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Ofentse Mazimba
Department of Chemistry,
University of Botswana, P. Bag
00704, Gaborone, Botswana

Kabo Wale
Department of Biological
Sciences, University of
Botswana, P. Bag 00704,
Gaborone, Botswana

Tebogo E. Kwape
Department of Biological
Sciences, University of
Botswana, P. Bag 00704,
Gaborone, Botswana

Shetonde O. Mihigo
Department of Chemistry,
University of Kinshasa, P.O.
Box 190 Kin XI, Kinshasa,
DR Congo.

Bokolo M. Kokengo
Department of Chemistry,
University of Kinshasa, P.O.
Box 190 Kin XI, Kinshasa,
DR Congo.

Correspondence:
Ofentse Mazimba
Department of Chemistry,
University of Botswana, P. Bag
00704, Gaborone, Botswana

Cinnamomum verum: Ethylacetate and methanol extracts antioxidant and antimicrobial activity

Ofentse Mazimba, Kabo Wale, Tebogo E. Kwape, Shetonde O. Mihigo, Bokolo M. Kokengo

Abstract

Cinnamomum verum is an evergreen tree that is widely used as cinnamon spice. The plant is medicinally used against gastrointestinal conditions and has anti-inflammation pharmacological activity. The antioxidant activities of the leaves and stem bark methanol and ethyl acetate extracts of plant material from the Democratic Republic of Congo were assayed using total phenolic content, diphenylpicrylhydrazyl radical (DPPH), ferric reducing, iron chelating and thiobarbituric acid (TBA) assays. The phytochemical analysis of methanol extracts indicated strong presence of alkaloids, flavanoids, tannins, phenols and saponins, while fatty acids were detected from the stem bark methanol extract only. The methanol solvent extracted high content of phenolics from the leaves and stem bark. The bark methanol extracts exhibited good DPPH activity ($IC_{50}= 76.5 \mu\text{g/mL}$) than the leaves extract ($IC_{50}= 100.2 \mu\text{g/mL}$). The methanol extracts of leaves ($IC_{50}= 98.5 \mu\text{g/mL}$) and stem bark ($IC_{50}= 72.3 \mu\text{g/mL}$) exhibited a good metal chelating capacity. In the TBA assay the leaf and bark methanol extracts displayed high activity (81-85 % inhibition). The extracts antimicrobial activities were tested against, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. The agar well diffusion was used for initial screening, while minimum inhibitory concentrations (MICs) were determined using agar dilution method for active extracts on the initial method. Ethylacetate stem bark extract was inactive while the methanol extracts had activities against some of the organisms. In conclusion, the methanol extract of both stem and leaves could be used as potential sources of new antioxidant and antimicrobial agents.

Keywords: *Cinnamomum verum*; Methanol extract; Antioxidant; Antimicrobial

1. Introduction

Cinnamomum verum, J Presl. (*Syn. Cinnamomum zeylanicum* Nees) (Lauraceae) is mainly used as a culinary herb like other cinnamons in the traditional Eastern and Western medicine [1]. *C. verum* is also traditionally used for bloating, nausea, flatulence, colic, and gastrointestinal tract spastic conditions [2]. Previous studies on the bark and leaves have identified cinnamaldehyde and eugenol as the most important components of the essential oils [3]. (*E*)-cinnamaldehyde is responsible for the anti-tyrosinase activity of cinnamon [4]. The bark and leaf aqueous and methanol extracts have good reducing power, radical scavenging activity and metal chelation properties. The methanol extracts inhibited the growth of prostrate and glioblastoma cancer cell lines and protected DNA against the hydroxyl radicals [1, 5]. The bark aqueous extract showed antibacterial activities against food borne pathogens, *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecali*, *Escherichia coli* and *Proteus mirabilis* [5]. A 62.5 mg/mL of Cinnamon extract inhibit *Bacillus sp.* and *S. aureus* on agar plates [6] while at 500 mg/mL *C. verum* bark aqueous extract exhibits complete inhibition of soybean borne *Aspergillus flavus* [7]. *C. verum* bark also showed inhibitory effect on the growth of *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *E.coli* in an agar diffusion test, with the minimal inhibitory concentrations (MICs) in the range of 4-16 mg/mL [8]. Activity against *Streptococcus agalactiae* using disk diffusion assay was 18 mm inhibition zone. The *in vivo* antimicrobial effect of *C. verum* was tested by feeding tilapia fish with *C. verum* extract and bark powder supplements, and it significantly reduced the mortality rate by 22 and 33 % respectively after four days of exposure to *Streptococcus agalactiae* [9]. These studies suggested that *C. verum* has potential as a natural food preservative given its antibacterial and antioxidant activity. Hence, given the importance of this spice plant, the current study evaluates the phytochemicals, total phenolic content, antimicrobial and antioxidants activities of the ethyl acetate (EtOAc) and methanol (MeOH) extracts of the leaves and stem bark from plant material sampled in the Democratic Republic of Congo.

2. Materials and methods

2.1. Crude extracts preparation

The plant materials (leaves and stem bark) of *Cinnamomum verum* were collected in Mvuazi/Mbanza-Ngungu, Bas-Congo Province, DR Congo in June 2012. The plant was identified/authenticated from a voucher specimen (H. Breyne N.30) deposited at the Herbarium of the INERA (Institut National d'études et Recherches Agronomiques) located at the University of Kinshasa. The dried leaves and stem bark cuttings were blended into fine powders for extractions. Thus, methanol and ethylacetate extracts were prepared by extracting 10 g of powder with 100 mL of solvent, stirring at 25 °C for 12 h. The extracts were filtered and the solvents evaporated using a rotary vacuum evaporator. The test samples were LE-leave ethylacetate (EtOAc), SBE-stem bark ethylacetate, LM-leave methanol and SBM-stem bark methanol extract.

2.2. DPPH radical scavenging activity

DPPH (Diphenylpicrylhydrazyl radical) free radical scavenging activity was determined by previously described method [10]. Briefly, DPPH solution (1.5 mL, 2 % in methanol) was incubated with 1.5 mL of extracts (100, 50, 25 and 10 µg/mL). The absorbance was read at 517 nm against a methanol blank after incubation for 0.5 and 3 hours. Ascorbic acid was used as a reference compound. The percentage of DPPH radical scavenging was calculated using the equation: %DPPH scavenged $[(A_0 - A_s)/A_0] \times 100$. Where A_0 is the absorbance of the control and A_s is the absorbance of the test compound. Measurements were carried out in triplicates. IC_{50} was determined from % Inhibition vs Concentration plots.

2.3 Iron metal chelating activity

The chelating effect of the extracts on ferrous ions was estimated using reported methods [1, 11] with slight modifications. The principle of this assay is based on the *O*-phenanthroline- Fe^{2+} complex formation and its inhibition in the presence of chelating agents. The reaction mixture containing *o*-phenanthroline (1 mL, 0.05% in methanol), $FeCl_2$ (2 mL, 0.1 %) and 2 mL of extracts (100, 200, 500 and 1000 µg/mL) was incubated at room temperature for 20 min and the absorbance was measured at 510 nm. EDTA (ethylenediaminetetraacetic acid) was used as a standard metal chelator. The ratio of inhibition of complex formation was calculated using the equation: Chelation activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$. Plots of % Chelation activity vs Concentration were made to determine IC_{50} using Microsoft office 2010 Excel™. The experiment was performed in triplicates.

2.4. Total phenolic content

The method followed was reported by Mazimba *et al.* [10]. Briefly, Test sample, 0.5 mL (standard, 0.01-0.05 mg/mL or extract, 1 mg/mL) and Folin-Ciocalteu reagent, 0.5 mL was shaken (5 min.) with 1 mL of 20% Na_2CO_3 solution and allowed to stand for 2 h. Finally, methanol (80 %, 5 mL) was added and the absorbance of the supernatant solution was recorded at 725 nm. The total phenolic content was expressed in milligrams of gallic acid equivalent (GAE) per gram of samples from the standard gallic acid plot. Analysis for each sample was performed in triplicate.

2.5. Phytochemical screening

The phytochemical tests of were referenced to the technical work described by several groups and carried out in duplicates [12-15].

2.6. Flavonoids

The extract (1 mL) was added to a concentrated sulphuric acid (0.2 mL) and 0.5 g of Mg. A pink or red coloration that disappear on standing (3 min.) indicates the presence of flavonoids. OR, Lead acetate solution (10 %) drops were added to the extract (1 mL). Formation of a yellow precipitate showed the presence of flavonoids.

2.7. Tannins

a. The extract (1 mL) was added to 2 mL of water followed by drops of dilute ferric chloride solution (0.1 %). A green to blue-green (catechic tannins) or a blue-black (gallic tannins) coloration were positive indicators.

2.8. Saponins

The extract (1 mL) was shaken vigorously with distilled water. A stable persistent froth for 20 min. was a positive indicator.

2.9. Alkaloids

The extract (1 mL) was treated with 3-5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 mL distilled water) and observed for the formation of reddish brown precipitate or colouration.

2.10. Anthocyanins

To the extract (1 mL) 2 mL of HCl (2M, 1 mL) and ammonia (4M, 1mL) were added. The colour change from pink-red to blue-violet indicates the presence of anthocyanins.

2.11. Coumarins

NaOH (2 mL, 10 %) was added to 1 mL of extract and formation of yellow color indicates the presence of coumarins.

2.12. Terpenoids

The extract (2 mL) was added to acetic anhydride (2 mL) and concentrated H_2SO_4 drops. Formation of blue, green rings indicated the presence of terpenoids.

2.13. Carbohydrates (Molisch's test)

Few drops of Molisch's reagent were added to the extract (1 mL), followed by 1 mL of conc. H_2SO_4 drops. The mixture was allowed to stand for two-three minutes. Formation of a red or dull violet colour at the interphase of the two layers was a positive test.

2.14. Fatty acids

The extract (0.5 mL) was mixed with 5 mL of ether. The solution was allowed to evaporate on filter paper. The appearance of transparence on dried filter paper indicated the presence of fatty acids.

2.15. Phenols

Ferric chloride test, an extract (1 mL) was treated with drops of ferric chloride (5%) and observed for the formation of deep blue or black colour.

2.16. Amino acids and Proteins

The extract (1 ml) was treated with drops of ninhydrin solution (1 % ninhydrin solution) and placed in a boiling water bath for 2 minutes. The formation of purple colour was a positive test.

2.17. Quinones

An extract (1 mL) was treated with conc. HCl drops and observed for the formation of yellow precipitate or colouration.

2.18. Oxalate

The extracts (2 mL) were treated with a few drops of glacial acetic acid. A greenish black colouration indicates presence of oxalates.

2.19. Reducing power assay

The Fe³⁺ reducing power of the extract was determined using established methods [16, 17]. Briefly, the extract (0.8 mL, 0.1 mg/mL) was mixed with phosphate buffer (0.2 M, pH 6.6, 1mL) and potassium hexacyanoferrate [K₃Fe(CN)₆] (1 %, 1mL), followed by incubating at 50 °C for 20 min. Trichloroacetic acid solution (10 %, 1 mL) was added and the resulting mixture was centrifuged at 3000 rpm for 10 min. The supernatant (1.5 mL) was mixed with distilled water (1.5 mL) and ferric chloride (FeCl₃) solution (0.1 %, 0.1 mL) and incubated for 10 min. The absorbance was measured at 700 nm as the reducing power. Increased absorbance (in absorbance units, AU) of the reaction mixture indicated greater reducing power. Values are averages of triplicates determinations.

2.20. Thiobarbituric acid (TBA) assay

The method followed was described by Rezaeizadeh *et al.* [18]. Briefly, extracts (1 mg/mL, 2 mL) were added to aqueous trichloroacetic acid (20 %, 1 mL) and thiobarbituric acid (0.67 %, 2 mL). After boiling for 10 min. the mixture was cooled and centrifuged at 3,000 rpm for 30 min. Absorbance of the supernatant was recorded at 532 nm. The antioxidant activity was calculated by percentage of inhibition as follows: % Inhibition = 100-[(A₁-A₀) ×100]. Where A₀ is the absorbance of the control and A₁ is the absorbance of the sample extracts. Measurements were done in triplicates.

2.21. Antimicrobial activity

Initial screening of the test samples was done to check for antimicrobial activity. Extract stock-solutions were prepared by placing 10 mg of plant extract into 1 mL of ethanol and diluted with 1:1 (v/v) Muller-Hinton medium. The mixture was filter-sterilised using 3 mL-syringe and blue-rimmed sterilising unit into 2 mL ependorf tube. Agar well diffusion method was used for initial screening at a concentration of 100 µg/mL [8, 19]. Minimum inhibitory concentrations (MIC) were determined for samples which showed activity [2, 8, 20] using the agar dilution method and inoculations at 0.5 McFarland turbidity standard. Gram-positive organisms (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NCIB 3610), Gram-negative organisms (*Pseudomonas aeruginosa* NCIMB 8295, *Escherichia coli* ATCC 8739) and a fungal yeast *Candida albicans* were obtained and confirmed at the Microbiology Laboratory, Department of Biological Sciences, University of Botswana. The microbial cultures were maintained on Mueller-Hinton medium (Oxoid, UK) and eighteen hour old cultures were used in the bioassay. Nystatin (Sigma-Aldrich, Czech Republic) and Gentamycin (Sigma-Aldrich, Czech Republic) were used as standards.

3. Results and discussion

3.1. Phytochemical Analysis

The phytochemical screening results in Table 1 shows the different components in the two plant parts extracts. Methanol is a good solvent for *C. verum* extraction as its leaves and stem bark extracts shows weak to strong presence of important phytochemicals. The methanol phytochemical profile has similar trend to the ethanol bark extracts [12]. The presence of alkaloids, flavanoids, steroids, tannins, triterpenoids and fatty acids is associated with medicinal values such as the anti-inflammatory, antidiabetic, analgesic and central nervous

system activities [12]. The detection of proteins, fatty acids and carbohydrates shows the nutritional value of the cinnamon spice [13, 22].

Table 1: Phytochemical screening of *C. verum* extracts

	LE	SBE	LM	SBM
Alkaloids	-	+	++	++
anthocyanins	-	+	+	+
Flavanoids	+	+	++	++
Tannins	-	+	++	++
Phenols	-	+	++	++
Coumarins	-	-	-	+
saponins	-	+	++	++
sterols	+	+	+	+
Terpenoids	+	+	+	+
quinones	-	-	+	+
Proteins	+	+	+	+
Fatty acids	+	+	-	++
carbohydrates	+	+	+	+
glycosides	-	-	+	+
oxalate	-	+	+	+

LE-leave ethylacetate, SBE-stem bark ethylacetate,

LM-leave methanol, SBM-stem bark methanol,

- = negative test, + = weak positive test,

++ = strongly positive test

3.2. Antioxidant activity

The methanol extract contained more phenolic compounds than ethylacetate extract from the powdered stems and leaves (Table 2). Likewise, the stem bark methanol extract (IC₅₀= 76.5 µg/mL) had better DPPH radical scavenging capacity than the leaves methanol extract (IC₅₀= 100.2 µg/mL) and the ethylacetate extracts of both the stem and leaves (IC₅₀= 213.5-233.2 µg/mL), after 3 hours of reaction time. The pattern of radical scavenging is similar those reported from previous studies; it was concentration dependent and the DPPH % inhibition increased with time [1, 10]. The stem bark and leaves methanol extract shows good activity in reducing Fe³⁺ to ferrous ion. Reductones in an extract exhibit their antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom [23] to convert radicals into stable and non-harmful products.

Table 2: DPPH scavenging and total phenolic content of *C verum*

Sample	TPC (GAE mg/g)	DPPH scavenging IC ₅₀ (µg/mL)	
		30 mins.	3 hours
LE	40.2 ± 0.31	289.8 ± 0.32	233.2 ± 0.35
SBE	75.2 ± 0.29	269.8 ± 0.35	213.5 ± 0.35
LM	180.8 ± 0.46	169.5 ± 0.43	100.2 ± 0.42
SBM	220.5 ± 0.53	109.6 ± 0.53	76.5 ± 0.42
Vitamin-C	-	55.88 ± 0.12	30.23 ± 0.12

Data is mean for triplicate determinations, ±S.D, n=3

The reducing power order of test samples was Vitamin C > SBM > LM > SBE > LE. The reducing power (0.86-0.94 AU; Table 3) of *C. verum* methanolic extracts at 0.1mg/mL was higher than those of seeds and pulps (0.2-0.25 AU) of *Cassia fistula*, a plant with good antioxidant properties [16]. The methanol extracts of leaves (IC₅₀= 98.5 µg/mL) and stem bark (IC₅₀= 72.3 µg/mL) exhibited good metal chelating capacity compared to the standard chelator, EDTA (IC₅₀= 50.98

$\mu\text{g/mL}$). The chelation of redox active metals is important because these are active in generating ROS species [24]. The ability of extracts to stop ferrous ion moving single electrons in Fenton type reactions indicates the secondary antioxidant potential of an extract. The methanol extracts exhibit good activities on the TBA assay at 85.8 % inhibition at a dose of 1mg/mL. This activity is better than the reported Vitamin E inhibition activity at 63.41 %, but it is lower than the activity of butylate hydroxyanisole (BHT) (96.22 %), which is a synthetic standard antioxidant [18].

The ethylacetate extracts of the stem bark and roots show weak activities on all the antioxidant assays. Given the low total phenolic content of these extract, the different levels between the methanol and ethyl acetate extract activities may be due to their differences in the phenolic contents, and also in the phenolic acid components [25]. Cinnamon has been shown to contain *trans*-cinnamic acid and *trans*-cinnamaldehyde [26] and their derivatives. Methanol, a polar solvent, extracts more polar components together with the non-polar constituents of the plant [27], hence it shows better antioxidant activities than ethylacetate. Studies have shown that fruit and vegetable possess a large spectrum of biological activities that are principally due to their antioxidant property. These control reactive oxygen species from exogenous factors and thus prevent free radical damage and lipid peroxidation [27, 28].

Total phenolic content has good positive correlation with DPPH radical scavenging and ferric reducing power ($R^2 = 0.99$). This was in accordance with literature report which has reported high correlation between antioxidant activity and total phenolic content [5, 10, 18].

Table 4: Antimicrobial activity of *C. verum* extracts

Sample	ZI (mm) [MIC ($\mu\text{g/mL}$)]				
	<i>S. aureus</i>	<i>E. Coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>C. albicans</i>
LE	-	-	-	10 [2.5]	12 [2.5]
SBE	-	-	-	-	-
LM	11 [2.5]	9 [2.5]	-	9 [2.5]	10 [2.5]
SBM	9 [2.5]	10 [2.5]	9 [2.5]	-	9 [2.5]
Gentamycin	20 [0.02]	22 [0.02]	21 [0.02]	19 [0.02]	nt
Nystatin	nt	nt	nt	nt	22 [0.02]

-: No inhibition zones (ZI) at 100 $\mu\text{g/mL}$; nt: not tested.

Previous studies have indicated antibacterial activity against Gram positive (*S. aureus*, *B. cereus*, *E. faecali*) and Gram negative (*E. coli*, *P. mirabilis*) bacterial strains [5]. Cinnamon oil shows activities against *P. aeruginosa*, *B. subtilis*, *P. vulgaris*, *K. pneumoniae* and *S. aureus* [21], *H. pylori* and *C. freundii* [29]. The spice EtOAc extract inhibition zones were reported to be 7 mm at 100 $\mu\text{g/mL}$ and 12 mm at 250 $\mu\text{g/mL}$ against *V. vulnificus* and *M. luteus* [30]. In this study zones of inhibition were not observed for concentration below 100 $\mu\text{g/mL}$, which conforms to previous observation that plant extracts show activity at higher concentrations [31]. Another study has shown *in-vitro* that the bark methanol extracts restrained tumor cell growth (1), while showing very low acute toxicity in animals, LD₅₀ 4.16 g/kg body weight [32]. The aqueous bark extract improve the semen quality of diabetic mice [33] and is not toxic to fish [9]. Cinnamon anti-inflammatory and anti-nociceptive activities have been reported [32]. In this study the methanol showed good activity against *S. aureus* which is associated with food spoilage. The current study and previous studies shows that *C. verum* is an effective antioxidant and antibacterial spice. The activities of this plant are probably due to its phytochemicals identified in Table 1, thus the isolation of the pure secondary metabolites

Table 3: *C. verum* reducing power, metal chelating and thiobarbituric acid antioxidant assays

Sample	Reducing Power (AU at 700 nm)	Metal chelation IC ₅₀ ($\mu\text{g/mL}$)	TBA % Inhibition
LE	0.36 \pm 0.12	289.3 \pm 0.14	60.8 \pm 0.39
SBE	0.41 \pm 0.13	140.0 \pm 0.16	69.5 \pm 0.50
LM	0.86 \pm 0.12	98.5 \pm 0.15	81.2 \pm 0.42
SBM	0.94 \pm 0.12	72.3 \pm 0.14	85.8 \pm 0.44
Vit-C	0.99 \pm 0.21	-	25.6 \pm 2.8
EDTA	-	50.98 \pm 0.17	-

Data is mean for triplicate determinations, \pm S.D, n=3

3.3. Antimicrobial activity

The susceptibility of selected pathogenic microorganisms against different plant extracts are presented in Table 4. The different plant extracts were screened for antimicrobial activity using the agar well diffusion assay. The ethylacetate stem bark extract was inactive on agar diffusion assay at 100 $\mu\text{g/mL}$ against all the tested microbes, *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis*, and *C. albicans*, while the leaves EtOAc extract was active against *B. subtilis*, and *C. albicans*.

The methanol extracts were active against four out of the five tested strains with 9-10 mm inhibition zones. The leaves were inactive against Gram negative *P. aeruginosa*, while the bark was inactive against *B. subtilis*. The extracts showed varied moderate antimicrobial activities against Gram-positive, Gram-negative bacteria and fungi when compared to standards (MIC = 0.02 $\mu\text{g/mL}$). Minimum inhibitory concentrations (MIC) of the leaves EtOAc and methanol extracts and stem bark methanol extract were found to be 2.5 $\mu\text{g/mL}$ against *C. albicans*.

responsible for the extracts activity will be a good addition to the cinnamon literature.

4. Conclusion

The study evaluates the antioxidant and antibacterial activities of the leaves and stem bark methanol and ethylacetate extracts. The results shows that methanol was an effective extractive solvent as it extracted higher concentration of phenolics that exhibited better antioxidant activities, ferric reducing power and metal chelating capacity. The leaf ethylacetate extracts shows positive antifungal activity against, *C. albican*. The methanol extracts of both leaves and bark had broad spectrum moderate antimicrobial activities. The antioxidant and antibacterial activities of cinnamon indicate that routine flavouring of food with cinnamon spice could help the body to relieve oxidative stress and fight microbial infections.

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6. Conflict of interest

The Authors declares “no conflict of interest”.

7. References

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