

## ***Callitris columellaris* var. *campestris* Silba Leaf Extract: Phytochemicals, Phenolic Content, Free Radical Scavenging, Antioxidant and Antimicrobial Potentials**

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**Abstract:** *Callitris columellaris* var. *campestris* is one of the major medicinal plants well used for different health disorders. The purpose of this study was to investigate the total phenolic content, free radical scavenging, antioxidant and antimicrobial potentials of the phytochemicals in the leaf crude extract of *C. columellaris* var. *campestris*. Using GC and GC-MS 58 organic compounds were identified representing 96.0% of the total composition of the leaf crude extract, out of which *m*-cymene (14.0%), (3S,4R,5R,6R)-4,5-bis(hydroxymethyl)-3,6-dimethylcyclohexane (7.3%), (+)-*epi*-bicyclosesquiphellandrene (7.2%) and 9-phenylthioxomethylthio-9-borabicyclo[3.3.1]nonane (5.0%) were detected as principal components. The quantification of total phenolic content was found to be 2,198  $\mu\text{mg}^{-1}$  of gallic acid equivalent; the study showed that the leaf extract possessed high content of phenolic compounds. The extract also exhibited excellent antioxidant potentials for human systems susceptible to free radical-mediated reactions and gave DPPH inhibitions between 55-95% with  $\text{IC}_{50}$ :  $3.5\mu\text{gml}^{-1}$ , the extract was found to be twice more active than the synthetic antioxidant (ascorbic acid). The free radical scavenging and antioxidant potentials were also found to increase in a dose dependent manner. This might be due to the presence of low molecular mass phenolic compounds in the plant. The results obtained in the present study clearly showed that the extract scavenges free radicals, ameliorating damage imposed by oxidative stress in different disease conditions and serve as a potential source of natural antioxidant. The antibacterial properties of the extract were tested against the representative multi-drug resistant bacteria strains and the results showed high degree of sensitivity. This plant has excellent pharmacological properties that could be used to resolve some major health problems.

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### **1. Introduction**

Secondary metabolites from plants have been a source of therapeutic agents for human benefits and impressive numbers of modern drugs have been isolated from plant origin. Natural products from plants are regarded as a significant antioxidant and antibiotic sources in human health. They are used to treat chronic and degenerative infectious diseases such as atherosclerosis, diabetes, inflammation, stroke, cancer, rheumatoid arthritis, brain dysfunction and heart disease in humans. Recently, there has been a global interest in medicinal natural products for the treatment of ailments that defile orthodox medicine principally because many diseases have developed resistance to conventional drugs. Moreover, there is continuous need of exploration and development of cheaper, effective and pharmacologically active new plant based drugs with better therapeutic potential and with no side effects (Sharma and Kumar, 2009).

*Callitris columellaris* var. *campestris* silba (*Cupressaceae*) is a small to medium-sized medicinal plant, which usually grows to about 18-30m tall and

0.45-0.9m in diameter. *C. columellaris* var. *campestris* is a distinct evergreen plant with several applications. The therapeutic benefits of *Callitris* are usually attributed to their ability to cure many diseases (Veronica, 2007).

To the best of our knowledge, no study has reported the total phenolic content, free radical scavenging, antioxidant and antimicrobial potentials of the leaf extract of this plant so far. Therefore, the present research was therefore undertaken for the first time with the aim of looking into these quantitative and qualitative parameters in the leaf of *C. columellaris* var. *campestris* from Nigeria.

### **2. Material and Methods**

#### **Plant Materials and Extraction of the Phytochemicals**

The leaves of the plant were collected from its natural habitat at Afforestation Research Station Jos, Nigeria and the plant was authenticated as *C. columellaris* var. *campestris*. Air-dried leaves of the plant were extracted with methanol to give gel-like

extract; the concentrated crude extract collected was stored in vial at low temperature.

### GC and GC-MS Analyses

The leaf extract of *C. columellaris* var. *campestris* was analysed with GC and GC-MS fitted with a FID. Separation of compounds was performed with capillary column (30 m x 0.25 mm inner diameter, with 0.25  $\mu\text{m}$  film thickness). A split-splitless injector heated at 250°C and a flame ionization detector (FID) at 240°C. The oven temperature was programmed from 40°C to 150°C at 3°C/min rate, then held isothermal for 10 min and finally raised to 250°C at 10°C/min. Helium (99.9%) was used as carrier gas at a flow rate of 1.0 ml/min. The injection volume was 1.0  $\mu\text{l}$  (split ratio 1:20). The GC was interfaced with mass selective detector, mass spectrometer in EI mode at 70 eV. The mass spectra were generally recorded over 40-500 amu that revealed the total ion current (TIC) chromatograms. The same column and analyses conditions were used for both GC and GC-MS. Identification of the individual component was made by matching their recorded mass spectra with the NIST library provided by the instrument software, and by comparing their calculated retention indices (RI) with literature. Relative area percentage of each individual component of the extract was expressed as the percentage of the peak area relative to the total peak area. RI value of each component was determined relative to the retention times (RT) of a series of *n*-alkanes with linear interpolation on the column.

### Determination of Total Phenolic Content

Total phenolic content of the leaf extract of *C. columellaris* var. *campestris* was determined using the Folin-Ciocalteu method. 1 ml of the methanolic extract was mixed with 46 ml distilled water and 1 ml of Folin Ciocalteu reagent, then 3 ml of (2% w/v)  $\text{Na}_2\text{CO}_3$  solution was added after 3 minutes and the mixture was allowed to stand for 2 hours for incubation in dark with intermittent shaking, the absorbance of the reaction mixture was measured on a UV-Visible spectrophotometer at 760 nm against a blank (containing all reagents except the test sample). The total phenolic content was expressed as gallic acid equivalents (Saeed *et al.*, 2012).

### *In vitro* 2,2'-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging and Antioxidant assay

The free radical scavenging and antioxidant activities of the extract against the stable free radical DPPH were measured. Briefly, Three different concentrations (1000, 100 and 10  $\mu\text{gml}^{-1}$ ) of the extract dissolved in methanol were incubated with a methanolic solution of DPPH. After 30 minutes of incubation at room temperature in the dark, the absorbance of the resulting mixture at 517nm was measured spectrophotometrically. Ascorbic acid was

used as reference compound. The assay was carried out in triplicate. The percentage inhibition (I%) for each concentration was calculated by using the absorbance (A) values according to the following formula:

$$I\% = [(A_{\text{blank}} - A_{\text{ext}})/A_{\text{blank}}] \times 100$$

Where:  $A_{\text{blank}}$  is the absorbance of blank solution and  $A_{\text{ext}}$  is the absorbance of the extract. The dose-response curve was plotted and  $\text{IC}_{50}$  value for the extract was calculated (Ozturk *et al.*, 2011).

### *In vitro* Antimicrobial assay

The antibacterial potentials of the extract were evaluated *in vitro* by agar-well diffusion method against representative multi-drug resistance Gram-positive organisms (*Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus viridans*), Gram-negative organisms (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*). The bacteria isolates were first sub-cultured in nutrient agar for 24 hours to prepare bacteria suspension. All the microbial cultures were adjusted to 0.5 McFarland standards, 20 ml of agar media was poured into each Petri plate, and plates were swabbed with 10 $\mu\text{l}$  inocula of the test microorganisms, and kept for 15 minutes for adsorption. Using sterile cork borer of 6 mm diameter, wells were bored into the seeded agar plates, and these were loaded with 10  $\mu\text{l}$  of different concentrations of (1000, 100 and 10  $\mu\text{gml}^{-1}$ ) of each compound reconstituted in the dimethylsulfoxide (DMSO). The plates were allowed to stand in the refrigerator for 1 hour to allow proper diffusion of the extract into the medium and incubated at 37°C for 24 hours. Antibacterial potential of leaf extract was evaluated by measuring the zones of growth inhibition against the organisms. Augmentin (AUG) and Gentamicin (GEN) were used as controls (Irshad *et al.*, 2012).

### 3. Results and Discussion

The composition of the extract of *C. columellaris* var. *campestris* was determined by the use of analytical GC and GC-MS techniques which led to the identification of 58 different constituents, representing 96.0% of the extract (Table 1). The study revealed that terpenoids and phenolic compounds afforded major portion of the leaf extract with *m*-cymene (14.0), (3S,4R,5R,6R)-4,5-bishydroxymethyl-3,6-dimethylcyclohexane (7.3%), (+)-*epi*-bicyclosesquiphellandrene (7.2%), and 9-phenylthioxomethylthio-9-borabicyclo[3.3.1]nonane (5.0%) in appreciable amounts. The remaining chemical constituents were detected in lesser amounts. This result on the phytochemicals in the leaf extract of *C. columellaris* var. *campestris* was

quite different from the chemical compositions of the other related species belonging to the same *Cupressaceae* family. In *C. neocaledonica* essential oil, guaiol, bulnesol,  $\alpha$ -eudesmol,  $\beta$ -eudesmol,  $\gamma$ -eudesmol and elemol were the principal compounds, while in *C. sulcata* essential oil the major constituents were guaiol, bulnesol,  $\alpha$ -eudesmol,  $\beta$ -eudesmol (Waikedre *et al.*, 2012).

**Table 1: Chemical composition of Leaf Extract of *Callitris columellaris* var. *campestris***

Compounds	% Composition	RI
1,6-dimethyl-9-(1-methylethylidene)-5,12-dioxatricyclo[9.1.0.0(4,6)]dodecan-8-one	0.6	626
nordextromethorphan	0.3	902
(3S,4R,5R,6R)-4,5-bis(hydroxymethyl)-3,6-dimethylcyclohexane	7.3	967
(+)-4-carene	1.0	1004
2-isobutyl-1,4-dimethylbenzene	4.5	1009
<i>p</i> -cymene	0.4	1011
3-acetoxy-25-methoxy-9,19-cyclolanostan-24-one	0.5	1012
L-limonene	0.4	1018
1-methyl-1-silabenzocyclobutene	3.4	1020
<i>o</i> -cymene	1.0	1029
1-methylpyrrolo[1,2-a]pyrazine	1.0	1038
<i>m</i> -cymene	14.0	1042
4-ethyl- <i>m</i> -xylene	2.0	1050
5-ethyl- <i>m</i> -xylene	3.6	1074
2-ethyl- <i>m</i> -xylene	1.0	1078
methyl-14-methylpentadecanoate	0.5	1079
3-ethyl- <i>o</i> -xylene	0.4	1095
6-butyl-1-nitrocyclohexene	0.7	1117
3-hydroxypseudocumene	0.9	1131
umbellulone	2.0	1171
1-nitrocyclohexene	0.8	1174
N-3-chloro-5-methylphenylacetamide	0.3	1225
patchoulane	0.6	1376
$\beta$ -cubebene	3.0	1390
di- <i>epi</i> - $\alpha$ -cedrene	0.6	1394
$\alpha$ -Ionene	0.4	1428
<i>Z,E</i> - $\alpha$ -farnesene	0.6	1458
$\alpha$ -curcumene	3.0	1473
12-methyl- <i>E,E</i> -2,13-octadecadien-1-ol	0.8	1487
elixene	0.3	1492
5-hydro-2-indolecarboxylic acid	0.8	1502

$\alpha$ -farnesene	0.7	1509
(+)- <i>epi</i> -bicyclosquiphellandrene	7.2	1521
1S- <i>cis</i> -calamenene	1.0	1526
durenediamine	0.4	1527
3,4-dimethyl-3-cyclohexen-1-carboxaldehyde	0.5	1541
epizonarene	2.5	1545
7-butyl-1-hexylnaphthalene	0.1	1572
<i>cis</i> -8-ethyl-bicyclo[4.3.0]non-3-ene	3.0	1578
caryophyllene oxide	0.8	1582
<i>cis</i> - $\alpha$ -copaene-8-ol	0.7	1625
DL-2-phenyl-1,2-propanediol	3.0	1633
$\alpha$ -cadinol	1.0	1653
$\alpha$ -bisabolol	0.6	1662
1-hexadecyne	0.8	1664
isoamyl cinnamate	0.5	1718
5-nitro-1-phenyl-1-hexen-3-ol	0.3	1886
palmitic acid	2.0	1962
androst-5-en-4-one	0.6	2043
diazoprogesterone	0.5	2050
cinnamyl cinnamate	1.7	2056
9-phenylthioxomethylthio-9-borabicyclo[3.3.1]nonane	5.0	2083
phytol	0.7	2111
<i>trans</i> -4-hexyloxychalcone	0.7	2160
stearic acid	0.5	2167
<i>trans</i> -tatarol	1.7	2234
<i>E</i> -9-eicosene	0.8	2287
ferruginol	2.0	2332
<b>Percentage Total</b>	<b>96.0</b>	

RI = Retention Index

#### Total Phenolic Content (TPC)

Total phenolic content analysis revealed the presence of high quantity phenolic compounds in the extract. This was found to be 2,198  $\mu\text{gmg}^{-1}$  gallic acid equivalents. The leaf extract gave a higher TPC when compared with the previous studies on the related species such as methanolic leaf extract of *Cupressus sempervirens* (*Cupressaceae*) with TPC value of 86.3  $\mu\text{gmg}^{-1}$  (Ali *et al.*, 2012), this shows that the TPC of *C. columellaris* var. *campestris* is 26 times more than that of *Cupressus sempervirens*. *C. columellaris* var. *campestris* exhibited the high TPC due to the presence of low molecular mass phenolic compounds such as ferruginol, 3-hydroxypseudocumene, isoamyl cinnamate, 5-nitro-1-phenyl-1-hexen-3-ol, nordextromethorphan, *trans*-tatarol and DL-2-phenyl-1,2-propanediol. This report is indicating that total phenolic content is directly proportional to antioxidant and pharmacological properties of the leaves of the plant. Therefore, these secondary metabolites contribute significantly to the

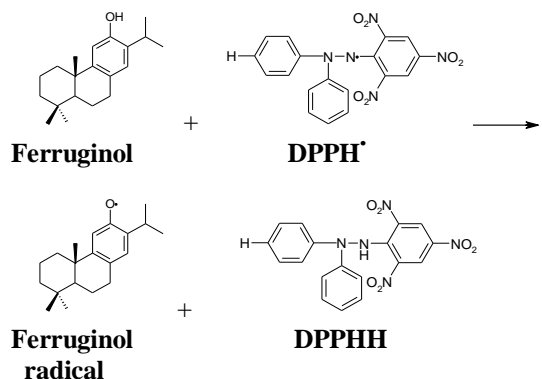
total antioxidant and therapeutic potentials of the plant. Phenolic compounds in the extract were oxidized by Folin-Ciocalteu reagent which reduced to a mixture of blue oxides of tungsten,  $W_8O_{23}$ , and molybdenum,  $Mo_8O_{23}$  after oxidation of the phenolic compounds (Walch *et al.*, 2011). Phytophenolic compounds are very important because their hydroxyl groups which are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals are implicated in several diseases. Plant phenolic compounds have been widely consumed for many years as dietary components with no side effect, they play important beneficial roles in mammalian systems, they are especially important in prevention of cancers, cardiovascular diseases, and other degenerative diseases. Ferruginol a natural diterpene phenolic compound has recently received attention for its extensive pharmacological properties, including anti-tumor, antibacterial, cardio-protective and gastroprotective effects. It showed *in vitro* human colon, breast, and lung tumor reduction and reduction in oncogene transformed cells as well (Son *et al.*, 2005; Wei *et al.*, 2009).

#### DPPH Free Radical Scavenging and Antioxidant Potentials

The extract of *C. columellaris* var. *campestris* was subjected to screening for their possible free radical scavenging and antioxidant properties by DPPH method. DPPH is a stable free radical which can readily experience reduction in the presence of an antioxidant. The percentage inhibitions of the extract at various concentrations (1000, 100 and 10  $\mu\text{gml}^{-1}$ ) were  $72 \pm 0.06$ ,  $51 \pm 0.006$  and  $50 \pm 0.001\%$  respectively; while the  $IC_{50}$  values was found to be  $3.5 \mu\text{gml}^{-1}$  in comparison to ascorbic acid which gave  $55 \pm 0.00$ ,  $83 \pm 0.001$  and  $95 \pm 0.00$  as the percentage inhibitions and  $IC_{50}$  value of  $7.0 \mu\text{gml}^{-1}$ . The free radical scavenging and antioxidant properties of the extract were found to be twice more active than the synthetic antioxidant (ascorbic acid) as shown in Table 2 below. Moreover, the extract of *C. columellaris* var. *campestris* inhibited the DPPH free radicals than some other related species such as *C. neocaledonica* and *C. sulcata* essential oils that scavenged at  $250 \mu\text{gml}^{-1}$  (Waikedre *et al.*, 2012). Phenolic compounds (eg ferruginol) reduce the DPPH radical by their hydrogen donating and single electron transfer abilities (Ndhlala *et al.*, 2010). The resonance effect of phenolic compounds make the release of hydrogen a free radical easy, while the inductive effect on benzene ring pushes the electrons toward oxygen free radical, resulting in the molecule becoming stable.

#### Hydrogen Atom Transfer (HAT)

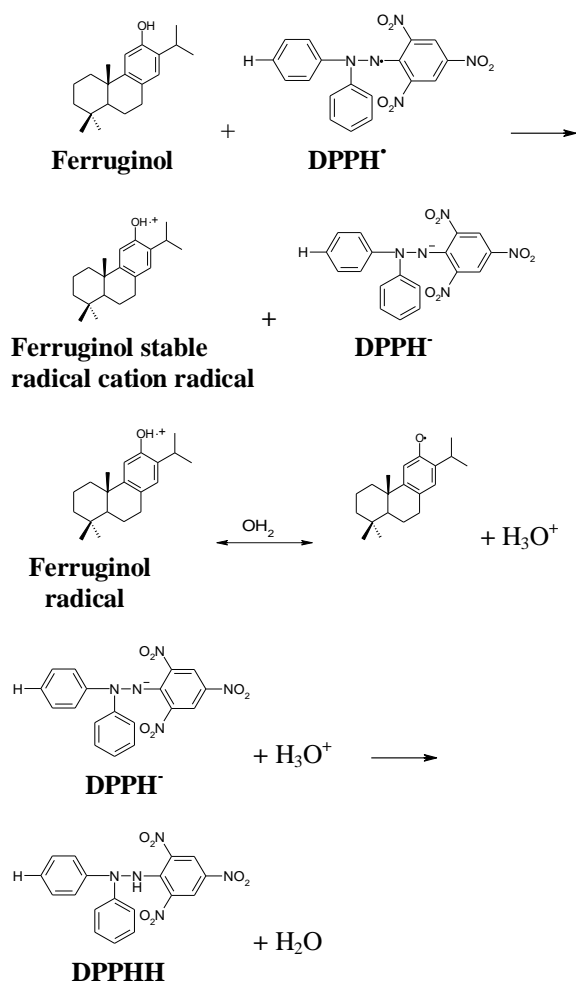
The HAT mechanism measures the ability of an antioxidant to quench free radicals by donating hydrogen. HAT-based mechanisms are more relevant to radical chain-breaking antioxidant capacity. In order to determine how phenolic antioxidants in the extract (eg ferruginol) inherently donate their hydrogen atom to radicals, the environment in which they do so is also an important mechanistic consideration. The HAT mechanism is pH and solvent dependent. Solvent can affect the rate of hydrogen atom donation of a phenol by hydrogen bonding to the phenolic hydrogen. Phenolic antioxidant reacts directly with a free radical which is neutralized, and a radical form of phenolic antioxidant appears. A numerical parameter associated with this mechanism is bond-dissociation enthalpy (BDE), the lower BDE parameter characterizes better antioxidant property (Huang *et al.*, 2005; Al-Amiery *et al.*, 2013).



#### Single Electron Transfer (SET)

The SET method measures the ability of antioxidant to transfer one electron to reduce free radical. SET involves two components in the reaction, *i.e.* the antioxidant and oxidant. Colour change in the probe occurs when it removes an electron from the antioxidant, with the degree of colour change being proportional to the concentration of antioxidants in the reaction mixture. The reaction end-point is reached when the colour change stops. SET-based assays involve one redox reaction in which the oxidant is also the probe for monitoring the reaction. SET mechanism measures the abilities of phenolic antioxidants in the extract (eg ferruginol) to transfer one electron to reduce radicals which changes colour when reduced. The degree of colour change is correlated with the antioxidant potential. SET reactions are pH-dependent and relatively slow and can require a long time to reach completion. Antioxidant capacity is based on the relative per cent of the decrease in product rather than kinetics. A numerical parameter related to the SET mechanism is

adiabatic ionization potential (Wright *et al.*, 2001; Prior *et al.*, 2005).



Other phenolic compounds (3-hydroxypseudocumene, isoamyl cinnamate, 5-nitro-1-phenyl-1-hexen-3-ol, nordextromethorphan and *trans*-totarol) in this plant followed the same mechanism. The high scavenging property of the extract may be due to hydroxyl groups existing in the phenolic compounds' chemical structure that can provide the necessary component as a radical scavenger. Inhibition of proliferation, induction of apoptosis, suppression of inflammation, modulation of immune responses, and protection against oxidative stresses, and many signaling pathways have been suggested as the molecular targets of plant phenolic compounds, most of the health effects of plant phenolic compounds can be attributed to their cytoprotective activities against environmental and endogenous stresses imposed by electrophiles/oxidants. Antioxidants through their scavenging power are useful for the management of reactive oxygen related diseases such as

neurodegenerative disorder and cancer (Asgarirad *et al.*, 2010).

**Table 2: Antioxidant Potentials of the Leaf Extract of *C. columellaris* var. *campestris***

Extract and Reference Compound	DPPH IC <sub>50</sub> $\mu\text{gml}^{-1}$
<i>C. columellaris</i> var. <i>campestris</i>	3.5
Ascorbic acid	7.0

### *In vitro* Antimicrobial Potentials

The antimicrobial potential of the extract is as shown on Table 3. The organisms used in the antimicrobial study were good representatives of the Gram-positive and Gram-negative bacteria, and are known pathogens of man. Leaf extract of *C. columellaris* var. *campestris* exhibited different degrees of antibacterial potentials. The values of antibacterial activities of the extract were very high, the highest inhibitory effect of the extract were observed against *S. viridans* (30 mm), followed by *S. aureus* (20 mm), *P. aeruginosa* (20 mm) and *E. coli* (18 mm), moderate inhibition against *P. mirabilis* (13 mm), *S. typhimurium* (10 mm), low inhibition against *S. agalactiae* (9 mm) and resistant to *K. pneumoniae*. The bacteria (except *P. aeruginosa*) were found to be resistant to Augmentin (AUG), but the organisms (except *S. agalactiae*) were sensitive to Gentamicin (GEN) synthetic antibiotics. The results of the present study were, however, better than that of closely related species in *Cupressaceae* family such as leaves extracts of *Thuja occidentalis* which has lower activities against *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis* and *S. typhi*, with zones of inhibition ranged from 6-12mm in hot water extract, 4-9 mm in cold water extract and 5-11 mm in methanolic extract (Shah *et al.*, 2014). The emergence of multi-resistant bacteria strains is a major source of concern and has been linked to the frequent use of synthetic antibiotics that make the organisms to become resistant to such drugs (Baharoglu *et al.*, 2013). The factors responsible for the high susceptibility of the organisms to the extract were due to the high percentage of terpenoids and low molecular weight phenolic compounds, if a compound has high molecular weight, the rate of its diffusion is always slow, amount diffused is reduced and also take longer time, whereas an extract of low molecular weight diffuses faster and at a quicker rate, therefore acts faster on the targeted organisms (Orji *et al.*, 2012). Moreover, Gram-positive bacteria are known to be more susceptible to the drugs than Gram-negative bacteria. The result obtained is evidence that the extract produced marked inhibitory effect on the tested organisms. The extract could be used to treat different systemic infections. In this

regard the secondary metabolites in this medicinal plant would play an important role as safe natural antibiotics.

**Table 3: Zones of Inhibition (mm) showing the Antimicrobial Properties of Leaf Extract of *C. columellaris* var. *campestris***

Conc Organism	<i>C. columellaris</i> var. <i>campestris</i>			AUG	GEN
	1000	100	10	30µg	10µg
<i>S. agalactiae</i>	09	09	08	-	-
<i>S. aureus</i>	20	20	20	-	20
<i>S. viridans</i>	30	30	30	-	18
<i>E. coli</i>	18	18	17	-	17
<i>K. pneumoniae</i>	-	-	-	-	19
<i>P. mirabilis</i>	13	13	13	-	16
<i>P. aeruginosa</i>	20	20	11	10	11
<i>S. typhimurium</i>	10	10	10	-	18

**Key note:** - = Resistant, 6-9 mm = low inhibition, 10-15 mm = moderate inhibition and  $\geq 15$  mm = high inhibition.

### Conclusion

The present study showed that *C. columellaris* var. *campestris* contains considerable amount of low molecular weight phenolic compounds and terpenoids with excellent antioxidant properties. Moreover, it was also demonstrated that the crude extract of *C. columellaris* var. *campestris* is a potential antimicrobial agent. These results indicated that the extract could serve as safe antioxidants and antiseptic supplements. Also, consumption of food produced with natural products from such aromatic plant like this could prevent the risk of free radical dependent diseases. These properties could be capitalized on to develop some drugs for the control of free radical induced health disorders. However, there is a need of establishing more systemic *in vivo* and clinical studies to validate the human consumption of phytochemicals in this medicinal plant.

**Conflict of interest:** We have no conflict of interest.

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