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Isolation and characterization of propitious bioactive compounds from *Cassia singueana* L.

Bilkisu A. Adedoyin[1,3,2]*, Oluremi I. Adeniran[1], Aminu B. Muhammed[2], Sani M. Dangoggo[2], Lutfun Nahar[3], George P. Sharples[3] and Satyajit D. Sarker[3]

[1]Department of Chemistry, University of Abuja, FCT, Nigeria.
[2]Department of Pure and Applied Chemistry, Faculty of Science, Usmanu Danfodiyo University, Sokoto, Nigeria.
[3]Medicinal Chemistry and Natural Products Research Group, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, United Kingdom.

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ABSTRACT

Cassia singueana L. is widely used in traditional medicine for the treatment of various ailments. The present work was set to investigate the antioxidant and antimicrobial activity of this plant and its secondary metabolites. Three bioactive compounds, lupeol, eugenol and octadecadienoic acid methyl ester, were isolated from the root extract of the plant using bioactivity-guided normal phase column chromatography. The antioxidant effect was studied using the *in vitro* assays, *e.g.,* 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), superoxide anion and metal chelation methods. The compounds were screened for their antimicrobial activity against four bacteria, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pneumonia* and a fungus, *Candida albicans* using the agar diffusion method. The MIC/MBC of the extracts were determined by the micro-dilution method. Chemical structures of the isolated compounds were determined using FTIR and NMR spectroscopy. Whilst all three compounds showed certain levels of antioxidant activity, the most prominent antimicrobial activity was observed only against *S. aureus*. In general, results from the work provided some evidence to support the traditional medicinal uses of *C. singueana*.

Keywords: Detection, elucidation, phytochemical, *Cassia singueana.*

*Corresponding author. E-mail: hamatullah1986@gmail.com, bilkisu.adedoyin@uniabuja.edu.ng.

INTRODUCTION

The oxidation of biological molecules by toxic reactive oxygen species (ROS) have been reported as the cause of DNA mutation leading to damage of target cells and tissues, thereby resulting in cell senescence and death (Atawodi et al., 2011). However, active radical scavenger of ROS can serve as a possible prevention for the free radicals generated by these species and the diseases they cause. This resulted in the global interest in searching for and studying natural antioxidants in past few decades (Adedoyin et al., 2013; Usman et al., 2013). Infections caused by bacteria can be prevented, managed and treated through anti-bacterial group of compounds (antibiotics). However, the increasing drug resistance of pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants (Odugbemi, 2008). The devastating nature of diseases caused by oxidative stress has been a great concern all over the world. Recently there is an increase of interest in developing new types of medicinal agents of plant origin with greater potency and reduced side effect against bacteria and oxidative stress (Dugo, 2001).

Cassia singueana plant has been reported to have great medicinal value particularly in the treatment of ulcer, malaria, pile, and diabetes mellitus (Ode et al., 2010). Methanol extract/fractions of the plant leaves has been report to exhibit significant antinociceptive, antipyretic, antiplasmodic, antiulcer, antioxidant, anticancer,

hepatoprotective and antimicrobial activities. Fractions from leaf extracts of *C. singueana* exhibited significant anti-histaminic properties (Ode et al., 2010; Ottu et al., 2013; Ibrahim et al., 2013; Olajide et al., 2011; Adzu et al., 2003). The stem bark was reported to contain anthraquinone and tetrahydroanthracene derivatives with antimicrobial and antispasmodic activities (Adedoyin et al., 2013; Usman et al., 2013; Odugbemi, 2008; Oloyede et al., 2010; Dugo, 2001; Ode et al., 2010; Ottu et al., 2013; Ibrahim et al., 2013; Olajide et al., 2011; Adzu et al., 2003; Ode et al., 2010; Hishe et al., 2018). The ethnomedicinal use of the *C. singuena* leave in the treatment of ulcer was validated scientifically (Ottu et al., 2013; Ibrahim et al., 2013; Olajide et al., 2011; Adzu et al., 2003; Ode et al., 2010). Despite the medicinal potentials, no study has reported any chemical components of the root of this plant. In this paper, we report the isolation of bioactive compounds with antioxidant and antimicrobial activities from the root of *C. singueana* linn.

MATERIALS AND METHODS

Collection and identification of the plant material

The root *C. singueana* plant used in this experiment was collected from the nursery of the Ahmadu Bello University Zaria. The taxonomic identification of the plant was carried out at the herbarium section of the Department of Biological Science A.B.U Zaria, Nigeria. Voucher No. 001242 was deposited at the herbarium. All chemicals and solvents used were of analytical and HPLC grade.

Preparation of the extract

The root of the plant was air dried at room temperature for three weeks and pulverized to a uniform powder of 40-mesh size using wood milling machine. The powdered sample (200 g) was subjected to successive extraction with 1000 ml of each of n-hexane, ethyl acetate and methanol using percolation method for two weeks. Each extract was filtered and concentrated using a rotary evaporator (40°C, 41 mmHg). The final dried samples were stored in labelled sterile bottles and kept at -20°C.

Isolation and purification of compound

Methanol fraction (10 mg) was dissolved in methanol and the solution was spotted on TLC plates. The plates were developed using several solvent systems; Hexane/ethyl acetate (9:1) and ethyl acetate/methanol (7:3) gave better separation of the components, and were used in the TLC monitoring of the Column Chromatography. An aliquot of the methanol fraction (3 g) was subjected to column chromatography on a silica gel (60 to 120 mesh) with gradient elution using hexane and ethyl acetate (Ottu et al., 2013; Ibrahim et al., 2013; Olajide et al., 2011; Adzu et al., 2003; Ode et al., 2010; Hishe et al., 2018; Sobeh et al., 2017; Ior, 2014; Ibrahim and Islam, 2014; Ottu et al., 2013; Ibrahim et al., 2013; Mutasa et al., 1990; Guetchueng et al., 2018). Eluents were collected in 20 ml beakers and TLC was used to monitor the fractions. A total of 33 collections were made and pooled into 10 major fractions, based on their TLC profiles. Fraction 3, 5 and 7 indicated significant proportion of the compound of interest and were further subjected to purification by preparative TLC using the solvent system methanol/ethyl acetate (7:3). A single homogenous spot was obtained on TLC with two different solvent systems hexane/ethyl acetate (9:1) and (7:3). This compounds, coded (X_1, X_2 and X_3), were subjected to spectral analysis.

Spectroscopic characterization

IR, ^{1}H NMR and ^{13}C NMR spectroscopic methods were used to elucidate the structure of the isolated compounds. The IR spectrum was recorded on FTIR-6890s (Agilent technologies) and the NMR spectra were recorded on a Bruker AVANCE-300 Japan (100 MHz and 400 MHz) in MeOD with TMS as internal standard.

Bioactivity assay of the isolated compounds

Brine shrimp lethality assay

The brine shrimp toxicity of X_1, X_2 and X_3 were determined using brine shrimp larvae (*Artemia salina)* method (Wansi et al., 2016; Al-Groshi et al., 2018; Geroushi et al., 2011; Geroushi et al., 2010; Tahsin et al., 2017; Garba et al., 2009). A drop of dimethyl sulphoxide (DMSO) was added to both test and control vials to enhance the solubility of the sample. Brine shrimp eggs (70 g) were hatched in a beaker containing 250 ml of seawater. The beaker was placed beside a window for light and proper ventilation at room temperature. After 48 h the brine shrimp larvae were collected by dropping pipette. About 1 mg portion of each sample were dissolved in 2 ml of methanol. Aliquots of solutions of the test compounds (50, 5 and 1 µg/L) were drawn into vials, two drops of DMSO were added and made up to 2 ml with distilled water corresponding to concentrations of 1000, 100 and 10 µg/ml, respectively. Each dosage was prepared in triplicates including the control. Ten shrimp larvae were added to each vial. The number of the surviving shrimp at each dosage and the control was recorded after 24 h and the LC_{50} was computed using Finney Probity Analysis computer programme (Garba et al., 2009).

Antimicrobial activity

Escherichia coli (NCTC 10418) *Pseudomonas aeruginosa* (NCTC10662), *Staphylococcus aureus* (NCTC6571), *Streptococcus pneumoniae* (NCTC7465) and *Candida albicans* (NCIMB 3179) were used in the *in vitro* antimicrobial assay. *DMSO* was used to dissolve essential samples/drug and to serve as a negative control. Levoxin, ofloxacin and peflotab were used as the standard drugs (positive controls). The agar diffusion method using Muller Hilton Agar (MHA) (Bano et al., 2002; Garba and Okeniyi, 2012) was used to determine the antimicrobial activity of the isolates (X_1, X_2 and X_3) at different concentrations (1.0, 0.5 and 0.25 µg/ml). Agar (35 g) was dissolved in 1 L of distilled water and autoclaved at 121°C for 15 min, cooled and poured into sterile Petri-dishes to solidify. A sterile cork-borer was used to make holes on each seeded agar plate for each concentration of the isolates and the control. Plate count agar (PCA) plates were inoculated with 100 µl of standardized inoculum (1.5×10^8 CFU/ml) of each selected microbe (in triplicates) and spread with sterile cotton swab. The isolate solutions (10 µ/ml) and the control were introduced into the holes containing the bacterial or fungal inoculum and the plates were incubated micro aerobically at 37°C for 24 h. The diameters of zones of inhibition were recorded after 24 h using a transparent ruler. The minimum inhibitory concentration (MIC) and MBC (minimum bactericidal concentration) of the isolates were determined by microdilution agar method as described in the literature (Geroushi et al., 2011; Andrews, 2001). Active cultures for MIC determination were prepared by transferring a loopful of cells

from the stock cultures to flasks and inoculated in MHA medium and incubated at 37°C for 24 h. A 2-fold serial dilution of the isolates were prepared in sterile distilled water to achieve a decreasing concentration ranging from 160 to 1.25 mg/ml in 9 sterile tubes labelled 1 to 9. Sterile cork-borer of 8 mm diameter was used to bore well in the presolidified MHA plates and 100 µl of each dilution was added aseptically into the wells that had microbe isolate seeded with the standardized inoculum (1.5×10^8 CFU/ml) and incubated at 37°C for 24 h. The lowest concentration of a sample showing a clear zone of inhibition after the macroscopic evaluation was considered as the MIC. In the determination of MBC, a 100 µl aliquot from the tube showing MIC was placed on MHA plate and spread over the plate and also incubated. After incubation at 37°C for 24 h, the plates were examined for the growth of a bacterium to determine the concentration of the sample at which 99.9% killing of bacterial isolates was achieved.

Antioxidant activity

The antioxidant property of the isolated compounds of *C. singueana* was determined by three different assays as outlined below.

DPPH free-radical scavenging activity

The 2,2-diphenyl-l-picry hydroxyl radical (DPPH) free-radical scavenging activity of the essential oils was assessed using the method described by Lugasi et al. (1999) and Marijana et al. (2011). DPPH (39.4 mg) was dissolved in 100 ml of methanol to give a 1M solution. The solution was allowed to stand for 10 min and the absorbance at 517 nm was measured. About 2 ml of the solution (0.25, 0.50, 1.00 and 1.50 mg/ml) of the isolates (X_1, X_2 and X_3) in methanol was prepared, 2 ml of 1M DDPH was added to 0.5 ml of each of the test solution. The mixture was shaken and left to stand for 10 min, and the absorbance of the solutions was measured at 517 nm against that of control. The percentage inhibition was calculated using the following equation. The same procedure was followed using butyrate hydroxyl anisole (BHA), ascorbic acid and α-tocopherol, which were used as positive controls.

$$Inhibition = \frac{(A_{DPPH} - A_S)}{A_{DPPH}} \times 100\%$$

A_{DPPH} and A_S are the respective absorbance of the neat DPPH and test solutions, respectively.

Superoxide scavenging activity

The scavenging effect of the isolates (X_1, X_2 and X_3) towards superoxide anion radicals were measured using the method published by Nishimiki et al. (1972). A volume of 1 ml of nitroblue tetrazolium solution (156 µM in 100 mM phosphate buffer, pH 7.4), 1 ml of nicotine amide dinucleotide solution (468 µM in 100 mM phosphate buffer, pH 7.4), and 1 ml of sample solution (0.25, 0.5 and 1.0 mg/ml) in methanol were mixed. The reaction started with the addition of 100 µl of phenazine methosulphate solution (60 µM in 100 mM phosphate buffer, pH 7.4) to the mixture and after 5 min at room temperature, the absorbance at 560 nm was measured. BHA, α-tocopherol and ascorbic acid were used as positive controls. The percentage inhibition of scavenging effect of anion superoxide anion was calculated the equation below.

$$Inhibition = \frac{(A_{blank} - A_{Sample})}{A_{blank}} \times 100\%$$

Where, A_{blank} is the absorbance of the blank in absence of sample, and A_{sample} is the absorbance in the presence of the sample.

Metal chelating activity

The chelation of ferrous ions by the isolates (X_1, X_2 and X_3) was estimated by the method described by Dinis et al. (1994). The reaction mixture contained 0.5 ml of the sample in methanol (0.25, 0.5, 1.0 and 1.5 mg/ml), 1.5 ml of deionized water and 0.5 ml of 2 mM of $FeCl_2$ solution. After 30 min, 1.0 ml of 5 mM ferrozine solution was added. After 10 min of incubation at room temperature, the absorbance at 562 nm was measured. Ascorbic acid, BHA, and α- tocopherol were used as positive controls. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated from the following equation.

$$\%\ activity = \frac{(A_{control} - A_{Sample})}{A_{control}} \times 100\%$$

Where, $A_{control}$ is the absorbance of the blank in absence of the sample, and A_{sample} is the absorbance in the presence of the sample.

RESULTS AND DISCUSSION

Compound X_I was obtained as white needles with melting point 213°C, The IR spectrum of X_1 showed characteristic absorption bands at IR v_{max} cm^{-1}: 3250.45, 2916.10, 2849.28, 1641.97, 1578.02, 1470.83, 882.73. The absorption signal at 2916.10 and 2849.28 cm^{-1} stretching absorption bands due to C-H stretching of alkanes. An intense broad band at 3250.45 cm^{-1} due to O-H stretching of a hydroxyl group and 1641.97 and 1578.02 cm^{-1} (C=C) stretching of vinyl alkenes. There were also 1470.83 cm^{-1} due to C-O to scissoring and 882.73cm^{-1} which was due to unsaturated out of plane bending of the terminal -CH_2 vibration. The ^{1}H NMR($CDCl_3$, 400MHz): δ 4.70, 4.55(2H, *s*, H-29a, 29b), 3.2(1H, *m*, H-3), 0.79, 0.81, 0.86, 0.98, 1.07, 1.28, 1.35 (each 3H, *s*); ^{13}C NMR($CDCl_3$, 100MHz): δ 151.0(C-20), 109.0(C-29), 79.0(C-3), 55.5(C-5), 50.5(C-9), 48.3(C18), 48.0(C-19), 43.0(C-17), 42.9(C-14), 40.9(C-8), 40.0(C-22), 38.9(C-4), 38.7(C-1), 38.1(C-13), 37.2(C10), 35.5(C-16), 34.2(C-7), 29.9(C-21), 28.0(C-23), 27.4(C-2), 27.1(C-15), 25.2(C-12), 21.0(C-11), 19.5(C30), 18.5(C-6), 18.0(C-28), 16.1(C-25), 16.0(C-26), 15.5(C-24), 14.8(C-27).

The ^{1}H-NMR spectrum of compound X_1 showed signals due to seven methyl groups at 0.79 (3H), 0.81 (3H), 0.86 (3H), 0.98 (3H), 1.07 (3H), 1.28 (3H) and 1.35 (3H) ppm, respectively, an olefinic methyl group at 1.71 (3H) ppm and a broad doublet due to a terminal methylene protons at 4.68 ppm (IH, *J* = 11.61 Hz) which are typical of triterpenoid (Andrews, 2001). The spectrum also showed another doublet at 3.20 ppm (*J* = 6 and 9 Hz) due to a methine proton joined at the carbon atom to which the hydroxyl group was bonded. A sextet of one proton at δ 2.37 ascribable to 19β –H is characteristic of lupeol. The H-3 proton showed a multiplet at δ 3.2 while a pair of broad singlets at δ 4.55 and δ 4.70 (IH, each) was

indicative of olefinic protons at (H-29 a & b). The ^{13}C NMR experiments which showed seven methyl groups at [δc: 28.0 (C-23), 18.0 (C-28), 16.1 (C-25), 16.0 (C-26), 15.5 (C-24), 14.8 (C27) and 19.5 (C-30)]; the signals due to an exomethylene group at [δc: 109.3 (C-29) and 151.0 (C20)]; ten methylene, five methine and five quaternary carbons were assigned with the aid of DEPT experiment. The deshielded singnal at δc 79.0 was due to C-3 which is bonded to the hydroxyl group. The confirmation of the structure of X_1 was accomplished through the 2D NMR experiments (COSY and HMBC).

The COSY spectrum of X_1 indicated some cross signals such as between δ_H 2.38, H-19 and one sp^3 methylene proton signal (δ_H 1.38, H-21) and another sp^3 methine proton signal (δ H 1.79, H-18); and between oxygenated methine proton signal (δ_H 3.2, H_30 and sp^3 methylene signal (δ_H 1.60, H-2). In the HMBC spectrum, the methine proton signal at δ_H 3.2 (H-3) showed cross signals with a methyl carbon signal (δc 27.0, C-23) by J_2 correlation and a methyl carbon signal (δc 19.5, C-6) by J_3 correlation. The sextet methyl signal at δ_H 2.38 (H-19) showed cross signals with two methylene carbon signals δc 28.9 (C-21) and δc 108.3 (C-29)], a methine carbon signal [δ_c 49.3 (C- 18), a methyl carbon signal [δ_c 18.9 (C-30)] and a quaternary carbon signal [δ_c 151.2 (C-20)]. The two broad singlets of olefinic proton at δ_H 4.55 and 4.70 showed cross signals with a methylene carbon signal [δ_c 47.0 (C-19) and δ_c 19.5 (C-30)] by J_3 correlation. The data compared well to reported data on Lupeol (Ahmed et al., 2007). Therefore, the structure of X_1 was tentatively suggested to be Lupeol (Lup-20(29)-en-type triterpene (Figure 1).

Compound X_1 (lupeol) isolated from the methanol extract of *C. singueana* root has earlier been isolated from plants such as *Cassia hirta* (Bano et al., 2002) and *Tacazzea apiculat*a (Ahmed et al., 2007).

Compound X_2 was obtained as an off-white pasty substance with a characteristic aroma and melting point -11 to -9°C. IR v_{max} cm^{-1}: 3500.7, 3000 .20, 2910.43 2849.28, 1600.02, 1440.53, 1290.27, 886.73, 882.73. The infrared spectrum of compound X_2 shows broad stretching absorption peaks at 3500.7cm^{-1} indicating (O-H) O-H stretch of phenol, 3000.20 cm^{-1} (C-H) stretch of aromatic, 2,910.43 cm^{-1} (C-H) terminal methyl stretch, 1600.02 cm^{-1} (C-C) of in ring aromatic and 1290.27 cm^{-1} (C-O) stretch of alcohol or phenol. There are also 1440.53 (C-H) bending and 886.73 out of plane bending of terminal CH_2, respectively. The total ion chromatogram of compound X_2. The mass spectrum shows that the molecular ion of compound X_2 is 164.

^1H NMR($CDCl_3$, 400MHz): δ 6.92 (1H, *d*, H-6), 6.78 (1H, *d*, H-5), 6.74 (1H, *s*, H-3), 5.94 (1H, *m*, H-8), 5. 54 (2H) 5.09 (1H, *d*, H-9a), 5.07 (1H, *d*, H-9b), 3.88 (2H, *d*, H-7) , 3.86 (3H, *s,* OCH_3); ^{13}C NMR($CDCl_3$, 100MHz): δ 146.4 (C-1) 143.8 (C-2) , 138.2 (C-4), 110.0 (C-3), 121.3 (C-5), 115.6 (C-6), 131.2 (C-8), 114.1 (C-9) 55.8 (C-10) and 39.9 (C-7).

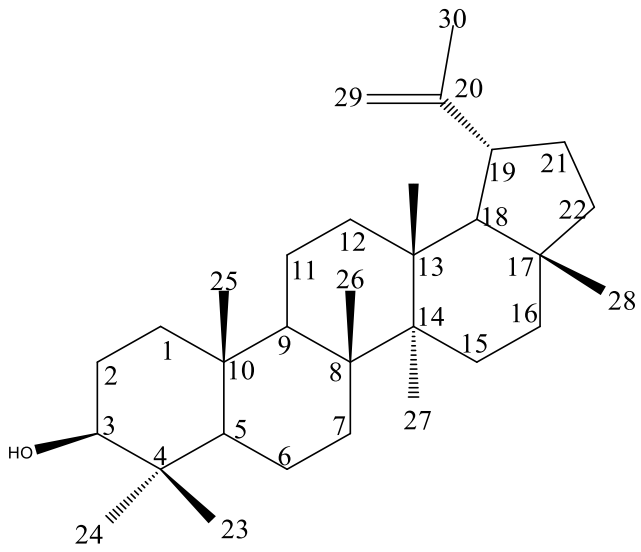

Figure 1. Proposed structure of X1; Lupeol [Lup-20(29)-en-type triterpene].

The ^1H-NMR showed the presence of 12 protons in the molecule. The presence of 3 protons signals downfield at 6.74, 6.78, 6.94 ppm (1H each) indicates the presence of an aromatic ring with 3-protons on the ring in the molecule. The signal downfield at 5.14 ppm (1H) signifies the presence of hydroxyl group attached to the aromatic ring. The presence of a singnal at 3.86 ppm (3H) suggests the presence of a methoxy group on the aromatic ring. Furthermore, the doublet signal at 3.28 ppm (2H) indicates the presence of CH_2 attached to an aromatic ring. The downfield doublet signal at 5.07 and 5.08 (1 H each) suggests the presence of an exocyclic double bond which was supported by the presence of one proton multiplet at 5. 54 ppm for methine proton. These values were in agreement with the observation of Awasthi et al. (2008), Grice et al. (2010) and Ahmed et al. (2007).

The $^{13}CNMR$ spectrums of compound X_2 indicate the presence of ten carbons in the molecule located at 146.4, 143.8, 138.2, 131.2, 12.3, 115.6, 114.1, 111.2, 55.8 and 39.9 ppm respectively representing carbon C-1 to C-10, respectively. The DEPT experiment confirmed the signal at δ 146.4 (C-1) 143.8 (C-2) and 138.2 (C-4) to be quaternary carbons, signal at δ 110.0 (C-3), 121.3 (C-5), 115.6 (C-6) and 131.2 (C-8) ppm signifies four tertiary carbons in the molecule. The signal at δ 39.9 (C-7) and 114.1 (C-9) represent the secondary carbons and at δ 55.8 (C-8) suggested to be primary carbon in the molecule. Signals at 146.4, 143.8 and 138.2 ppm, respectively represent three quaternary carbons. There were also four CH peaks out of which three appeared in the aromatic region (115.6, 121.2 and 111.2 ppm), the remaining peaks (131.2, 114.1, 55.8 and 39.9 ppm) belonged to side chain attached to the benzene ring. The

signal at 146.4, 143.8 and 138.2 ppm respectively are suggested to be position in which the hydroxyl, methoxyl, and vinyl side chain are located in the molecule. The detailed results compared to recent literature (Tahsin et al., 2017; Awasthi et al., 2008; Ahmed et al., 2007).

From the ^{1}HNMR, out of the three signals due to aromatic protons, one is singlet at δ 6.74 ppm and the remaining two aromatic proton at 6.78 and 6.94 ppm are doublets. These could suggest that position of Carbon 1, 2 and 4 on the aromatic ring are substituted. Hence the downfield quaternary carbon at δ 146.4 (C-1),143.8 (C-2) and 138.2 (C-4) respectively, then δ 146.4 (C-1) and 143.8 ppm (C-2) should be assigned to aromatic carbons (1 and 2) bearing a hydroxyl and methoxy groups, respectively while the upfield quaternary carbon at 138.2 ppm (C-4) was signal for allylic group attached to the ring at position 4 (Awashi et al., 2008; Ahmed et al., 2010) suggesting the compound X_2 to be phenolic compound and GC-MS NIST library (supplementary data – S2) all supported the suggested structure as 2-methoxy-4-(2-propenyl)phenol or 4-allyl-2-methoxyphenol (eugenol). From IR, NMR and GC-MS data and their data obtained which compared well with the available literature (Ahmed et al., 2007; Grice et al., 2010) the structure of compound X_2 tentatively suggested to be eugenol (Figure 2).

Compound X_2 isolated from the methanol extract of *C.singueana* was tentatively identified as eugenol which has been earlier isolated from other plants such as Syzygium *aromaticum*, *Ocimum sanctum* (Prakash and Gupta, 2005), *Cymbopogon ambiguus* (Awasthi et al., 2008) and *Tacazzea apiculat*a (Ahmed et al., 2007).

Compound X_3 was obtained as pale yellow oily pasty substance, soluble in n-hexane ethyl acetate and methanol with melting point 31 to 35°C. IR ν_{max} cm^{-1}: 3470.28, 2922, 2852.47, 1729.57 and 1625 cm^{-1}. The infrared spectrum of compound X_3 showed stretching absorption peaks at 2922 and 2852.47 cm^{-1} indicating (C-H), 1729.57 cm^{-1} (C=O), 1625 cm^{-1} (C=C) terminal methyl, the were also 1464.87 cm^{-1} (C-O) scissoring and 782.73 cm^{-1} out of plane bending of the terminal -CH_2 respectively. The C–O stretching vibration at 1038.24 cm^{-1} due C-O stretching of carbonyl compounds. The mass spectrum shows that the molecular ion of compound X_3 is 298.

The ^{1}HNMR spectrum of compound X_3 reveals the presence of signals at 3.669 and a doublet at of –CH_2 protons at 2.308 ppm. These two peaks are the distinct peaks for the confirmation of methyl esters in the molecule. Other observed peaks were at 0.894 ppm indicating the presence of terminal methyl protons, a strong signal at 1.625 and 1.314 ppm signifies the presence of methylene protons of the long carbon chain and 5.399 ppm due to olefinic methane protons (Monterio et al., 2009; Samio et al., 2010).

The prominent signals recorded in the ^{13}C-NMR spectrum of compound X_3 include 174.2, 130.1, 129.2, 51.8, 51.8, 34.1, 32.08, 29.8, 27.4, 25.08, 22.82 and 14.0 ppm respectively. Signals at 174.2 and 51.4 ppm

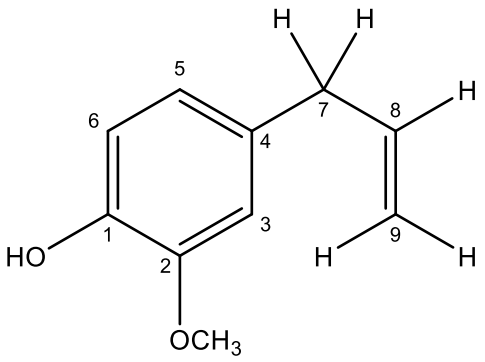

Figure 2. 4-allyl-2-methoxyphenol [Eugenol] **.**

representing characteristic peaks of ester carbonyl (–C=O) and C–O respectively signifying the presence of carbonyl esters in the molecule. The peaks at 130.1 and 129.2 ppm indicate the presence of unsaturation in the molecule. Other peaks are related to the terminal carbon of methyl groups at 14.0 ppm and methylene carbons of long carbon chain in the range of 22.82 to 34.1 ppm (Guetchueng et al., 2017; Al-Groshi et al., 2018; Monterio et al., 2009).

The ^{1}HNMR spectrum of compound X_3 shows signals at δ 3.633 (3H) related to methyl protons of carbonyl ester group and at 2.635 ppm (2H) of –CH_2 protons are suggested distinct peaks for the confirmation of methyl esters in the compound. The signals at 5.37 and 5.42 ppm (2H each) represent the methine protons of an olefinic group in the molecule, signal at δ 0.866 (3H) related to methyl proton of a long carbon chain. Other protons includes peaks at δ 2.251, 1.962, 1.964, 1.562, 1.427, 1.425, 1.275, 1.252, 1.277 and 1.241 ppm (2 H each) represent the methylene protons of the long carbon chain. The methylene protons between 1.241 to 2.251 ppm and a terminal methyl at 0.866 ppm indicate that the compound has long chain –CH_2 molecule, supported by the appearance of a prominent doublet signal at 2.635 ppm (2H) due to the carbonyl group. The appearance of the methylene protons at 1.562 ppm (2H) due to an olefinic group attached to –CH_2 carbon along the chain (Monterio et al., 2009; Samio et al., 2010; Okeke et al., 2001). The carbonyl group of the fatty acid is partly responsible for the appearance of the proton at 2.65 ppm.

This is supported by ^{13}CNMR with the appearance of signals at 174.2 ppm due to an ester carbonyl group. The ^{13}CNMR spectrum of compound X_3 r eveal the presence of signals located at 174.2, 51.82, 130.10, 129.10, 14.0 and 22.82–32 ppm represents C1-C19 respectively (Figure 3). Signals at 174.2 (C-2) and 51.82 (C-1) representing characteristic peaks of ester carbonyl (C=O) and C–O respectively. The peaks at δ 130.10 (C-9 and C-13) and 129.10 (C-10 and C-13) ppm indicate the position of the olefinic carbons in the molecule. Signal at δ 14.00 (C-19) ppm suggested to be methyl group carbon and methylene carbons of long

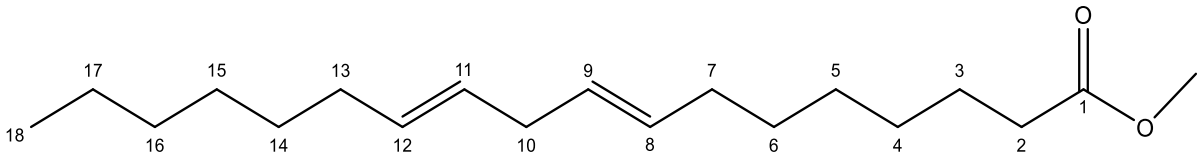

Figure 3. 8-11-Octadecadienoic acid methyl ester [Methyl 8,11-octadecadienoate].

carbon chain are include signals at δ 32.08 (C-8 and C-14), 27.4 (C-6 and C-16), 25.8 (C-11), 28.9 (C-15), 25.08 (C-17), 22.82 (C-18), 27.3 (C-7), 29.7 (C-5) 29.3 (C-4) and 32.8 (C-3) (Chin et al., 2006).

From ^{1}HNMR and ^{13}CNMR spectra of compound X_3 (S6 and S7), it can be observed that proton at δ 0.866 (3H) signifies methyl protons of (C-19) at 14.00 ppm in the spectrum. Furthermore, the methine protons at δ 5.42 and 5.37 are related to the unsaturated carbons at δ 130.1 and 129.2 ppm respectively. Methylene protons at 2.25 ppm are due to C-11 with the signal at 32.8 ppm located between the olefinic carbons and methyl protons at δ 3.633 (3H) are suggests for the methyl protons at 51.28 ppm (C-1) respectively (Figure 3.). The appearance of these signals suggests that compound X_3 is likely fatty esters (Ahmad et al., 2005). The information on IR, NMR, and GC-MS NIST library tentatively suggest that structure of compound X_3 to be 8-11-Octadecadienoic acid, methyl ester (Figure 3).

The data obtained are also in accordance with the available literature (Monterio et al., 2009). Compound X_3 isolated from the methanol extract of *C. singueana* was octadecanoic acid methyl ester which has been earlier isolated from plants such as hemp plant (Okeke et al., 2001) and *Cassia apiculata* (Prakash and Gupta, 2005).

Lupeol (X_1) is commonly found in most plants, Ahmed et al. (2007) reported high antimicrobial and antioxidant activity of lupeol.in *Cassia apiculata*. Eugenol (X_2) and octadecadienoic acid (X_3) are of limited occurrence. Although the compounds X_3 isolated (methyl 8,11-octadecadienoate) showed moderate antimicrobial and antioxidant activities against the selected pathogens and free radicals. Moreover, the free radical scavenging effect of the crude extracts and eugenol isolated from the methanolic root extract might have contributed significantly to the activities exhibited by the plant.

Cytotoxic analysis of the isolated compounds

The compounds isolated were tested for their, cytotoxicity, antimicrobial and antioxidant properties. The brine shrimp test of the isolated compound showed that compounds X_1 and X_2 were highly active (Table 1).

Table 1. Brine shrimp test of the isolated compound.

Compounds obtained	BST LC_{50} (µg/cm^3)*
X_1	23.56 (5.83-85.57)
X_2	57.52 (15.91-225.61)
X_3	100.00 (6.73-1486.75)

* Bracket values = High-Low 95% Confidence interval. (BST, LC_{50} 23.56 and 57.52 µg/cm^3, respectively). Compound X_3 least active (BST LC_{50} 100.00 µg/cm^3).

Antimicrobial activity of isolated compounds

The results of antimicrobial analysis of the isolated compounds are presented in Table 2a, 2b and 2c respectively. The activity assay carried out on the compounds showed that compound X_3 showed activity against S. *typhi* and *A. niger* with zone of inhibition of 29 and 30 mm, respectively. Compounds X_1 and X_2 were active against all test microbes with zone of inhibition range of 20 to 30 mm (Tables 2a-c). The antimicrobial efficacies of the isolated compounds are compared with those of the reference standards. The antimicrobial activities of the isolated compounds were slightly lower than those of reference standards. These are comparable to the reference standard at the same concentration (Table 2a). The isolated compounds showed high activities of inhibitions ranging from 20 to 30 mm against *S. aurenus* and *S. pneumonia* at the concentration of 800 µg/cm^3 while amoxicillin and erythromycin show high activities of inhibition of 22 to 34 mm. However, compound X_2 shows the same inhibition of 30 mm with amoxicillin against *S. aureus* at these concentrations.

Chloramphenicol and Gentamycin were used as reference standards for gram negative bacteria. Antimicrobial activities of the isolated compounds against *E. coli, S. typhi*, and *P. aeruginosa* was high (zone of inhibition range of 24 to 30 mm) at the concentration of 800 µg/cm^3 which were also comparable with those of the reference standards (Table 2b). Although, the reference standards show slightly higher activity with the zone of inhibition of 25 to 35 mm, however compound X_3 showed a significantly high activity with a zone of inhibition of 29 mm against *S. typhi* compared to chloramphenicol and gentamycin with 25 and 26 mm respectively against *S. typhi* (Table 2b).

The results of antifungal activity of the isolated compounds were also compared with those of biocoten and peflotab ointments as reference standards. Isolated compounds showed high activities with the zone of

Table 2a. Antimicrobial activity of isolated compounds against gram positive bacteria.

Test samples	Concentration (µg/cm^3)	Zone of inhibition *Staphylococcus aureus*	Diameter (mm) *Streptococcus pneumonia*
Amoxicillin®	200	17±0.2	17±0.4
	400	22±0.3	22±0.4
	600	28 ±0.1	27±0.3
	800	33±0.2	30±0.4
	C	NI	NI
Erythromycin®	200	18±0.5	17±0.1
	400	22±0.4	25±0.2
	600	26±0.3	30±0.3
	800	30±0.4	34±0.3
	C	NI	NI
X_1	200	10±0.2	11±0.2
	400	15±0.3	17±0.3
	600	22±0.4	21±0.4
	800	30±0.3	28±0.4
	C	NI	NI
X_2	200	10±0.3	12±0.4
	400	17±0.5	18±0.5
	600	24±0.4	25±0.7
	800	28±0.2	29±0.9
	C	NI	NI
X_3	200	08±0.3	09±0.4
	400	12±0.1	13±0.2
	600	16±0.3	17±0.4
	800	20±0.1	21±0.3

*Key: C = Control, NI = No inhibition.

Table 2b. Antimicrobial activity of isolated compounds against gram negative bacteria.

Test samples	Concentration (×10^2 µg/cm^3)	Zone of inhibition *Escherichia coli*	Diameter (mm) *Pseudomonas aeruginosa*	*Salmonella typhi*
Gentamycin®	200	17±0.34	12±0.41	10±0.41
	400	22±0.23	17±0.44	15±0.44
	600	29 ±0.31	23±0.3	20±0.3
	800	35±0.22	30±0.3	25±0.3
	C	NI	NI	NI
Chloramphenicol®	200	14±0.51	10±0.11	11±0.11
	400	20±0.42	17±0.21	16±0.21
	600	26±0.32	23±0.33	20±0.33
	800	33±0.44	30±0.32	26±0.32
	C	NI	NI	NI
X_1	200	10±0.21	11±0.22	10±0.61
	400	15±0.31	17±0.29	15±0.21
	600	22±0.35	21±0.34	20±0.51
	800	29±0.27	30±0.39	24±0.32
	C	NI	NI	NI

Table 2b. Continues.

	200	10±0.43	12±0.01	12±0.11
	400	17±0.21	18±0.47	17±0.21
X_2	600	24±0.22	23±0.71	22±0.33
	800	28±0.21	28±0.11	27±0.32
	C	NI	NI	NI
	200	08±0.33	09±0.41	08±0.41
	400	12±0.12	12±0.22	13±0.44
X_3	600	16±0.32	16±0.43	17±0.3
	800	21±0.11	22±0.33	29±0.3
	C	NI	NI	NI

Table 2c. Activity of isolated compounds against fungi.

Test samples	Concentration (µg/cm^3)	Zone of inhibition *Aspergillus niger*	Diameter (mm) *Candida albicans*
	200	10±0.14	10±0.55
	400	16±0.30	17±0.41
Biocoten®	600	20 ±0.51	23±0.09
	800	26±0.25	27±0.10
	C	NI	NI
	200	13±0.61	11±0.42
	400	20±0.31	19±0.22
Canesten®	600	24±0.08	23±0.03
	800	29±0.45	30±0.90
	C	NI	NI
	200	10±0.21	10±0.29
	400	15±0.33	15±0.23
X_1	600	21±0.35	19±0.37
	800	27±0.77	28±0.49
	C	NI	NI
	200	10±0.44	12±0.01
	400	17±0.61	19±0.47
X_2	600	20±0.82	23±0.71
	800	26±0.91	28±0.11
	C	NI	NI
	200	08±0.31	07±0.49
	400	13±0.19	12±0.83
X_3	600	19±0.52	16±0.73
	800	30±0.16	21±0.85
	C	NI	NI

inhibition of 26 to 30 mm at the concentration of 800 µg/cm^3 which is quite comparable to the refrence standards showing zone inhibition of 26 to 30 mm, respectively (Table 2c).

Antioxidants activity of the isolated compounds

The results of the Radical Scavenging Activity assay of the isolated compounds (Figure 4) showed that the free

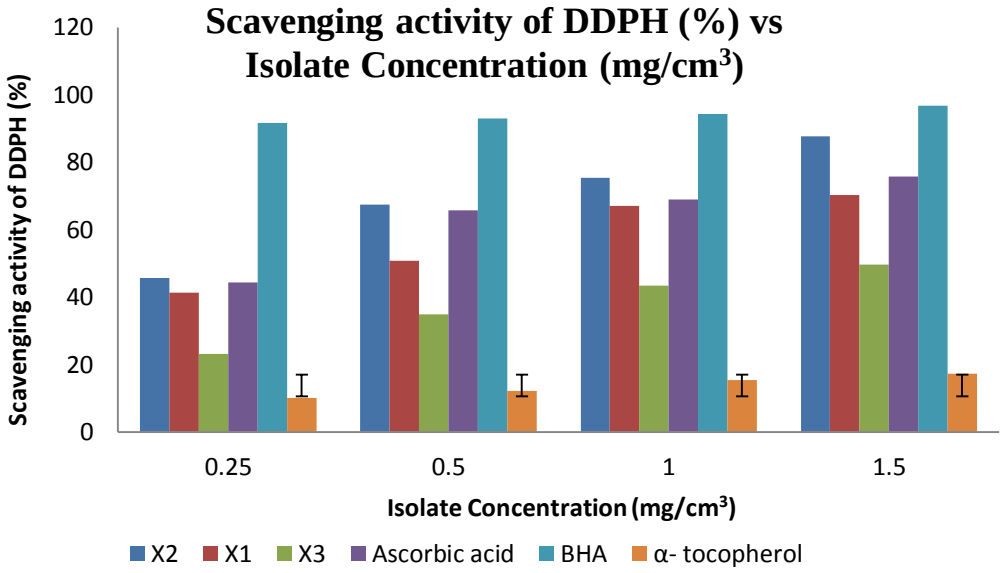

Figure 4. Comparison of DPPH radical scavenging ability of the isolated compounds. Key: BHA = Butylated hydroxyanisole .

radical scavenging activities of the isolated compounds increased with increase in the concentration of the later. However, it was observed that compound X_2 (83.5%) possesses better antioxidant activities than compounds X_1 and X_3 (67.7% and 45%) respectively (Figure 4). For instance, the percentage inhibition shown by Compound X_2 in DPPH at the concentration of 1.5 mg/cm^3 was 83.5% which is close to that of BHA (89.6 %) and higher than those of ascorbic acid and α- tocopherol 75.8 and 17.3%, respectively.

The result of the scavenging capacity of the isolated compounds against the superoxide anion free radicals shows that the effects of the compounds increased with increasing concentration (Figure 5). The highest scavenging activity of the compounds in this assay was 74.2 % at the concentration of 1.5 mg/cm^3 exhibited by compound X_2. The effectiveness of the isolated compounds as anion superoxide scavenger range in the following descending order; BHA > X_2 ≥ Ascorbic acid > α-tocopherol. X_1 > X_3.

Figure 6 shows the results of the metal chelating capacity of the isolated compounds against ferozine. The effectiveness of antioxidants in metal chelating ranged in the following descending order: BHA>X_2 > X_1 > Ascorbic acid > X_3 > α-tocopherol.

Median effective concentration (EC_{50}) of the isolated compounds on free radicals

The EC_{50} obtained for isolated compounds (Table 3) were 2.50 µg/cm^3 for compound X_2, 6.7 µg/cm^3 for compound X_1 and 136 µg/cm^3 for compound X_3, respectively. These were compared with those of standard antioxidants used (BHA, Ascorbic acid, and α-tocopherol). Compound (EC_{50} = 2.52 µg/cm^3) poses similar activity with BHA (EC_{50} = 2.5 µg/cm^3) and exhibit better activity than ascorbic acid (EC_{50} = 6.9µg/cm^3) and α-tocopherol (EC_{50} = 35 µg/cm^3) respectively. Compound X_1 (EC_{50} = 6.7 µg/cm^3) also possesses similar activity with Ascorbic acid (EC_{50} = 6.9 µg/cm^3) but lower activity compared to BHA (EC_{50} = 2.5µg/cm^3). However, compound X_3 (EC_{50} = 34 µg/cm^3) exhibit lower activity compare to compound X_1 and X_2, respectively.

Brine shrimp toxicity of the isolated compounds of C. singueana flowers (LD50 = 18.7 µg/ml) indicated that compound X1 and X2 (LD50 = 23.56 and 57.52 µg/ml, respectively) possess higher cytotoxicity. Compound X_1 (Lupeol) recorded scavenging activity 67% (EC_{50} = 6.9 µg/cm^3) which indicate good antioxidant property. Compound X_3 (8,11-octadecanoic acid, methyl esters) possess low antioxidant activity (32.4 %, EC_{50} 20.3.µg/cm^3). The scavenging activity of the compound X_2 (Eugenol) in the DPPH assay (Figure 4) shows that the compound can significant ($P < 0.05$), scavenge DPPH radical (EC_{50} 2.52 µg/cm^3). In the superoxide anion assay, the antioxidant activity of methanol extract of *C. singueana* root was obtained to be 77.5% (EC_{50} = 27.3.0 µg/cm^3).The results obtained show increase in inhibitory activity of superoxide anion radical scavenging effects with increasing concentrations. The isolated compounds exhibited good to moderate activity, i.e lupeol (EC_{50} = 30.0 µg/cm^3). Eugenol (EC_{50} =15.0 µg/cm^3) and 8, 11-octadecanoic acid, methyl ester (EC_{50} = 38.1 µg/cm^3), however good effects were recorded against Eugenol (EC_{50} = 2.52 µg/cm^3) (Figures 4 to 6), this might also be attributed to the higher polarity of compound X_2.The antioxidant activity of eugenol (X_2) has long been known

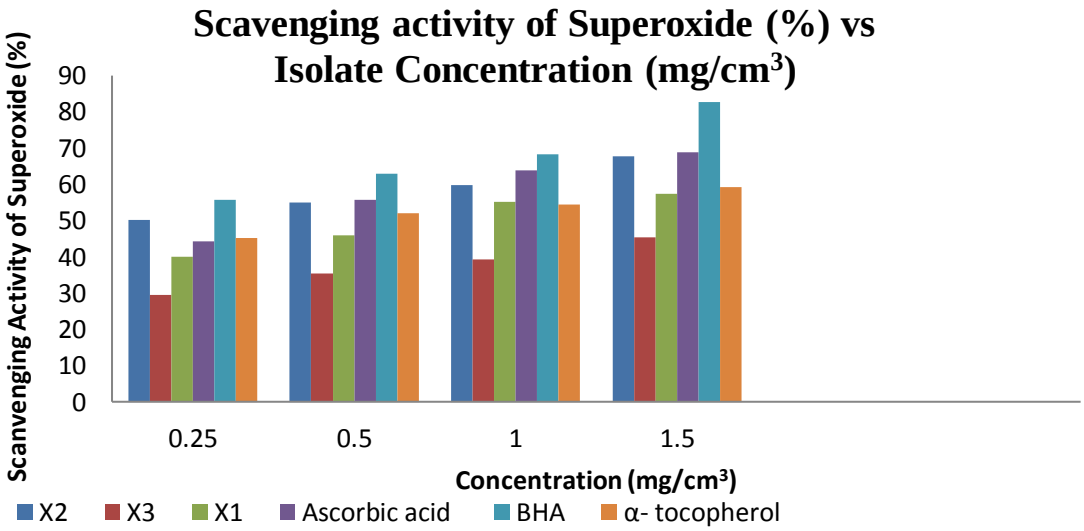

Figure 5. Comparison of superoxide radical scavenging ability of the isolated compounds. Key: BHA = Butylated Hydroxy Anisole.

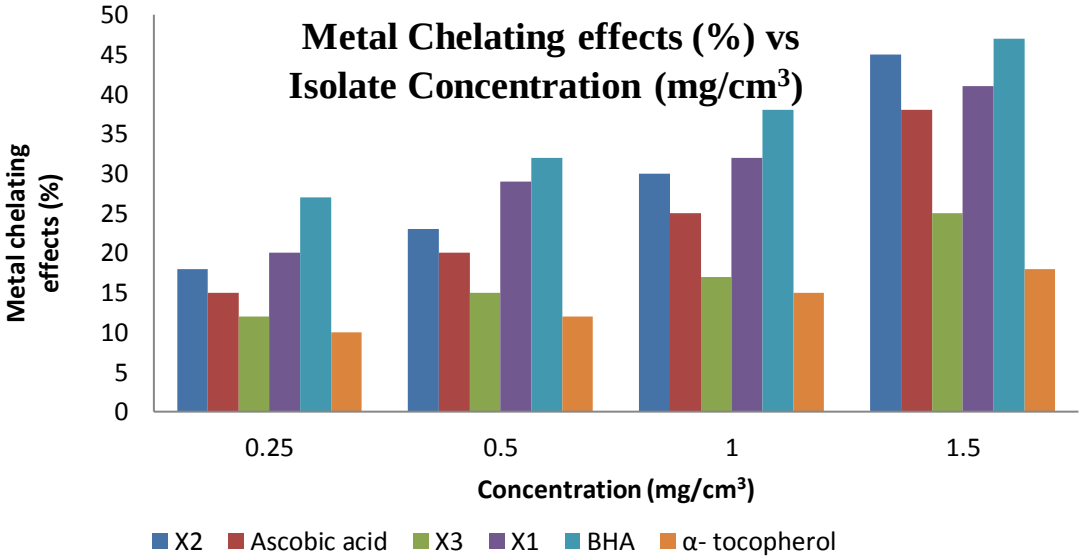

Figure 6. Comparison of metal chelating effects of the isolated compounds on ferrous ions. Key: BHA= Butylated Hydroxy Anisole.

Table 3. Median effective concentration [EC_{50}] of the isolated compounds.

Compound EC_{50} (µg /cm^3)	DPPH assay	Superoxide anion assay	Metal chelation assay
X_1	6.7	15.0	20.3
X_2	2.52	5.4	35.0
X_3	34.0	38.1	60.0
BHA	2.5	2.8	15.0
Ascorbic acid	6.5	15.0	75.0
α-tocopherol	34	125.0	250

Key: X_1 = Compound X_1, X_2 = Compound X_2, X_3 = Compound X_3, BHA = Butylated Hydroxy Anisole, DPPH = 2.2-Diphenyl-l-Picryhydroxyl radical.

(Lee et al. 2004). Ahmed et al. (2007) showed that the antioxidant activity of eugenol (X_2) and related monomeric and dimeric compounds vary in the order eugenol > tetrahydrodieugenol > dihydroeugenol> eugenol. The presence of eugenol in *C. singueana* is a good reason for the antioxidant properties of its extracts and also a good basis for its uses in traditional medicine for the treatment of numerous diseases. Although our study shows that the compounds Lupeol isolated -from *C. singueana* also have good inhibitory action against the DPPH radicals although less than that of eugenol (Figure 4). Lupeol (X_1) and long-chain fatty acid esters (X_3) have been reported as antibacterial and anti-inflammatory agents (Geetha and Varalakshmi, 2001) and antilithic effect (Sudhahar et al., 2008; Mensor et al., 2001) with remarkable inhibitory activity against drugs.

CONCLUSION

In this study, three known compounds, which heretofore have not been reported in *C. singueana* were isolated for the first time from the root extract of the plant. The study also revealed the ability of the compounds to inhibit microbial growth, however, one of the three compounds [4-allyl-2-methoxyphenol – (Eugenol)] showed a broad-spectrum antimicrobial activity; it competes favourably with standard antimicrobial agents at certain concentrations. In addition, it exhibited a good antioxidant activity. Taken together, this result may suggest that potential efficient and cost-effective natural antioxidant and broad-spectrum antimicrobial agent may be developed from the plant.The antimicrobial and antioxidant properties, and brine shrimp toxicity of the isolated compounds from *C. singueana* as observed in the present study might provide some scientific rationale behind some of the traditional medicinal uses of this plant; particularly, the high level of antioxidant property of lupeol could contribute to its traditional uses as an anti-inflammatory agent.

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