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Characterization of (2E,6E)-3,7,11-Trimethyldodeca-2,6,10-Trien-1-Ol with Antioxidant and Antimicrobial Potentials from *Euclea crispa* (Thunb.) Leaves

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Keywords: *Euclea crispa* leaves; (2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-ol; farnesol; antioxidant activity; antimicrobial activity

Abstract. (2E,6E)-3,7,11-Trimethyldodeca-2,6,10-trien-1-ol or farnesol is a natural isoprenoid possessing a range of beneficial biological activities as anti-candidiasis, anti-tumor and anti-hyperglycaemia. The present study, for the first time, isolated and identified farnesol from *Euclea crispa* leaves' extract, subsequently, validated its antioxidant and antimicrobial potentials. The isolated compound was confirmed by spectroscopic techniques including ultraviolet-visible (UV/Vis), fourier-transform infrared (FTIR), and ^{1}H and ^{13}C magnetic resonance (NMR) spectroscopies. By *in vitro* investigations, farnesol exhibited a considerable antioxidant activity with IC_{50} values of 113.79, 109.59, and 116.65 µg/mL for 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, and nitric oxide radical scavenging assays, respectively. Additionally, farnesol performed a potent reducing power capacity which was in line with ascorbic acid, an outstanding antioxidant. By the disc-diffusion assay, farnesol exposed the superior antimicrobial activity against various disease-causing microorganisms. Significantly, at the concentration of 50 µg/mL, the compound effectively inhibited the growth of *Escherichia coli* and *Aspergillus niger* with inhibition zones of 12 and 11 mm, respectively. Findings from this research suggest that *E. crispa* leaf is a potential source of farnesol, a powerful antioxidant and antimicrobial agent.

Introduction

Phytocompounds from medicinal plants afford the opportunities for new drugs as a consequence of their unmatched availability of chemical diversity. Rising insist for chemical diversity in screening programs and in search of therapeutic agent from the natural compounds has grown in worldwide [1]. Identification of new compounds from medicinal plants leads to design and development of novel drug agents for various disease and disorders. Reactive oxygen species (ROS) are generated during the normal metabolic process and they are implicated in pathology of many diseases including cancer, inflammatory disease, diabetes, hypertension and neuronal diseases by inactivating enzymes and damage the important components cell, origin points of injury [2]. Synthetic antioxidants are toxic and possess side effects by the mutagenic effects. Antioxidative compounds from the medicinal plants embrace a significant role in biological system which provides resistance

capacity besides oxidative stress by ROS. Thus, phytocompounds with antioxidative potential might be helpful to manage pathological disorders in human [3]. In contrast, the antimicrobial resistance is expanding rapidly on available synthetic antibiotics which cause the many adverse effects. This multidrug resistance is a known health problem around the world, an imperative aspect of the search for antimicrobial activity of natural compounds from the medicinal plant. It has been broadly used as alternative drug agent in the treatment of infectious diseases for the decades [4].

Sesquiterpenes are a class of terpenes that consist of three isoprene units which are natural or synthetic compounds with a wide application in medicine, pharmaceutics, cosmetics, and agriculture. But in most cases, only small amount of sesquiterpenes are available from natural sources [5]. (2E,6E)-3,7,11-Trimethyldodeca-2,6,10-trien-1-ol (farnesol) is a sesquiterpene alcohol that is produced in both plants and animals. Farnesol has been known as a signalling molecule that plays an important role in the metabolism of the fungus *Candida albicans* [6]. Recently, farnesol is found as a potential anti-candidiasis [7], anti-tumor [8,9], and antidiabetic [10] agent. Additionally, the compound has been proven to be an antioxidant metabolite in the protective regulation of *C. albicans* against oxidative stress [7]. Besides, farnesol is widely used in perfumery and as a body deodorant thanks to its anti-bacterial activity [11]. However, biological activities including antioxidant and antibacterial properties related to human diseases of farnesol have not been adequately known.

E. crispa is a South African medicinal plant which usually called as blue guarri in English, bloughwarrie in Africans [12]. This hardy evergreen plant species belongs to the family of Ebenaceae. *E. crispa* is traditionally used for extended ailments like; leprosy, gonorrhoea, scabies, diarrhoea and wound infections [13]. The extract from *E. crispa* leaves also contains growth inhibitory activity against both bacteria and fungi. The root decoction of *E. crispa* suppresses the cough and its crude extract is used in the treatment of leprosy [14]. The leaf extract of *E. crispa* shows the potential antimicrobial activity [15]. The ethanolic extract of *E. crispa* leaves possesses many important secondary metabolites such as alkaloids, flavonoids, terpenoids and phenolic compounds. The crude extract and partially purified ethyl acetate fractions from *E. crispa* subsp. *crispa* (Ebenaceae) leaves possess antimicrobial activity [16]. Therefore, the aims of the present were to isolate, structurally characterize phytocompound from *E. crispa* leaves and analyze their antioxidant and antimicrobial activities.

Materials and Methods

Collection of Plant Material

The leaves of *E. crispa* were collected from the area of Phuthaditjhaba, near to university of the Free State Campus, South Africa and this material was authenticated by Professor A.O.T. Ashafa, Department of Plant Sciences in the same Campus (Reference number: 6404000-400), then the plant materials were dried under the shade condition, powdered and stored in air tighten container at 4ºC for future analysis.

Preparation of Solvent Extract

Powdered material of *E. crispa* leaves was extracted by the method of exhaustive extraction [17]. Briefly,400g of the plant leaf powder was soaked in 2000 mL of ethanol contained flask and it was kept on the rotating shaker for 72 hours at 25ºC (average room temperature). Finally, the collected extract was concentrated through rotary evaporator (RE-2A evaporator) set at 40ºC. Further it was stored at 4ºC for future studies.

Compound Isolation

E. crispa leaf extract (15g) was fractionated through silica-gel column chromatography (3 × 30 cm) and effectively fractionated from petroleum ether to chloroform by the following combinations 100:0%, 80:20%, 60:40%, 40:60%, 20:80% and 0:100. 20 mL test tubes were used to collect the column fractions. The collected fractions were analyzed by thin layer chromatography (TLC) to identify the single compound.

Structural Prediction of Isolated Compound

Fourier transform infrared (FTIR) spectrometer instrument (Shimadzu FTIR-8400S) was employed to identify the functional groups, which are present in the phytocompound. Shortly, the

sample compound was mixed with KBr salt and then densed into a slight pellet. IR spectrums were observed between 4000-400 cm^{-1} on a Shimadzu FTIR-8400S. Further, structural characterization studies were done by spectroscopic techniques of ^{1}H NMR and ^{13}C NMR. Perkin-Elmer polarimeter (model 341) was used to identify the optical rotation of the substance. NMR spectrums of dissolved compound mixed with $CDCl_3$ were recorded through Bruker DRX-500 NMR Spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C); the deuterated drug signals were used as reference [18].

Antioxidant Assays

The antioxidant potential of isolated compound was determined through standard assays such as α,α-diphenyl-β-picrylhydrazyl (DPPH), nitric oxide, hydroxyl scavenging assays, reducing power and FRAP assays.

The DPPH scavenging assay offers a significant approach to evaluate the antioxidant capacity of the compound. DPPH scavenging potential of isolated phytocompound was analyzed according to Blois method [19]. In brief,100 μM of DPPH was dissolved in ethanol, 1 mL of this mixtureadded to 1 mL of different concentration (50-250 μg/mL) of isolated phytocompound (dissolved in ethanol) were mixed vigorously and stands at room temperature for 30 min. The decreased absorbance values were observed at 517 nm against blank solution (ethanol). The DPPH scavenging capacity was calculated by reduction of radical percentage. Each test was analyzed in triplicate. Decreased absorbance values signified higher free radical scavenging activity.

Nitric oxide was produced through sodium nitroprusside (SNP) and it was analyzed by Garratt method [20]. Briefly, the effective combination contained 10 mM of SNP, phosphate buffer (pH 7.4) and different concentration (50-250 μg/mL) of isolated phytocompound (dissolved in ethanol) in the volume of 3mL. It was incubated at room temperature for 150 min, then 1 mL of sulfanilamide was mixed in 0.5 mL of the incubated solution and it was allowed to stand for 5 min. Finally, 1 mL of naphthyl ethylenediamine dihydrochloride (NED, 0.1% w/v) was added and the mixture was incubated for another 30 min at room temperature. The pink chromophore was produced during the diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was analyzed by spectrophotometrically at 540 nm against the blank solution. Each test was performed in triplicate.

Hydroxyl radical scavenging capacity of phytocompound has analyzed through proposed method of Elizabeth and Rao [21] with slight modification. Freshly prepared 1 mL of reaction mixture includes 2-deoxy-2-ribose (2.8 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), $FeCl_3$ (100 μM), EDTA (100 μM), H_2O_2 (1.0 mM), ascorbic acid (100 μM) and different concentration (50–250 μg/mL) of isolated phytocompound (dissolved in ethanol) was incubated at 37°C for 1 hr, followed by 0.5 mL of the reaction mixture was added to 1 mL of 2.8% trichloroacetic (TCA) and 1 mL 1% aqueous thiobarbituric acid (TBA) then it was incubated at 90°C for 15 min (for the colour development). The absorbance was calculated at 532 nm against a suitable blank solution. Each test was analyzed in triplicate.

The reducing power activity of phytocompound was measured through Oyaizu [22] proposed method. In brief, the different concentration (50-250 ug/mL) of the isolated phytocompound (0.5 mL dissolved in ethanol) were added to 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium hexacyanoferrate (0.1%), then it was incubated at 50°C in the water bath for 20 min. Later, 0.5 mL of 10% TCA was added in the mixture. 1 mL of top section solution was added in 1 mL of distilled water, and then 0.1 mL 0.01% of $FeCl_3$ solution was added. Then the mixture allowed stands for 10 min at 25°C. The absorbance was calculated at 700 nm against the appropriate blank solution. Each test was analyzed in triplicate. Superior absorbance values specify greater reducing power.

The antioxidant capacity of phytocompound was measured using FRAP assay proposed by Benzie and Strain [23]. A prospective antioxidant might decrease the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}). Finally, it produced a blue complex of Fe^{2+}/2,4,6-tripyridyl-s-triazine (TPTZ), which enhanced the absorption at 593 nm. In brief, FRAP reagent was prepared by the mixture of solution contains 10 μM of TPTZ, 40 μM of HCl, 20 μM of $FeCl_3$ at 10:1:1 ratio and 0.3 M of acetate buffer. It was prepared freshly and stands at 37°C. 800 μL of reagent was mixed with 80 μL of sample solution contains various concentration of phytocompound (50-250 μg/mL dissolved in ethanol). This

mixture was shaken vigorously and stands for 30 min at 37°C. The absorbance has taken at 595 nm. All analyses were done in triplicate.

Antimicrobial Activity

The pathogenic microorganisms of *Staphylococcus aureus* (MTCC 96), *Streptococcus pyogenes* (MTCC 737), *Escherichia coli* (MTCC 452), *Klebsiella pneumoniae* (MTCC 418), *Aspergillus niger* (MTCC 584) and *Aspergillus terreus* (MTCC 1782), were purchased from Microbial type culture collection centre, Pune, India. These organisms were subcultured on to their selective media for further studies.

Antimicrobial potential of the phytocompound has determined through Bauer et al. [24] proposed method. Shortly, the sterile disc (5 mm) was dipped in various doses of isolated phytocompound (5, 10 and 15 mg/mL) and dried at 40ºC. Each disc was drenched in DMSO used for control and chloramphenicol was used as reference drug. Inoculate [10^8 CFU] has broaden on the sterile of nutrient agar medium plate using cotton swabs after that, the plates were incubated at 35ºC for 20 min. The discs were placed in the plates and it were incubated at 37ºC for 24 hrs. Correspondingly antifungal activity was measured by afore mentioned method in Sabouraud Dextrose Agar (SDA) medium. The standard streptomycin (10 µg/disc) was used as positive control. Each plate was incubated at 25°C for 72 hrs. The zone of inhibition was calculated in mm. Each test was performed in triplicate.

Statistical Analysis

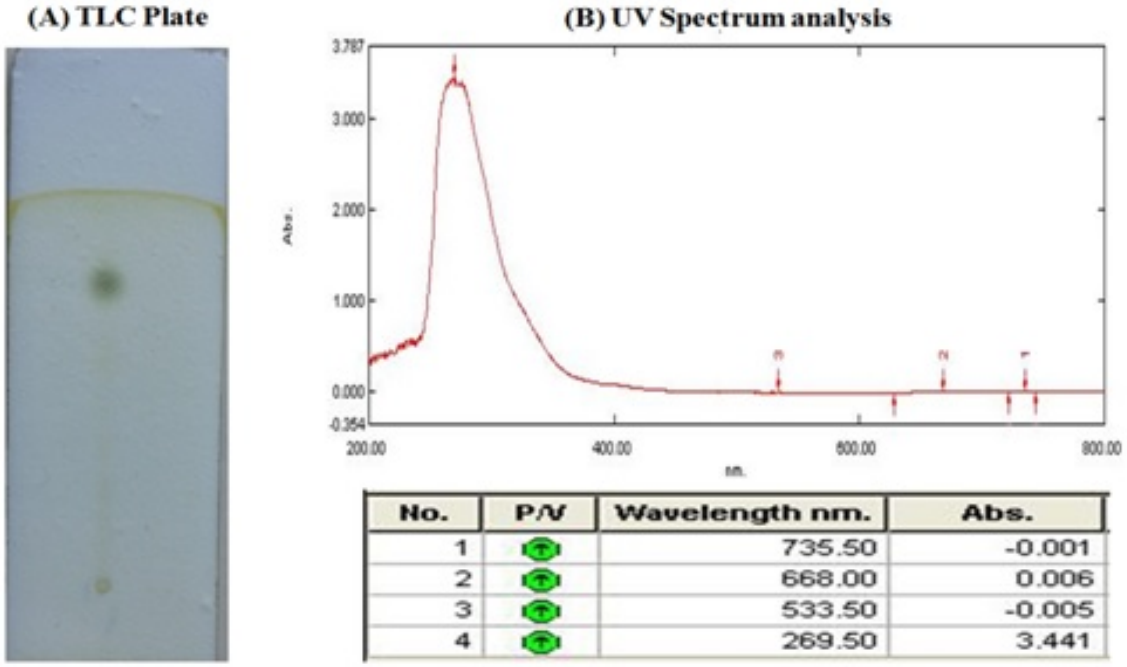
The obtained results from the assays were showed as mean ± standard deviation (SD). The Statistical evaluations were measured through statistical package program (SPSS 10.0, IBM, Armonk, New York, United States).

Results and Discussion

Isolation and Structural Characterization of Phytocompound

Plants are being natural resource for the pharmaceutical, cosmetics and food industries [25]. The modern research explored that, phytocompounds have significant antioxidant and antimicrobial properties. In worldwide, approximately 80% of people using natural medicine for their personal care [26]. Discovery of active compounds through chromatographic and spectroscopic methods might provide the qualitative and quantitative information about herbal medicine compositions [27].

Totally 126 fractions were isolated through silica gel column chromatography, among that 240 mg of single compound (soluble in 95% ethanol) was identified from 92-109 fractions with retardation factor value 0.75 cm (analyzed by TLC) which is showed in (Figure 1A). The group of absorbance bands are found at λmax of 269 nm and the maximum absorbance of 3.441 were recorded in UV-visible spectroscopy (Figure 1B) authenticated the single compound was present in those identified fractions. The functional group characteristic of Fourier Transform Infra-Red (FTIR) spectrum exposed the absorption bands (Supplementary Data, Figure S1) at 2933 cm^{-1} for C-H stretch 2666 cm^{-1} for O-H stretch 1712 cm^{-1} for C=O stretch 1448 cm^{-1} for -C-H bending 761 cm^{-1} for =C-H bending. However, this study never claimed that those functional groups are present in the proposed chemical compound. The origin of those functional groups is presumably due to impurities present with the chemical compound investigated in the study. Upon extensive purification procedures carried out for the chemical compound, it was noted that, removal of the impurities is very critical (data not shown) from the interested compound. Meanwhile, the presence of the impurities did not harm any scientific claims.



No.	P/V	Wavelength nm.	Abs.
1		735.50	-0.001
2		668.00	0.006
3		533.50	-0.005
4		269.50	3.441

Figure 1. (A) The pure fraction was primarily identified through TLC analysis and (B) UV-Spectrum of farnesol

The ^{1}H-NMR spectra (Supplementary Data, Figure S2) exhibited as a group of signals at δ1.62, δ1.70 and δ1.77 were due to methyl groups attached to unsaturated carbon atoms. The group of signals at δ2.02 were due to methylene groups attached to unsaturated carbon atoms. The multiple signal at δ4.12 was due to an oxymethylene group. The signal at δ5.14 suggested the presence of unsaturated protons. The signal at δ5.45 was due to an unsaturated proton attached to an oxymethylene carbon. In the ^{13}C-NMR spectra (Supplementary Data, Figure S3), the two groups of signals at δ134.88 suggested the presence of tetra substituted unsaturated carbons and δ125.06 were due to tri-substituted unsaturated carbon atoms. The signal at δ59.01 was due to an oxymethylene carbon. The signal at δ39.77 was due to carbon atoms in the trans-trans position to the double bond. Similarly, the signal at δ32.22 and δ31.99 were due to methylene carbons present in the trans-cis and cis-cis positions respectively. The other signals were due to methylene and methyl group carbon atoms. The signals reflecting triad sequences of dimethyl allyl, cis and trans isoprene units as well as cis isoprene unit that links to the hydroxyl group. It suggests presence of three trans and five cis isoprene units. It belongs to the group of medium chain length prenologues. Furthermore, it exhibited the proportions of cis and trans isoprene residues cis(α):cis:trans(ω):trans groups in the ratio 1:6:1:3. In the ^{13}C-NMR, the intensity of the signals at δ39.77, δ32.22 and δ31.99 were in the ratio 3:5:1. Thus, all the above spectral studies revealed that the isolated compound was characterized.The assumed structure of phytocompound is (2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (farnesol), its molecular weight is 222.36 g/mol and molecular formula is $C_{15}H_{26}O$(Figure 2).

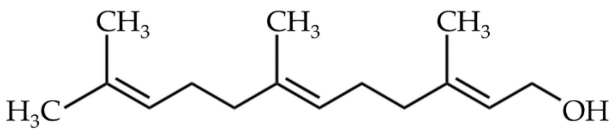


Figure 2. The structure of farnesol

Antioxidant Activity

Generally, the antioxidants are complicated to protect the organisms against the ROS produced free radicals which leads to various disease and disorders in the human disease management system [28]. Novel methods are proposed to measure the antioxidant activity of phytocompounds, but the standard scavenging assays referred based on their free radical scavenging capacity [29].

Table 1. Antioxidant potential of farnesol

Sample	IC_{50} values (µg/mL)		
	DPPH scavenging assay	**Nitric oxide scavenging assay**	**Hydroxyl scavenging assay**
Farnesol	113.79±0.82	116.65±0.03	109.59±0.26
Ascorbic acid	101.94±0.52	107.62±0.12	93.28±0.19

DPPH, 2,2-diphenyl-1-picrylhydrazyl

DPPH Scavenging Activity

DPPH assay is the well-known procedure to determine the antioxidative properties of plant extracts or phytocompounds [30]. Usually, the free radicals are scavenged by an antioxidant which donates an electron or hydrogen ion to a radical and consequently, a constant molecule is produced [31]. The dose-dependent DPPH scavenging effect of farnesol expressed the comparable and significant IC_{50} values of 113.79±0.82 µg/mL when compared with ascorbic acid value of 101.94±0.52 µg/mL (Figure 3 and Table 1).

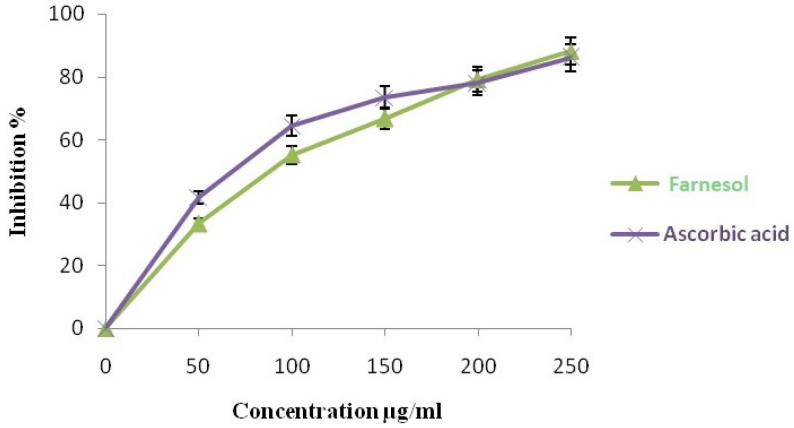


Figure 3. DPPH scavenging potential of farnesol

Nitric Oxide Scavenging Activity

Nitric oxide is well-known pro-inflammatory mediator, which is concerned in variety of physiological events like, smooth muscle relaxant, inhibition of platelet aggregation and regulation of cell mediated toxicity [32]. However, over production of nitric oxide may leads to pathogenesis of some inflammatory diseases. Therefore, nitric oxide inhibitory agent could be favorable for management of inflammatory reaction [33]. The nitric oxide scavenging activity of farnesol holds the significant IC_{50} value of 116.65±0.03 µg/mL (Figure 4 and Table 1) when compared with ascorbic acid as the standard drug (107.62±0.12 µg/mL) which indicates that, isolated farnesol from the *E.crispa* leaves extract might have the capable to inhibit the nitric oxide.

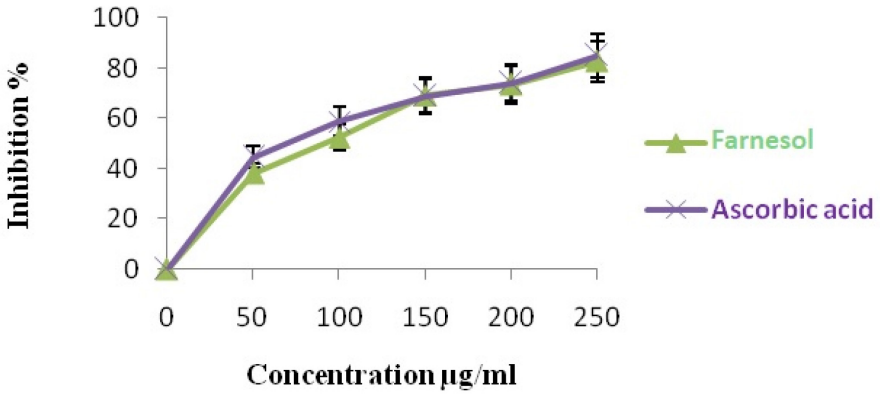


Figure 4. Nitric oxide scavenging capacity of farnesol

Hydroxyl Scavenging Activity

Production of hydroxyl radicals by Fenton reaction which degrades deoxy ribose and cause the oxidative DNA damage [33]. The isolated phytocompound farnesol and reference drug of ascorbic acid exposed their significant IC_{50} values were 109.59±0.26 µg/mL and 93.28±0.19 µg/mL respectively (Figure 5 and Table 1). The major ROS of hydroxyl radicals are causing lipid peroxidation and different biological damages [34]. This assay can suggest that, farnesol might capable to eliminate the hydroxyl radicals from the sugar module of malondialdehyde like oxidant and barred the oxidation reaction.

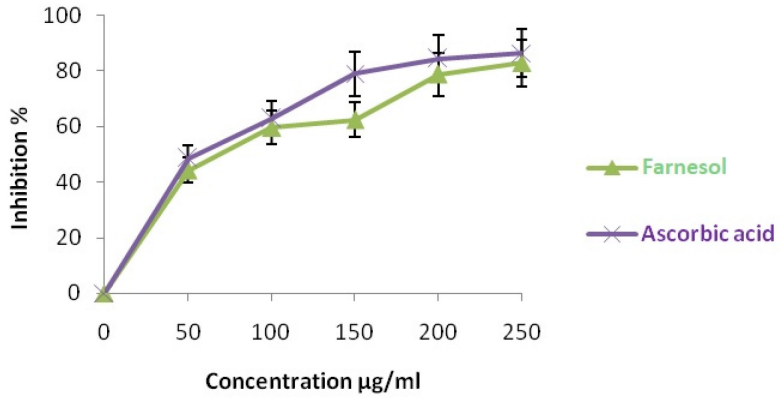


Figure 5. Hydroxyl scavenging potential of farnesol

Reducing Power Activity

Mostly, this method can be used to assess the capability of an antioxidant to donate an electron [35]. In this assay, the capacity of farnesol reducing power capacity has analyzed through transformation of Fe^{3+} to Fe^{2+} in the presence of farnesol and reference drug of ascorbic acid. At the concentration of 250 µg/mL, the greatest absorbance of farnesol and reference drug were 0.67±0.04 and 0.78±0.03, respectively (Figure 6), which indicates that, isolated farnesol from *E. crispa* leaves extract has considerable reducing power activity. Hence, farnesol could serve as electron donors and it may react with free radicals to change the constant products. It may lead to stop the free radical chain reaction.

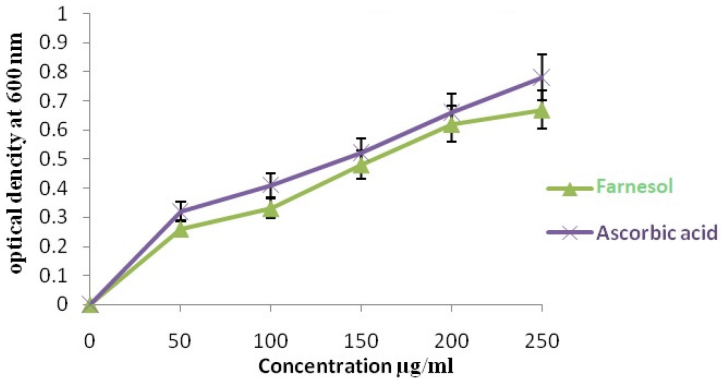


Figure 6. Reducing power capacity of farnesol

FRAP Assay

This assay is mainly based on decreasing effects of an antioxidant responding through a ferric tripyridyltriazine complex and generates the colored ferrous tripyridyltriazine. The reducing power of plant extract or compound associated with their exerting action through terminating free radical chain by a hydrogen atom donation [36]. Isolated phytocompound of farnesol from the *E. crispa* leaf extract exposed the highest absorbance of 0.75±0.02, while ascorbic acid has 0.88±0.05 at the concentration of 250 µg/mL (Figure 7) which indicates that, the farnesol might have considerable reducing power.

Antioxidant activity on free radical scavenging assay on farnesol exposed the dose-dependent percentage of scavenging activities which is expressed as half-maximal inhibitory concentration (IC_{50}) value and it possesses strong antioxidant activity which indicates that the isolated farnesol might scavenge the free radicals.

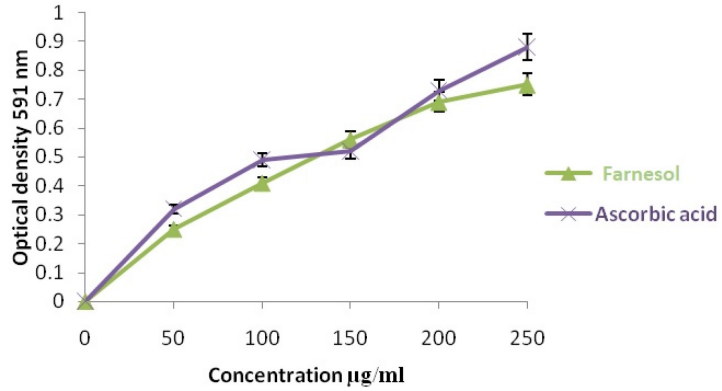


Figure 7. FRAP assay activity of farnesol

Antimicrobial Activity

Antimicrobial activity of farnesol and reference drugs were determined by disc diffusion method (Table 2) against a variety of microorganisms which are regularly meets in infectious diseases. The antimicrobial activity of farnesol expressed the highest inhibition zone against tested microorganisms such as, *Staphylococcus aureus* (9.0 ± 0.1 mm), *Streptococcus pyogenes* (9.0 ± 0.3 mm), *Escherichia coli* (12.0 ± 0.2 mm), *Klebsiella pneumoniae* (10.0 ± 0.1 mm), *Aspergillus niger* (11.0 ± 0.3 mm) and *Aspergillus terreus* (9.0 ± 0.2 mm). Multidrug resistance is growing rapidly by the microorganisms during recent decades. The modern researcher has urged to search the new drug sources which have improved therapeutic properties [37]. Thus, results indicated that farnesol had a significant antimicrobial activity against the disease-causing microorganisms and it could have the potential effects on those microorganisms.

Table 2. Antimicrobial capacity of farnesol

No.	Microorganism species	Inhibitory zone (mm)			
		Farnesol			Standard drug
		5 µg/mL	10 µg/mL	15 µg/mL	15 µg/mL
1	*S. aureus*	4.0 ± 0.3	7.0 ± 0.2	9.0 ± 0.1	14.0 ± 0.2
2	*S. pyogenes*	5.0 ± 0.1	7.0 ± 0.1	9.0 ± 0.3	12.0 ± 0.3
3	*E. coli*	7.0 ± 0.1	10.0 ± 0.3	12.0 ± 0.2	14.0 ± 0.3
4	*K. pneumoniae*	5.0 ± 0.2	8.0 ± 0.2	10.0 ± 0.1	14.0 ± 0.3
5	*A. niger*	6 .0 ± 0.2	9 .0 ± 0.1	11 .0± 0.3	15.0 ± 0.1
6	*A. terreus*	4.0 ± 0.1	7.0 ± 0.3	9.0 ± 0.2	13.0 ± 0.2

Note: The isolated farnesol from *E. crispa* leaves extract showed the highest inhibition zone against tested microorganisms at the concentration of 15 µg/mL.

Although antimicrobial and antioxidant activities of farnesol have been described previously, the present study might provide beneficial information about the free radical inhibitory effect of pure farnesol through various mechanisms. The presented dose-dependent curve for each test may play as an important suggestion for drug designers who target to treat particular oxidative stress as well as create a novel antioxidant agent based on natural farnesol. In the same manner, the growth inhibitory effect of farnesol on six various bacterial pathogens may be a potential option for producing targeted antibacterial medicines in the future. On the other hand, farnesol is a quorum-sensing molecule [38], therefore, further *in vivo* models and preclinical trials should be carried out in order to find out the optimal threshold of farnesol in healing a peculiar health problem.

Conclusions

Natural compound (2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (farnesol) was isolated and structurally characterized from *E. crispa* leaves extract. The antioxidant potential of farnesol exposed the significant scavenging activity on DPPH, nitric oxide, hydroxyl scavenging, reducing power, FRAP reducing assays. Moreover, it also possesses notable antimicrobial activity on tested microorganisms when compared with referenced antibiotic. Together this study can conclude that, farnesol holds both antioxidant and antimicrobial activity. In future, it might use to develop a novel drug for the hindrance and treatment of diseases which are associated with microbial infections and oxidative stress. Nevertheless, further *in vitro* and *in vivo* studies are needed to authenticate the current finding.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Data

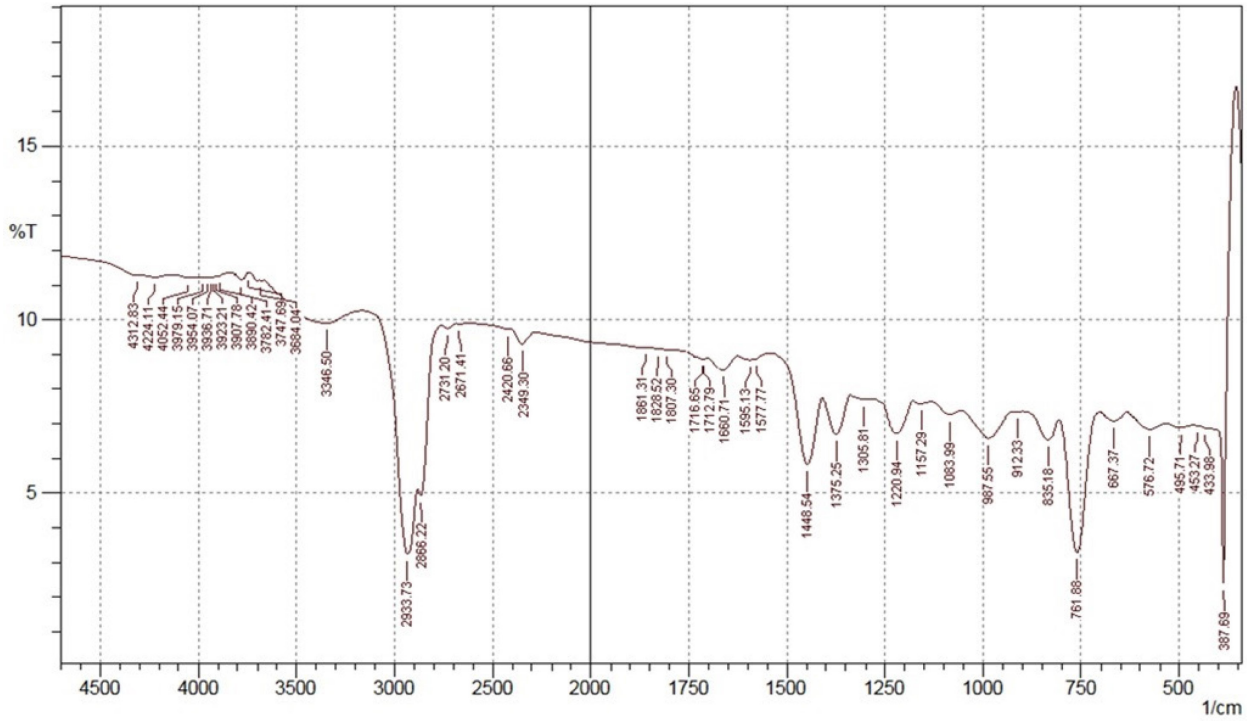

Figure S1: FTIR spectrum for farnesol which indicates presence of functional groups by the characteristic absorptions (1/cm)

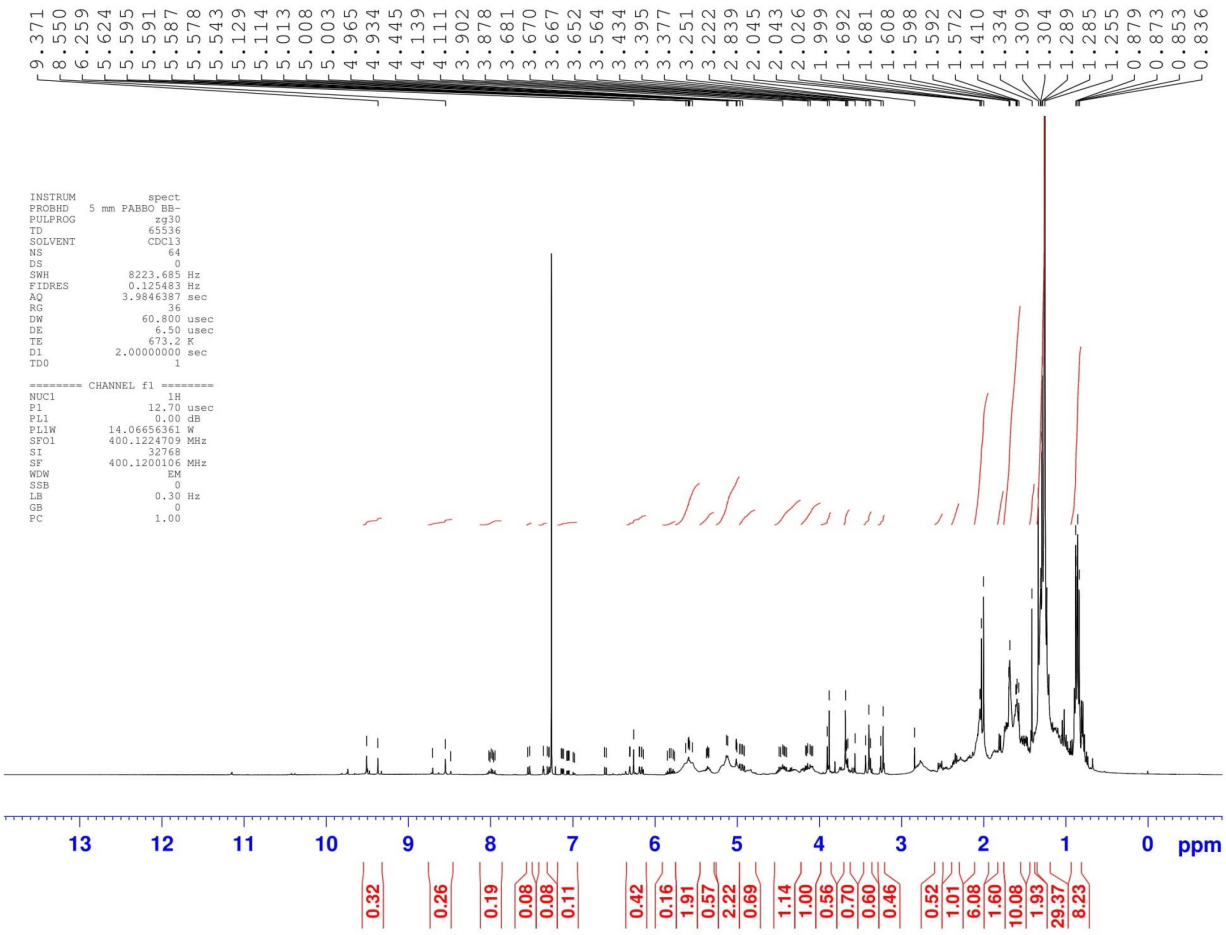

Figure S2: ^{1}H NMR spectrum analysis for farnesol

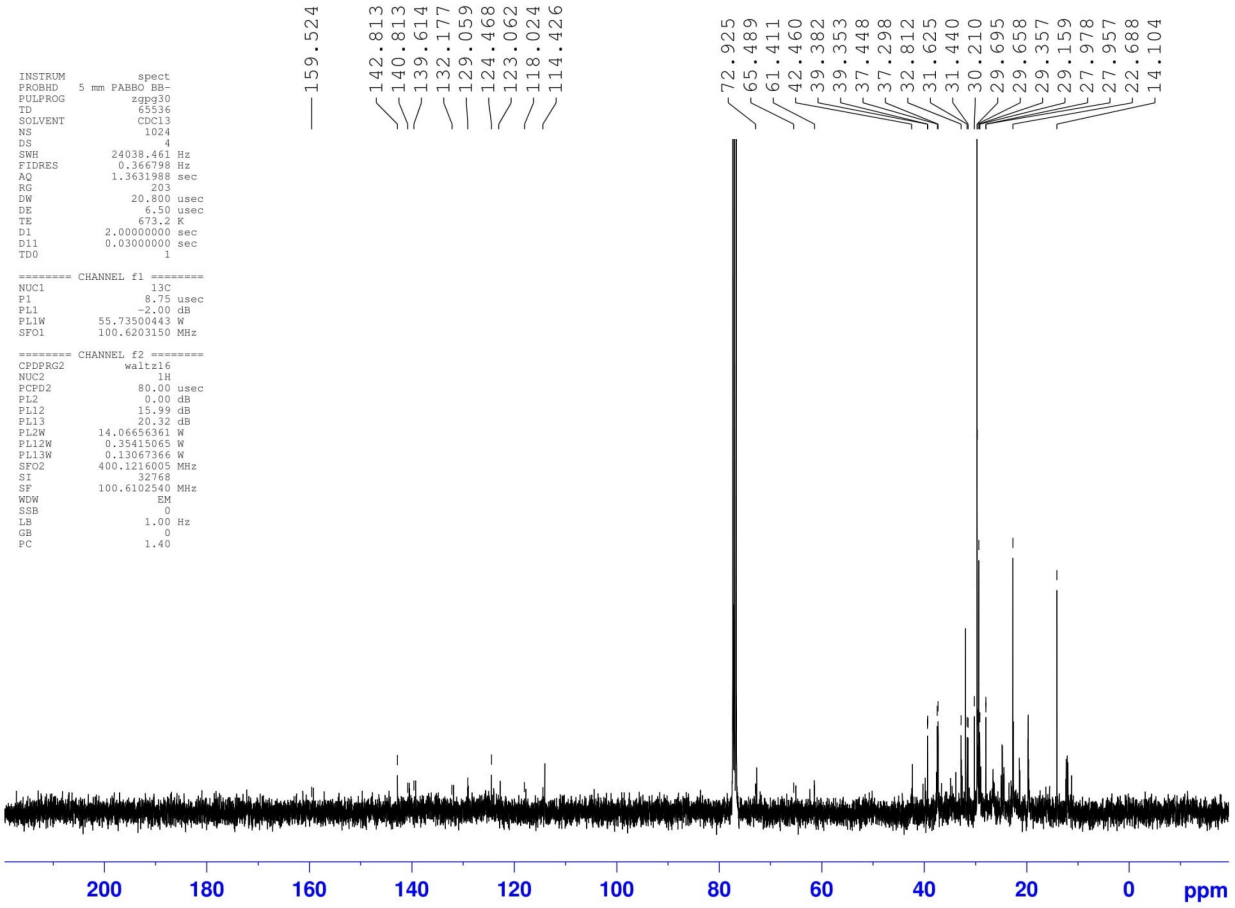

Figure S3: ^{13}C NMR spectrum analysis for farnesol

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