and acriflavin were the positive controls.

2.7. Time kill assay

Time kill assay was carried out for the *K. pneumoniae*, *E. coli* and *S. aureus* strains which were among the bacterial strains to which bactericidal effects were obtained from the MBC studies. Initial suspensions of 0.5 McFarland Standard equivalent to 1.0×10^8 CFU/mL suspension of each of these organisms were used. This assay was conducted following the standard procedure (White et al., 1996).

3. Results and Discussion

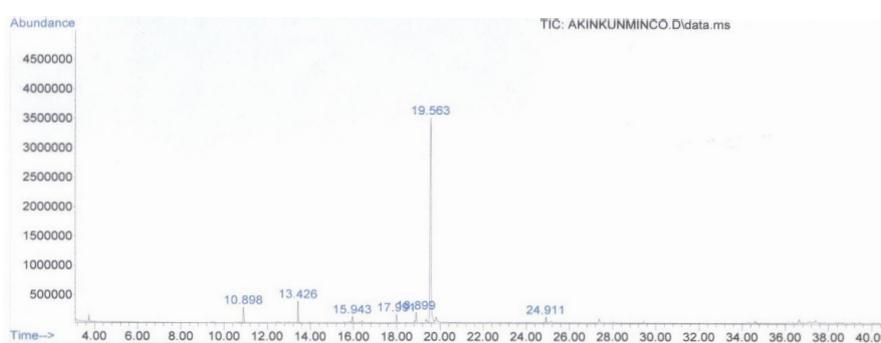
3.1. Chemical profiles of the oil samples in comparison with previous reports

Table 1 gives the Kovats retention indices and the relative percentages of the compounds present in the oil samples. For the fresh oil sample, the number of peaks was 6 with 5 identified constituents accounting for 97.5% of the oil profile (Fig. 3). Thymol (76.0%) was the main constituent followed by linalool (7.0%) and other minor constituents. In the case of the eight year

Table 1

Percentage composition of constituents in freshly extracted and eight year old stored *O. gratissimum* L. essential oil obtained by hydrodistillation as determined by GC-MS.

Constituents	Kovats retention index	% Composition	
		Fresh oil	Old oil
Acetic Acid, Butyl ester	1020.3	-	1.0
<i>o</i> -Cymene	1330.1	-	0.6
Eucalyptol	1338.7	5.3	3.9
Linalool	1445.2	7.0	14.0
Terpinen-4-ol	1555.1	2.4	1.8
α -Terpineol	1574.8	-	2.1
Neral	1648.9	6.7	8.4
Nerol	1668.7	-	1.8
Geranial	1691.4	-	11.7
Thymol	1723.6	76.0	46.2
Caryophyllene	1902.8	-	0.6
α -Selinene	1998.7	-	5.1

**Fig. 3.** Gas chromatogram of fresh *O. gratissimum* L. oil.

old oil, the total number of peaks was 17 out of which 12 were identified representing 97.11% of the oil (Fig. 4). Thymol (46.2%) was still the main constituent. Other major constituents were linalool (14.0%) and geranial (11.7%).

Comparing the chemical constituents of the fresh and old oils shows that thymol was present in the highest abundance in both samples. This indicates that the oil is of thymol chemotype confirming some earlier studies on the constituents of essential oil of *O. gratissimum* L. in Nigeria (Sofowora, 1970; Sainsbury and Sofowora, 1971) but different from some recent reports from Nigeria (Saliu et al., 2011; Owokotomo et al., 2012) and from other African countries (Unnithan and Sushen, 2017). Saliu et al. (2011) and Owokotomo et al. (2012) reported γ -terpinene and γ -terpinene/ α -pinene chemotypes, respectively from Nigeria. On the other hand, Unnithan and Sushen (2017) recently reported eugenol/methyl eugenol chemotype from Ethiopia. In another recent study, Olugbade et al. (2017) has reported that the varieties of *O. gratissimum* L. from Sierra Leone are thymol chemotypes like the Nigeria varieties they screened.

The thymol contents were 76.0% and 46.2% for the fresh and the old oil, respectively. This means that the quantity of thymol in the old oil is about 42% lower when compared with the fresh oil. On the other hand, the old oil contains 11.7% of geranial which was not detected in

the fresh oil although the stereoisomer neral (6.7%) was found in the fresh oil. This result suggests the possibility of isomerisation reactions in the oil.

Furthermore, the old essential oil contained 14.0% of linalool which was found to be 7.0% in the fresh oil. The inter-conversions of components of the essential oil of *O. gratissimum* L. on rapid autooxidation has been reported (Orafidiya et al., 2007). Although this investigation does not confirm that these differences were due to the storage of the oil, similar changes have been reported in the constituents of essential oils from other plants on storage (Wabner et al., 2006; Oladimeji and Orafidiya, 2007; Akinkunmi et al., 2015).

3.2. Comparison of the antimicrobial activities of the essential oil samples

Both the old and new essential oils were equally active and demonstrated variable activities against the bacterial strains in the MIC and MBC determinations as MIC ranges from 0.25-2.0% v/v for both the fresh oil and the old oil. MBC against the bacterial strains also range from 0.5-2.0% v/v and 0.5-4.0% v/v for the fresh and old essential oils, respectively (Table 2). The antimicrobial effects of the essential oils on both Gram positive and Gram negative bacteria were comparable.

The zone of inhibition (ZI) for the fresh essential oil against the bacteria tested ranged from 12.3 ± 0.8 mm

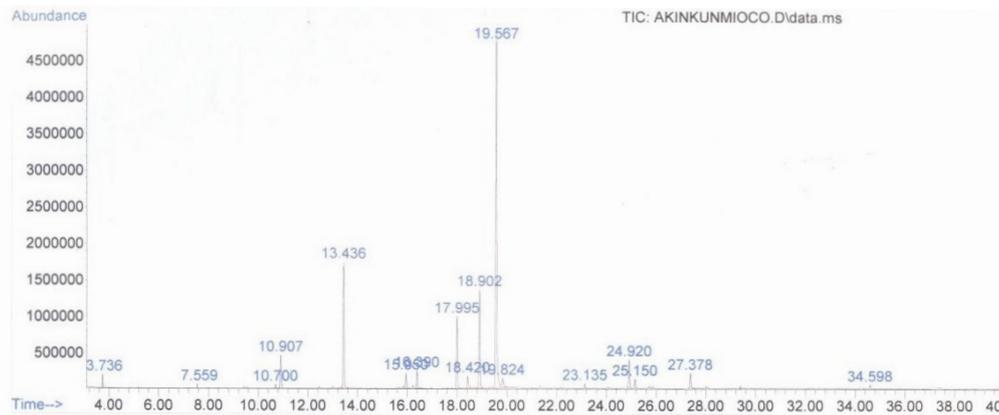


Fig. 4. Gas chromatogram of eight year old stored *O. gratissimum* L. oil.

Table 2

Antimicrobial activity of *O. gratissimum* L. essential oil using the disc diffusion method.

Organisms	Mean zone of inhibition \pm SD (mm)			Minimum inhibitory concentration (%v/v)			Minimum bactericidal concentration (%v/v)		
	Fresh oil	8-year old oil	Tobramycin (15 μ g/disc)	Fresh oil	8-year old oil	Streptomycin (μ g/L)	Fresh oil	8-year old oil	Streptomycin (μ g/L)
<i>E. coli</i> (ATCC 25922)	19.3 \pm 0.2	8.0 \pm 0.0	12.0	0.5	0.25	0.0625	1.0	2.0	0.5
<i>Pseudomonas fluorescens</i> (NCIB 3756)	35.3 \pm 0.3	0.0	8.0	0.5	0.5	0.25	0.5	0.5	0.5
<i>P. aeruginosa</i> (NCIB 950)	0.0	0.0	14.0	2.0	1.0	0.5	2.0	2.0	0.5
<i>Proteus mirabilis</i> (clinical strain)	20.0 \pm 0.0	0.0	22.0	1.0	1.0	0.25	1.0	2.0	0.5
<i>Bacillus cereus</i> (NCIB 6349)	19.3 \pm 0.8	0.0	13.0	1.0	1.0	0.25	1.0	1.0	0.5
<i>Staphylococcus aureus</i> (ATCC 29213)	17.0 \pm 0.0	8.0 \pm 0.0	15.0	1.0	1.0	0.0625	1.0	1.0	0.5
<i>Klebsiella pneumoniae</i> (clinical strain)	12.3 \pm 0.8	0.0	15.0	1.0	2.0	0.25	2.0	4.0	0.5
<i>Clostridium sporogenes</i> (NCIB 532)	30.0 \pm 0.0	10.0 \pm 0.0	14.0	0.25	0.25	0.25	0.5	2.0	0.5
<i>B. subtilis</i> (NCIB 3610)	30.0 \pm 0.5	0.0	13.0	1.0	0.5	0.25	1.0	0.5	0.5
<i>Candida albicans</i> (ATCC 24433)	15.0 \pm 0.5	17.0 \pm 0.0	24.0 \pm 0.5*	0.25	<0.0625	16.0**	0.25	<0.0625	16.0**
<i>Candida pseudotropicalis</i> (NCYC 8)	15.0 \pm 0.1	18.0 \pm 0.0	30.0 \pm 0.5*	0.25	<0.0625	16.0**	0.25	<0.0625	16.0**

*Acriflavin 6.3mg/mL/disc; **Concentration of Acriflavin (mg/L).

for *K. pneumoniae* to 30.0 \pm 0.0 mm for *C. sporogenes* and *B. subtilis*. Only *E. coli* (ZI=8.0 \pm 0.0 mm) together with *C. sporogenes* and *S. aureus* (ZI=10.0 \pm 0.0 mm) were slightly inhibited by the disc diffusion assay of the old oil. *P. aeruginosa* was not sensitive to both fresh and old essential oils using the disc diffusion method (Table 2).

The results of both diffusion and dilution assays indicate increasing inhibitory and fungicidal activities against the two *Candida* strains screened. The ZI was 15.0 mm for the fresh oil while it was 17.0 mm and 18.0 mm for the two fungi strains for the old essential oil. The MIC and MBC also increased from 0.25% v/v for the fresh essential oil to <0.0625% v/v for the old essential oil. In the time-kill studies, while it took only one hour to eliminate both the *E. coli* and *S. aureus* strains, it took three hours for the *K. pneumoniae* strain (Fig. 5 and Fig. 6).

The disc diffusion method gave different results for antimicrobial activities for the stored essential oil samples against the same organisms compared with the microbroth dilution in that broth dilution always show activity while the disc diffusion results indicated that the stored oil was not active against the bacterial strains. A possible reason for this effect may be due to more direct contact of the essential oils with of the organisms in the microbroth dilution method. In disc diffusion

method, barriers presented by the agar medium might have affected the diffusion of the highly viscous stored oil resulting in lowering the degree of contact and exposure of the organisms to the essential oil. These results suggested that agar disc diffusion test may not be suitable for this kind of investigations. The need to use a combination of techniques to obtain optimum information on the antibacterial activities of essential oil has been recommended (Alarc3n et al., 2015).

The MBC and MFC values of the two essential oil samples were close to the MIC. It can be inferred therefore that both the stored and fresh *Ocimum* essential oils has bactericidal and fungicidal properties. Similarly, the time-kill analysis results showed that the two essential oils had similar killing pattern and kill rapidly within three hours the *K. pneumoniae*, *E. coli* and *S. aureus* strains tested irrespective of their Gram character.

Each of these constituents found in the two essential oil samples, as already indicated, is a strong antimicrobial compound in its own rights (Onawunmi, 1989; Lamikanra, 2010). Therefore, the strong antimicrobial effects obtained for the two oil samples are not surprising. Furthermore, the comparatively higher number of antimicrobial constituents in the old oil suggests the possibility of higher interaction and synergism among the constituents in producing the antimicrobial effects

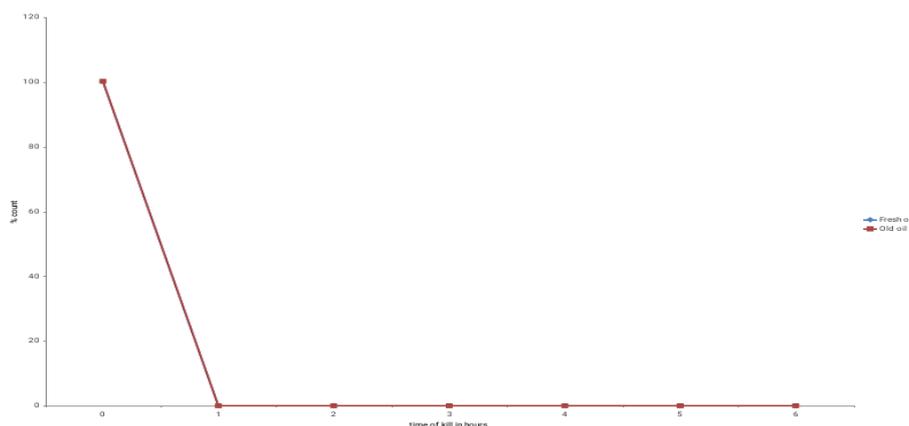


Fig. 5. Time-kill plot of *E. coli* and *S. aureus* exposed to the essential oils of *O. gratissimum* L.

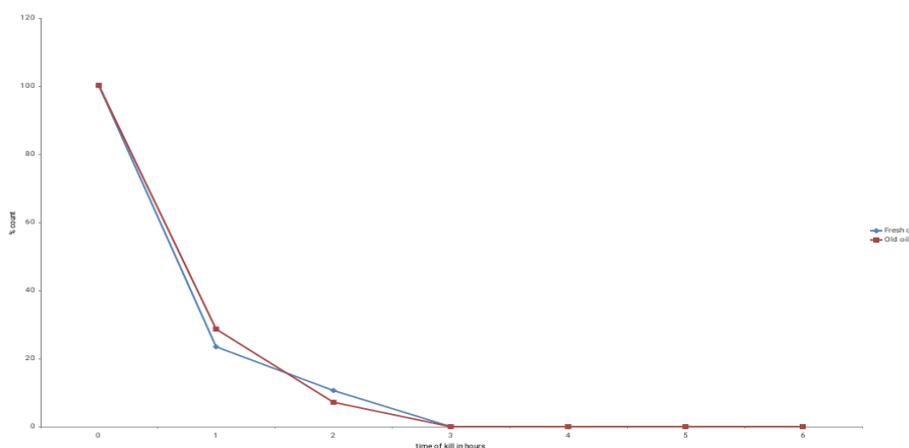


Fig. 6. Time-kill plot of *K. pneumoniae* exposed to the essential oils of *O. gratissimum* L.

observed. In view of this, the higher antimicrobial activities obtained for the old oil against some of the microbial strains is not surprising. The impressive antifungal activities of the old oil against the *Candida* strains are especially noteworthy. It lends credence to the constituents of the oil most of which have been reported earlier to possess strong antifungal properties. For example, the effective antifungal activities of citral have been documented in literature (Onawunmi, 1989). Results therefore indicate that the antimicrobial effects observed for the two oil samples are due to different proportions of antimicrobial compounds.

4. Concluding remarks

This study again confirmed the broad spectrum antimicrobial properties of *O. gratissimum* L. essential oil. Furthermore, it indicated that the essential oil still remained effective as antimicrobial oil after eight years of storage. It is shown that the impressive antimicrobial activities obtained for both essential oil samples were due to different proportions of constituents.

Conflict of interest

The authors declare that there is no conflict of

interest.

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