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Effect of Solvents on Phytoconstituents and Antimicrobial Activities of *Ocimum gratissimum* and *Eugenia caryophyllata* Extracts on *Listeria monocytogenes*

Solventlerin fitobileşenler ve *Ocimum gratissimum* ve *Eugenia caryophyllata* Ekstraktlarının *Listeria monocytogenes*'e Karşı Antimikrobial aktiviteleri Üzerine Etkileri

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Abstract

This study examined the effect of different solvents on active ingredients and antimicrobial performance of fresh leaves of *Ocimum gratissimum* and dried buds of *Eugenia caryophyllata*. Plant parts were extracted in a soxhlet apparatus using dietyl-ether, ethyl acetate, and ethanol. Phytoconstituents of the plants was determined using gas chromatography-mass spectrometer (GC-MS). The extract with high eugenol concentrations was prepared in seven treatments (160, 80, 40, 20, 10, 5, and 0 mg/mL) and tested against *Listeria monocytogenes* using agar well diffusion methods in triplicates. Minimum inhibitory concentration (MIC) and zone of inhibition were determined using standard technique. The data was subjected to descriptive statistics and one-way analysis of variance (ANOVA) at α0.05. The results showed that the phytochemical concentration of the extract varied significantly ($p<0.05$) with respect to solvents. Extract of *E. caryophyllata* using ethyl acetate had highest concentration of eugenol (71.32%) while *O. gratissimum* had 16.67%. The results revealed anti-*L. monocytogenes* activities of fresh leaf of *O. gratissimum* and dried buds of *E. caryophyllata* capacity with zones of inhibition of 13.33 mm and 19.00 mm respectively and MIC of 80 mg/ml. This establishes the possibility of using fresh leaf of *O. gratissimum* and dried buds of *E. caryophyllata* to prevent the growth of foodborne bacterias, especially *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, antibacterial activity, *Ocimum gratissimum*, *Eugenia caryophyllata*

Öz

Bu çalışmada farklı solventlerin aktif maddeler ile *Ocimum gratissimum*'un taze yaprakları ve *Eugenia caryophyllata*'nın kuru tomurcuklarının antimikrobial performansları üzerindeki etkileri araştırıldı. Bitki parçacıkları soxhlet cihazı içerisinde dietil eter, etil asetat ve etanol kullanılarak ekstrakte edildi. Bitkilerin fito-bileşenleri, gaz kromatografisi- kütle spektrometresi (GC-MS) kullanılarak saptandı. Yüksek öjenol konsantrasyonlu ekstrakt 7 işlemde hazırlandı (160, 80, 40, 20, 10, 5, and 0 mg/mL) ve *Listeria monocytogenes*'e karşı agar kuyucuk difüzyon metodu ile 3 kez tekrar edilerek test edildi. Minimum inhibitör konsantrasyonu (MIC) ve inhibisyon sahası standart teknik kullanılarak tespit edildi. Veriler istatistiki olarak hesaplandı ve $\alpha_{0.05}$ düzeyinde tek yönlü varyans analizi (ANOVA) yapıldı. Sonuçlara göre ekstraktın fito-kimyasal konsantrasyonu solventler ile ilişkili olarak anlamlı ölçüde ($p<0,05$) farklılık gösterdi. Etil asetat kullanılarak elde edilen *E. caryophyllata* ekstraktının en yüksek (%71,32); *O. Gratissimum*'un ise %16,67 oranında öjenol konsantrasyonu içerdiği gözlendi. Bulgulara göre, *Ocimum gratissimum*'un taze yaprakları ve *Eugenia caryophyllata*'nın kuru tomurcuklarının sırasıyla 13,33 mm ve 19,00 mm'lik inhibisyon sahaları ile *L. Monocytogenes*'e karşı antimikrobiyel aktivite gösterdiği ve MIC değerinin 80 mg/mL olduğu belirlendi. Bu sonuçlar, *Ocimum gratissimum*'un taze yaprakları ve *Eugenia caryophyllata*'nın kuru tomurcuklarının gıda kaynaklı bakterilerin, özellikle *L. Monocytogenes*'in gelişimini engellemek için kullanabileceklerini kanıtlamaktadır.

Anahtar kelimeler: *Listeria monocytogenes*, antibakteriyel aktivite, *Ocimum gratissimum*, *Eugenia caryophyllata*

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Introduction

The bacteria responsible for listeriosis are the genus Listeria. The genus has ten species which are *Listeria grayii, L. innocua, L. ivanovii, L. monocytogenes, L. seeligeri, L. welshimeri, L. marthii, L. rocourtiae, L. weihenstephanensis,* and *L. fleischmannii* species (Pusztahelyi et al., 2016). The bacteria are ubiquitous in nature and have been isolated from different materials (Moreno et al., 2014). The ability to survive in a wide range of environmental conditions makes it difficult to control. The genus has colony characteristics of beta-heamolysis, they are mostly gram positive rod, facultative and non-sporing organisms however, *L. monocytogenes* and *L. ivanovii* are capable of infecting humans and animals (Bayoub et al., 2010). Although, the organism has low morbidity (Miettinen, 2006), but a high mortality rate (up to 30%) (Miettinen and Wirtanen, 2006), making it of public health and food safety concern especially in aged, pregnant women and immune-deficient people (Pusztahelyi et al., 2016).

Several antibiotics have been used to control and treat listeriosis such as ampicillin, chloramphenicol, penicillin, amoxicillin, tetracycline, gentamicin, sulfamethoxazole, etc. singly or in combination with one another (Adetunji and Ishola, 2011). However, there are reports of Listeria species being resistant to most of the drugs creating a serious public health concern (Gupta et al., 2008) and thus a need for alternative therapy.

In Nigeria, use of medicinal plants in health management is very common locally, because traditional ways of treating diseases are well established and cheap. The healing ability of medicinal plants and oil from such plants has been attributed to several active ingredients in them that have antimicrobial, antibacterial, antioxidants and antiseptics properties (Naczk and Shahidi, 2006; Daayf and Lattanzio, 2008). In addition, the antimicrobial property of medicinal plants may differ depending on the forms which may be fresh, dried or extracted forms. In aquaculture, several plants have been used in the control, treatment and management of diseases e.g. *Eclipta alba, Zingiber officinale, Azadirachta indica* (Dugenci et al., 2003; Christybapita et al., 2007; Yin et al., 2008). Therefore its use in aquaculture is gaining wide acceptance. The extracts are used directly and/or included in feed to improve growth, enhance health conditions and wound-healing materials (Bello et al., 2013).

Plants from the Lamiaceae family are commonly used in trado-medicine from time immemorial. *Ocimum gratissimum* L (Lamiaceae), commonly also known as "Efinrin" in the Yoruba language and *E. caryophyllata* commonly also known as "kononfuru" have been used in the treatment of different diseases in Nigeria. The plant grows virtually in many farms, residential and industrial areas especially when it is found as weeds (Onajobi, 1986; Ilori et al., 1996). The clove (*Eugenia caryophyllata*), an aromatic plant belonging to the family of Myrtaceae, originated from the Molucca Islands in Indonesia and the southern Philippines, but is widely present in North central and south western parts of Nigeria (Omotayo et al., 2013).

Extracts from these plants has been used to treat viral conditions, malaria, cholera, tuberculosis, scabies, internal parasites, abdominal bloating, rheumatism, and stimulates uterine muscles to facilitate labour during child delivery (GHC, 1998; Karla et al., 1994a; Karla et al., 1994b). It also serves as mosquitoes repellent, mouthwash to prevent mouth odour and toothache while in food preservation it is used as spices (Omotayo et al., 2013; Harvey and John, 2015).

The antimicrobial properties of these plants have been associated with some chemical compounds in them such as eugenol, methyl cinnamate, camphor and thymol (Durling et al., 2007; Michiels et al., 2012). Eugenol is the active compound responsible for inhibition against many bacteria (Bayoub et al., 2010; Okoye and Madumelu, 2013). Also, it has been reported that eugenol constituting about 30 to 90% of *O. gratissimum* and *E. caryophyllata* (Ponce et al., 2003; Phillipe et al., 2012; Omotayo et al., 2013). However, concentrations of eugenol in the extract is affected with many factors such as solvents used for the extraction, plants parts among others (Bayoub et al., 2010). Since solvents used in the extraction process have effects on the quality and quantity of the compound (Zhou and Yu, 2004; Bayoub et al., 2010), the effect of different extraction solvents on quantity of active ingredients of *O. gratissimum* and *E. caryophyllata* needed to be investigated. This study therefore investigates the effect of different solvents on active ingredients of *O. gratissimum* and *E. caryophyllata* and its antimicrobial susceptibility performance against listeria species.

Materials and Methods

Plant collection and identification

The fresh leaves of *O. gratissimum* and *E. caryophyllata* were collected from a backyard at Agbowo and "Oja Oba" Ibadan respectively in the month of October, 2015. Ibadan is located on 10°23′0″N, 12°5′0″E. The plants were identified in the Herbarium Unit of Forest Research Institute of Nigeria (FRIN), Ibadan. The fresh *O. gratissimum* specimen was placed in Forest Herbarium Ibadan under voucher registration FHI 110548 while *E. caryphyllata* specimen was placed under voucher registration FHI 110603.

Extraction procedure

The extraction procedure was as described by Bayoub et al. (2010). Briefly, 5g of fresh leaves of *O. gratissimum* and dried buds of *E. caryophyllata* were weighed on a digital ScoutPro sensitive scale, covered with sterilized cotton wool and transferred into the Soxhlet apparatus. Exactly 170 mL of three different solvents (Ethanol, Ethyl-acetate and Diethyl Ether) were poured in a spiral tube of the equipment. Because of effectiveness (Bayoub et al., 2010) and being the commonest solvents used for extraction in the study area, ethanol, ethyl-acetate and diethyl ether were used for this study. The extraction lasted for six hours until no

further drop of extract in triplicates. The filtrate were concentrated on a rotary evaporator at 45°C for chemicals elimination, stripped into sterile bottles and transferred into laboratory for analysis. The extracts were weighed and recorded.

Phyto-chemical analysis

The extracts of fresh leaves of *O. gratissimum* and dried buds of *E. caryophyllata* were quantitatively examined for the presence of secondary metabolites using various analytical tests and reagents. The metabolites examined were Terpenoids (Odebiyi and Sofowora, 1978), Flavonoids (Harbone, 1998; Joshi et al., 2011), Tannins (Mayuri, 2012), Alkaloids (FDA, 2008; Joshi et al., 2011), Saponins (Harbone, 1988; Mayuri, 2012) and Steroids (Marcano and Hasena, 1991).

Phyto-constituents analysis

Constituents of the extracts' fresh leaves of *O. gratissimum* and dried buds of *E. caryophyllata* were examined using Gas Chromatography/Mass Spectrometry (GC/MS). Extracts were analyzed using Agilent 7890A Gas Chromatography with Agilent 5975 Mass Spectrometer detector (Avondale, PA USA) equipped with HP column of 5m long (0.25 m in diameter and 0.25 cm internal diameter) Agilent 190915-433HP-5M, 5% phenylmethylsilox (30 m×250 µm×0.25 µm) operated at ionization energy of 70eV, with splitless injector (at 300°C), 1.0 µm film thickness. An autosampler was used to inject 1 µL of each sample. The oven temperature was programmed from 35°C to 300°C held at 35°C for 5 minutes, then at a rate of 20°C per minute to 250°C for 5 minutes using helium carrier gas at a flow of 1mL per minute. The samples were run using full scan with a range of 50 to 750 mass units, and recorded using an HP Chemostation System. The extracts components were identified by comparing their relative retention times and mass spectra with those of authentic samples from analytical standards from the database (Bayoub et al., 2010). The library database (NIT XI) selecting only those structures that reached 90% or more probability made the structural assignments. The best two extracts with highest concentration were then used for susceptibility test.

***Listeria* species isolates**

Isolates were obtained from fish samples in the Ibadan metropolis. The isolates were screened using biochemical and molecular techniques using PCR method. The confirmed *L. monocytogenes* isolates were used for susceptibility tests. In brief, 756 samples from fish skin, muscle and visceral were pre-enriched in half-strength fraser broth. One gram (1 g) of fish tissues were added to universal bottles containing 9mL of sterile half-Fraser broth and supplements and incubated at 37°C for 24 hours (LQAD 2008). From the pre-enrichment culture media, 0.1 mL was transferred into 10mL of full strength Fraser broth with supplements added and was incubated at 35°C for 48hrs (secondary enrichment) (Kamat and Nair, 1993). From the secondary enrichment, the cultured media were streaked on PALCAM prepared plates using wire loop and incubated for 48hrs (Eruteya et al., 2014). The suspected colonies were subcultured on PALCAM agar and incubated for 24-48 hours to have pure colonies. Organisms that show the cultural and evidence of aesculin hydrolysis or black-halo formation on PALCAM plates by the *Listeria* bacteria were observed and subjected to both biochemical and molecular characterization. The tests were Gram staining, beta-haemolysis, catalase reaction, motility at room temperature and carbohydrate fermentation (Rhamnose, Xylose, Lactose, Fructose and Manitol) using standard methods. The organisms were stored on glycerol slant and backup with slant of PALCAM and stored in a freezer and refrigerator respectively until use. Biochemically characterized isolates were further subjected to molecular characterization using PCR method for detection of *L.monocytogenes* using primers specific to invasive associated protein (5'ACAAGCTG-CACCTGTTGCAG3'-F and 5'TGACAGCGTGTGTAGTAGCA3'-R) and haemolysis (5'GCAGTTGCAAGCGCTTGGAGTGAA3'- F and 5'GCAACGTATCCTCCAGAGTGATCG3'-R) as described by Swetha et al. (2012). Fifty-eight (58) isolates were biochemically confirmed to be Listeria species where molecularly 19 isolates were *L. monocytogenes* and 39 were other listeria species.

Inoculation of test organisms

The 9.9 mL of sterile water was dispensed into nineteen test tubes (equal to number of isolates). The listeria broth was serially diluted to a factor of 1:100 (Moreno et al., 2014) using sterile water. From the diluted broth, 0.1 mL of listeria broth (1×10^7 CFU/mL) (Bayoub et al., 2010) was added to each tube and used immediately (NCCLS, 2002; Moreno et al., 2014).

Concentration and application of the extracts

Agar well diffusion methods were adopted. From the phyto-constituents analysis, eugenol was not detected in ethanolic extracts in this study, hence it was not used for further study because eugenol is the active ingredient that responsible for antibacterial activities against many pathogens (Bayoub et al., 2010) but extracts using ethyl-acetate and diethyl ether show eugenol. From the both extracts using ethyl-acetate and diethyl ether, 2-fold dilution of 160 mg/mL were made to 5 mg/mL (Stokes and Ridgeway, 1980) in Dimethyl Sulfoxide (DMSO) (Gupta et al., 2008) to constitute 160, 80, 40, 20, 10, 5 and 0 mg/mL.

Antimicrobial assay

A cork borer of 6 mm in diameter was used to create wells on the Nutrient agar plates. Each well was filled with the extracts at the working concentration using sterilized droppers and incubated at 37°C for 24 to 48 hours (CLSI, 2011). The sensitivity of the extracts was investigated on *L. monocytogenes* using agar well diffusion method as described by Schillinger and Lucke (1989). Briefly, sterile disposable petri dishes were swabbed with 0.1 mL fresh 24 hours cultured Listeria broth (about 10^7 CFU/mL) using swab sticks in triplicates. The zones of inhibitions were measured with meter rule calibrated in millimeter. The chloramphenicol antibiotic was used as positive control containing the standard drugs of chloramphenicol (30 µg) in

a disc diffusion method which was placed on inoculated plates and incubated for 24 h at 37ºC.

Minimal inhibitory concentration assay

Following the previous screening of plant extracts, the presence of eugenol (an active compound that is capable of inhibiting *L. monocytogenes*) in an appreciable quantity in fresh leaves of *O. gratissimum* and dried buds of *E. caryophyllata* their minimum inhibitory concentrations (MIC) were determined for *Listeria monocytogenes*. Briefly, micro-dilution method using serially diluted (2-fold) plant extracts recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002) was performed and the MICs were determined by agar well diffusion method, inoculated plates were incubated at 37ºC for 18 h. The MICs were determined as the lowest concentration of the extracts inhibiting visible growth of each organism on the agar plate.

Statistical analyses

The results were subjected to simple percentages and Analysis of Variance (ANOVA) using Statistical Package for Social Sciences (SPSS) version 20. The means were separated using Duncan Multiple Range Test (DMST).

Results

Table 1 shows the results of the quantitative phytochemical analysis of the extracts. Analysis of the secondary metabolites in *O. gratissimum* shows that alkaloids were highest in extract using ethyl acetate (270.00±15.00 mg/100 g) while least (196.67±7.64 mg/100 g) was in diethyl ether extract. Highest terpenoids (616.67±5.77 mg/100 g), flavonoids (123.33±10.40 mg/100 g) and tannins (335.00±15.00 mg/100 g) were observed in diethyl-ether extract and least in ethanolic extract (338.33±12.58 and 20.00±5.00 mg/100 g respectively) while ethyl acetate recorded the least tannins (18.33±2.89 mg/100 g). More so, steroids (226.67±12.58 mg/100 g) and saponins (83.33±7.64 mg/100 g) were highest in ethanolic extract while the least of steroids was in ethyl acetate extract (76.67±12.58 mg/100 g) and the least saponins was in diethyl-etheric extract (21.67±5.77 mg/100g) as shown in Table 1. There were statistically significant difference in all the parameters tested among the diethyl ether, ethyl acetate and ethanol extracts (p<0.05).

Table 2 shows that extract of *E. caryophyllata* using ethyl acetate had highest terpenoids (123.33±2.87 mg/100 g), tannins (423.33±10.41 mg/100 g), saponins (83.33 mg/100 g) and flavonoids (165.00±13.23 mg/100 g) while the least tannins (13.33±2.89 mg/100 g), saponins (38.33±10.41 mg/100 g) and flavonoids (41.67±7.64 mg/100g) were observed in extract using diethyl-ether and least terpenoids (13.33±2.87 mg/100 g) was observed in ethanolic extract. However, ethanolic extract had highest alkaloids (340.00±15.00 mg/100 g) and steroids (193.33±7.61 mg/100 g) while the least were observed in extract using diethyl-ether (218.33±10.41 and 50.00±13.21 mg/100 g respectively) as shown in Table 2. There were statistically significant difference in all the parameters tested among the diethyl ether, ethyl acetate and ethanol extracts (p<0.05).

Active constituents of *O. gratissimum*

Table 3 shows the phyto-constituents of the *O. gratissimum* and *E. caryophyllata* extract. Eugenol was identified in the extract of *O. gratissimum* using diethyl-ether (5.63%) and ethyl-acetate (1.98%). Also, in *E. caryophyllata* extract, eugenol were identified in the extract using diethyl-ether (16.60%) and ethyl acetate (71.32%). However, ethanolic extract did not show any trace of eugenol in both plants. More so, phytol and thymol were among other compounds identified in *O. gratissimum* while phenol and complex siloxane were among other compounds identified in *E. caryophyllata*.

Antagonistic activity of *O. gratissimum* and *E. caryophyllata* extract against *L. monocytogenes*

Table 4 shows the MIC of the *O. gratissimum* and *E. caryophyllata* extract using ethyl acetate against *L. monocytogenes* and other *L.* species. Extract of *O. gratissimum* and *E. caryoplyllata* show

Table 1. Quantitative analysis of secondary metabolites in the extracts of *O. gratissimum*

Parameters (mg/100 g)	*O. gratissimum*		
	Di-ethyl ether	Ethyl acetate	Ethanol
Alkaloids	196.67±7.64[c]	270.00±15.00[a]	248.33±12.58[b]
Terpenoids	616.67±5.77[a]	383.33±10.41[b]	338.33±12.58[c]
Tannins	335.00±15.00[a]	18.33±2.89[c]	46.67±2.89[b]
Steroids	81.67±7.65[b]	76.67±12.58[b]	226.67±12.58[a]
Sapoinins	21.67±5.77[b]	30.00±5.00[b]	83.33±7.64[a]
Flavonoids	123.33±10.40[a]	28.33±10.41[b]	20.00±5.00[b]

Values are represented as mean±standard deviation of triplicates; means with different superscripts in the same row are statistically significant different between means at p<0.05.
O. gratissimum: Ocimum gratissimum

Table 2. Quantitative analysis of secondary metabolites in the extracts of *E. caryophyllata*

Parameters (mg/100 g)	*E. caryophyllata*		
	Di-ethyl ether	Ethyl acetate	Ethanol
Alkaloids	218.33±10.41[c]	233.33±10.41[b]	340.00±15.00[a]
Terpenoids	38.33±10.41[b]	123.33±2.87[a]	13.33±2.87[c]
Tannins	13.33±2.89[c]	423.33±10.41[a]	88.33±7.64[b]
Steroids	50.00±13.21[c]	76.67±10.41[b]	193.33±7.61[a]
Sapoinins	38.33±10.41[b]	83.33±7.64[a]	68.33±2.89[a]
Flavonoids	41.67±7.64[c]	165.00±13.23[a]	66.67±2.89[b]

Values are represented as mean±standard deviation of triplicates; means with different superscripts in the same row are statistically significant different between means at p<0.05.
E. caryophyllata: Eugenia caryophyllata

Table 3. Selected Phyto-constituents identified in *O. gratissimum* and *E. caryophyllata*

Solvents	Name of compound	RT (min.)	MF	MW	% Conc.	Qual. Peak (%)
O. gratissiumum						
Di-ethyl ether	Thymol	26.650	$C_{10}H_{14}O$	150	27.83	94
	Phytol	41.213	$C_{20}H_{40}O$	296	5.18	99
	Eugenol	28.307	$C_{10}H_{12}O_2$	164	5.63	98
Ethyl-acetate	Phytol	41.206	$C_{20}H_{40}O$	296	12.90	99
	Thymol	26.964	$C_{10}H_{14}O$	154	4.95	94
	Eugenol	28.299	$C_{10}H_{12}O_2$	164	1.98	98
Ethanol	Benzimidazol-5-amine	38.802	$C_7H_7N_3$	133	25.62	50
	Tetradecamethylcycloheptasiloxane	32.408	$C_{14}H_{42}O_7Si_7$	519	23.64	76
	Dodecamethylcyclohexasiloxane,	26.964	$C_{12}H_{36}O_6Si_6$	445	21.46	83
E. caryophyllata						
Di-ethyl ether	Eugenol	28.323	$C_{10}H_{12}O_2$	164	16.60	98
	9,12-octadecadienoic acid	41.567	$C_{18}H_{32}O_2$	280	8.03	99
	Phenol, 2-methoxy-4-(2-propenyl)-acetate	33.672	$C_{10}H_{14}O_3$	206	7.75	97
Ethyl-acetate	Eugenol	29.250	$C_{10}H_{12}O_2$	164	71.32	98
	Phenol, 2-methoxy-4-(2-propenyl)-acetate	34.089	$C_{10}H_{14}O_3$	206	28.68	97
Ethanol	Octamethylcyclotetrasiloxane	14.898	$C_8H_{24}O_4Si_4$	297	15.80	86
	Dodecamethylcyclohexasiloxane	26.972	$C_{12}H_{36}O_6Si_6$	445	14.24	86
	Tetradecamethylcycloheptasiloxane	32.392	$C_{14}H_{42}O_7Si_7$	510	20.87	60

RT: retention time; MF: molecular formula; MW: molecular weight, RT: retention time; MF: molecular formula; MW: molecular weight; % Conc: percentage concentration; Qual. Peak: Quality Peak; O. gratissimum : Ocimum gratissimum; E. caryophyllata: Eugenia caryophyllata
Minimum inhibitory concentration and zones of inhibition

no significant inhibition until the concentration reaches 10 mg/mL meanwhile extracts using diethyl ether solvent shows inhibition at similar dosage (10 mg/mL) for *E. caryophyllata* and *O. gratissimum* but greater inhibition (more than 10 mm) were observed at above 80 mg/mL against tested Listeria species.

Table 5 shows the zones of inhibition of the extracts. Using *O. gratissiumum,* the zone of inhibition observed on the two *L. monocytogenes* isolates ranged between 11.67±0.37 and 12.33±1.15 mm while other *Listeria* species had (14.00±0.00 mm). The positive control had zone of inhibition ranged between 22.33±1.52 and 26.14±3.00. There were statistically significant differences in zones of inhibition among the extracts ($p<0.05$). However, using *E. caryophyllata* zone of inhibition observed were between 13.67±1.53 and 19.33±1.53 mm against *L. monocytogenes* while 18.33±0.58 and 19.00±2.00 mm were observed in other *Listeria* species. There were statistically significant differences in zones of inhibition among the extracts ($p<0.05$).

Discussion

This study examined the effect of different solvents on active ingredients and antimicrobial performance of fresh leaves of *O. gratissimum* and dried buds of *E. caryophyllata.* The result of phytochemical analysis of *O. gratissimum* leaf showed that it contains alkaloid, terpenoids, tannins, steroids, sapoinin and flavoloids compounds in various quantities which are in agreement with reports of other researchers (Ladipo et al., 2010; Okoye and Madumelu, 2013). The presence of the metabolites further showed that *O. gratissimum* leaf has medicinal properties but the number of active compounds varied with solvents used for extraction. The amount of phytol and thymol recorded in this study was in agreement with other studies (Bayoub et al., 2010; Philippe et al., 2012). However Eugenol was highest (5.63%) in diethyl ether extract which agreed with the report of Philippe et al. (2012) but differed with the works of Matasyoh et al.,(2008) who found eugenol (68.81%) and methyl eugenol (13.21%) in *O. gratissimum* in Kenya and that of Saliu et al. (2011) eugenol (61.9%) in Nigeria. These differences in the chemical composition of constituents of *O. gratissimum* may probably be due to the difference of solvents used in the extraction, climate and period of harvest. For *E. caryophyllata,* alkaloids, saponins, flavonoids, terpenoids, tannins and steroids were present in different quantities with Eugenol, the major compound was found to

be extracted more with ethyl acetate (71.32%) which is in agreement with the works of Hema et al. (2010), Bayoub et al. (2010) who reported 77% to be eugenol in *E. caryophyllata*.

Table 4. Minimum Inhibitory Concentration of ethyl-acetate extract of *O. gratissimum* and *E. caryophyllata* the against Listeria species

	Concentrations (mg/mL)							+ve control
Isolates	**160**	**80**	**40**	**20**	**10**	**5**	**0 (-ve control)**	**(Chlo)**
Ethyl-acetate								
	Ocimum gratissimum							
LM 1	++	++	+	+	+	-	-	++
LM 2	++	++	+	+	+	-	-	++
OLS	++	++	+	+	+	-	-	++
	Eugenia caryophllata							
LM 1	++	++	++	+	+	-	-	++
LM 2	++	++	++	+	+	-	-	++
OLS	++	++	++	+	+	-	-	++
Diethyl ether								
	Ocimum gratissimum							
LM 1	++	+	+	+	+	-	-	++
LM 2	++	+	+	+	+	-	-	++
OLS	++	+	+	+	+	-	-	++
	Eugenia caryophllata							
LM 1	++	+	+	+	+	-	-	++
LM 2	++	+	+	+	+	-	-	++
OLS	++	+	+	+	+	-	-	++

++: Inhibition (more 10 mm diameter); +: Inhibition less than 10mm; -: No activity
LM 1: *L. monocytogenes* isolated from fish muscle; LM 2: *L. monocytogenes* isolated from fish skin; OLS: Other Listeria species isolated from different fish organs; -ve control: Plates with no extract and no antibiotics; +ve control: Plates tested with chloramphenicol (30 µg) antibiotics

The results further showed that extracts of *O. gratissimum* and *E. caryophyllata* are capable of inhibiting listeria species, which might be due to the presence of eugenol. The zones of inhibition recorded in this study shows that *O. gratissimum* and *E. caryophyllata* extracts have antibacterial effect against *L. monocytogenes* bacterium. The MIC was 80 mg/mL although there were high activities at 40 mg/mL but zone of inhibition diameters (13 mm) recorded in 80 mg/mL and above. This value falls within the recommendation of Clinical and Laboratory Standards Institute (CLSI) (2011). The positive control (chloramphenicol) had higher zone of inhibition values against *L. monocytogenes* than the *E. caryophyllata* extract but the variation is not significantly different ($p>0.05$). This result is in variance with the work of Bayoub et al. (2010) but similar to the record of Gupta et al. (2008). The results obtained show that the zones of inhibition values varied between the plants and the level of eugenol contained. From this study, dried buds of *E. caryophyllata* had a higher percentage of eugenol compared to fresh leaf *O. gratissimum* and that solvents (diethyl-ether and ethyl acetate) only affected the percentage of the active ingredient in the extract but did not affect the effectiveness of the extract against *Listeria* species.

The anti-listeria properties of ethyl acetate extracts are required tools in the control of listeria species. In the present study dried buds of *E. caryophyllata* extracts were found to be effective against *L. monocytogenes* and other *Listeria* species which was considered from the MIC (80 mg/mL) against *L. monocytogenes*. The result further suggests that dried buds of *E. caryophyllata* has a strong anti-listeria activity which could be attributed to the higher presence of eugenol. The result is similar to the findings of Nanasombar and Lohasupthawee (2005) and Bayoub et al. (2010) who reported eugenol content in *E. caryophyllata* between 70 and 90%. Furthermore, high content of tannin in *E. caryophyllata* also promote the antimicrobial activity of the plant. Similar result was recorded by Pounce et al. (2003) and Hema et al. (2010).

Table 5. Zone of inhibition of the antimicrobial activity of extracts (80 mg/mL)

Isolates	-ve control (mm)	+ve control (mm)	Diethyl ether	Ethyl acetate
			O. gratissimum (mm)	
L. monocytogenes (1)	0.00^{c}	24.67 ± 0.58^{a}	13.33 ± 1.52^{b}	11.67 ± 0.37^{b}
L. monocytogenes (2)	0.00^{c}	22.33 ± 1.52^{a}	12.33 ± 2.52^{b}	12.33 ± 1.15^{b}
OLS	0.00^{c}	26.14 ± 3.00^{a}	14.00 ± 1.00^{b}	14.00 ± 0.00^{b}
			E. caryophyllata (mm)	
L. monocytogenes (1)	0.00^{c}	24.67 ± 0.58^{a}	13.67 ± 1.53^{b}	16.00 ± 2.00^{b}
L. monocytogenes (2)	0.00^{c}	22.33 ± 1.52^{a}	17.00 ± 1.73^{a}	19.33 ± 1.53^{a}
OLS	0.00^{c}	26.14 ± 3.00^{a}	18.33 ± 0.58^{a}	19.00 ± 2.00^{a}

Values are represented as mean±standard deviation; means with different superscripts in the same row are statistically significant different between means at $p<0.05$.
-ve control: Plates with no extract and no antibiotics; +ve control: Plates tested with chloramphenicol (30 µg) antibiotics; LM 1: *L. monocytogenes* isolated from fish muscle; LM 2: *L. monocytogenes* isolated from fish skin; OLS: Other Listeria species isolated from different fish organs

In conclusion, from the results of this study, it could be concluded that fresh leaves of *O. gratissimum* and dried buds of *E. caryophyllata* have a favourable and promising anti-listeria activity which showed ethyl acetate extracts are capable of becoming valuable agents. These extracts from leaves of *O. gratissimum* and dried buds of *E. caryophyllata* could be used as a natural anti-listeria additive in fish production.

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