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 Antimikrobiyal 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 α=0.05. 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 bacteria, 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 tohumachersının antimikrobiyal performansları üzerindeki etkileri araştırıldı. Bitki parçacıkları soxhlet cihazı içerisinde dietyl-ether, etil asetat ve etanol kullanılarak tespit edildi. Bitkilerin fito-bileşenleri, gaz kromatografi- kütle spektrometresi (GC-MS) kullanılarak saptandı. Yüksek eugenol konsantrasyonlu ekstrakt 7 işlemde hazırlanı (160, 80, 40, 20, 10, 5, ve 0 mg/mL) ve Listeria monocytogenes’e karşı agar kuyucu difüzyon metodudaki 3 kez tekrar edilerek test edildi. Minimum inhibitory concentration (MIC) ve inhibisyon sahası standart tekniğe göre belirlendi. Veriler istatistiksel olarak analiz edildi ve ANOVA testi 0.05 düzeyinde istatistiksel anlamlı olduğunu gösterdi. Bulgulara göre, Ocimum gratissimum’un taze yaprakları ve Eugenia caryophyllata’nın kuru tohumachersının 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 tohumachersının gıda kaynaklı bakterilerin, özellikle L. Monocytogenes’in gelişimini engellemek için kullanılabilmesini 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. fleischmanii* 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-haemolysis, 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, gentamycin, 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, antioxidant, and antiseptic properties (Naczk and Shahidi, 2006; Daayf and Lattanzio, 2008). In addition, the antimicrobial property of medicinal plants may differ depending on the solvents 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 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 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. caryophyllata* 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...
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). The 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 19091-433HP-5M, 5% phenylmethysilox (30 mx250 µm x0.25 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-CACCTGTTCAGAG3’-F and 5’TGACAGCGTGTGATAGCA3’-R) and haemolysis (5’GCAGTTGCAAACCGCTTGAGTGA3’- F and 5’GCAACGTATCCTCAGAGTATG3’-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 Lücke (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 inhibition were measured with meter rule calibrated in millimeter. The chloromphenicol 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 37°C.

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 *L. 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 37°C 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 (383.33±12.58 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 tannins was in ethyl acetate extract (76.67±12.58 mg/100 g) and the least saponins was in diethyl-etheric extract (30.00±5.00 mg/100 g), respectively. Analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) version 20 showed that alkaloids were highest in extract using diethyl-etheric (218.33±10.41 mg/100 g) and the least saponins was in diethyl-etheric extract (83.33±7.64 mg/100 g) were observed 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/100 g) 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).

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. caryophyllata* show cally significant difference in all the parameters tested among the diethyl ether, ethyl acetate and ethanol extracts (p<0.05).

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

| Parameters (mg/100 g) | Di-ethyl ether | Ethyl acetate | Ethanol |
|----------------------|----------------|---------------|---------|
| Alkaloids 196.67±7.64 | 270.00±15.00 | 248.33±12.58 |
| Terpenoids 616.67±5.77 | 383.33±10.41 | 338.33±12.58 |
| Tannins 335.00±15.00 | 18.33±2.89 | 46.67±2.89 |
| Steroids 81.67±7.65 | 76.67±12.58 | 226.67±12.58 |
| Saponins 21.67±5.77 | 30.00±5.00 | 83.33±7.64 |
| Flavonoids 123.33±10.40 | 28.33±10.41 | 20.00±5.00 |

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) | Di-ethyl ether | Ethyl acetate | Ethanol |
|----------------------|----------------|---------------|---------|
| Alkaloids 218.33±10.41 | 233.33±10.41 | 340.00±15.00 |
| Terpenoids 38.33±10.41 | 123.33±2.87 | 13.33±2.87 |
| Tannins 13.33±2.89 | 423.33±10.41 | 88.33±7.64 |
| Steroids 50.00±13.21 | 76.67±10.41 | 193.33±7.61 |
| Saponins 38.33±10.41 | 83.33±7.64 | 68.33±2.89 |
| Flavonoids 41.67±7.64 | 165.00±13.23 | 66.67±2.89 |

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*
Adeshina et al. Antimicrobial Activities of Plants Extracts on *Listeria monocytogenes*  
*Acta Vet Eurasia* 2018; 44: 31-38

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

| Solvents    | Name of compound                  | RT (min.) | MF                | MW    | % Conc. | Qual. Peak (%) |
|-------------|-----------------------------------|-----------|-------------------|-------|---------|----------------|
| *O. gratissimum* |                                     |           |                   |       |         |                |
| Di-ethyl ether | Thymol                            | 26.650    | C₁₀H₁₄O           | 150   | 27.83   | 94             |
|              | Phytol                            | 41.213    | C₁₂H₂₆O₂           | 296   | 5.18    | 99             |
|              | Eugenol                           | 28.307    | C₃₀H₄₀O           | 519   | 1.64    | 98             |
| Ethyl-acetate | Phytol                            | 41.206    | C₂₀H₄₀O           | 154   | 4.95    | 94             |
|              | Eugenol                           | 28.299    | C₁₀H₁₄O₂           | 154   | 1.64    | 98             |
| Ethanol      | Benzimidazol-5-amine               | 38.802    | C₇H₇N₃            | 133   | 25.62   | 50             |
|              | Tetradecamethylcycloheptasiloxane  | 32.408    | C₁₄H₂₀O₇Si₇       | 445   | 21.46   | 76             |
|              | Dodecamethylcyclohexasiloxane      | 26.964    | C₁₂H₃₆O₆Si₆       | 519   | 23.64   | 76             |
| *E. caryophyllata* |                                  |           |                   |       |         |                |
| Di-ethyl ether | Eugenol                           | 28.323    | C₁₀H₁₂O₂           | 164   | 16.60   | 98             |
|              | 9,12-octadecadienoic acid         | 41.567    | C₁₆H₃₂O₂           | 280   | 8.03    | 99             |
|              | Phenol, 2-methoxy-4-(2-propenyl)-acetate | 33.672 | C₁₆H₂₉O₅ | 206 | 7.75 | 97           |
| Ethyl-acetate | Eugenol                           | 29.250    | C₁₀H₁₂O₂           | 164   | 71.52   | 98             |
|              | Phenol, 2-methoxy-4-(2-propenyl)-acetate | 34.089 | C₁₀H₁₂O₂ | 206 | 28.68 | 97           |
| Ethanol      | Octamethylcyclotetrasiloxane      | 14.898    | C₈H₁₆O₄Si₄        | 297   | 15.80   | 86             |
|              | Dodecamethylcyclohexasiloxane     | 26.972    | C₁₂H₃₆O₆Si₆       | 445   | 14.24   | 86             |
|              | Tetradecamethylcycloheptasiloxane | 32.392    | C₁₄H₄₂O₇Si₇       | 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. gratissimum*, 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.

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

| Isolates | Concentrations (mg/mL) | +ve control (Chlo) |
|----------|------------------------|--------------------|
|          | 160 80 40 20 10 5 0    |                    |
| Ocimum gratissimum |                     |                    |
| LM 1     | ++ ++ + + + - - +     | ++                 |
| LM 2     | ++ ++ + + + - - +     | ++                 |
| OLS      | ++ ++ + + + - - +     | ++                 |
| Eugenia caryophyllata |             |                    |
| LM 1     | ++ ++ + + + - - +     | ++                 |
| LM 2     | ++ ++ + + + - - +     | ++                 |
| OLS      | ++ ++ + + + - - +     | ++                 |

++: Inhibition (more 10 mm diameter); +: Inhibition less than 10 mm; -: 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

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

| Isolates | -ve control (mm) | +ve control (mm) |
|----------|------------------|------------------|
| O. gratissimum |                   |                  |
| L. monocytogenes (1) | 0.00c             | 24.67±0.58a      |
| L. monocytogenes (2) | 0.00c             | 22.33±1.52a      |
| OLS      | 0.00c             | 26.14±3.00a      |
| E. caryophyllata |                   |                  |
| L. monocytogenes (1) | 0.00c             | 24.67±0.58a      |
| L. monocytogenes (2) | 0.00c             | 22.33±1.52a      |
| OLS      | 0.00c             | 26.14±3.00a      |

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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