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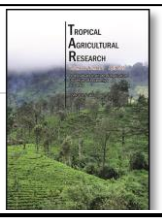

Comparison of High-Performance Liquid Chromatography (HPLC) Profiles and Antimicrobial Activity of Different *Cinnamomum* Species in Sri Lanka

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ABSTRACT

The demand for Ceylon cinnamon (*Cinnamomum verum* syn. *C. zeylanicum*) is consistent due to its superior characteristics. Besides that, Sri Lanka is home to seven other wild species: *Cinnamomum capparu-coronde, Cinnamomum citriodorum, Cinnamomum dubium, Cinnamomum litseifolium, Cinnamomum ovalifolium, Cinnamomum rivulorum,* and *Cinnamomum sinharajaense.* Since cinnamon bark contains more than seventy bioactive compounds, it possesses various pharmacological and health benefits, including antimicrobial activity. Even though there is some literature on the antimicrobial activity and chemical composition of *C. verum,* wild species have not been studied yet. The present study evaluated the antimicrobial activity and biochemical compounds of *Cinnamomum* species found in Sri Lanka using bark methanol extract and crude. Trans-cinnamaldehyde, cinnamyl alcohol, cinnamyl acetate, coumaric acid, and coumarin were quantified using HPLC techniques. The antimicrobial properties were evaluated by disc diffusion assay against two standard pathogenic strains, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. There were no detectable coumarin or coumaric acid levels in any of the species. The highest trans-cinnamaldehyde content and antimicrobial activity were observed in *C. verum,* followed by *C. sinharajaense* and *C. capparu-coronde.* Further, *C. ovalifolium*, *C. dubium*, and *C. rivulorum* did not show positive inhibition zones against the two pathogenic strains and detectable levels of trans-cinnamaldehyde. Moreover, the results showed a strong positive correlation between antimicrobial activity and trans-cinnamaldehyde content. The results suggest that the antibacterial activity of *C. sinharajaense* and *C. capparu-coronde* should be tested against a broader range of pathogenic strains and could be useful in future breeding programs.

INTRODUCTION

The Genus *Cinnamomum* consists of several economically valuable species. Among them, *C. verum* J. Presl (syn. *C. zeylanicum* Blume), *C. cassia* (L.) J.Presl (syn. *C. aromaticum* Nees), *C. tamala* (Buch.-Ham.) T.Nees & C.H.Eberm., *C. pauciflorum* Nees, and *C. camphora* (L.) J.Presl are popular worldwide (Rani et al., 2017). Of them, Sri Lanka contributed the largest share of true cinnamon, also known as Ceylon cinnamon, at 5.4 billion USD in 2019 (Global cinnamon market share, 2019).

The historical evidence suggests that the upcountry rain forest of Sri Lanka was the original home of cultivated Ceylon cinnamon (Ravindran et al., 2003). The Dutch invaders brought it to cultivation in 1700 (Ravindran et al., 2003). Interestingly, some wild populations of cultivated species exist in the upcountry and mid-country wet zones of Sri Lanka (Liyanage and Senanayake, 2010).

Sri Lankan rainforests are also home to seven other wild *Cinnamomum* species. They are *C. capparu-coronde* Blume, *C. citriodorum* Thwaites, *C. dubium* Nees., *C. litseifolium* Thwaites, *C. ovalifolium* Wight., *C. rivulorum* Kosterm, and *C. sinharajaense* Kosterm. Of them, *C. citriodorum* and *C. rivulorum* are categorized as endangered, while other wild species are included in the vulnerable category except for *C. dubium,* which is categorized as a near threatened species (MOE, 2020). Some species, such as *C. sinharajaense, C. rivulorum, C. litseifolium,* and *C. ovalifolium,* are naturally grown in isolated habitats restricted to specific geographical locations and conditions (Liyanage and Senanayake, 2010).

Literature on cultivated species in Sri Lanka over the last several years was reviewed by Suriyagoda et al. (2021). The article clearly states the superiority of C. verum in terms of biochemical properties and antimicrobial activity over the others. However, literature on wild species is still minimal. The recent work by Bandusekara et al. (2020) investigated a morphological index and a key for the morphological identification of species in the field. Further, Chandrasekara et al. (2021) shared that the universal barcoding regions *rbc*L, *mat*K, and *trn*H-*psb*A do not discriminate against *Cinnamomum* species in Sri Lanka.

The wild species *C. dubium* and *C. capparu-coronde* are utilized in traditional medicine and as food additives, and there is no evidence for the utilization of other wild species. However, some preliminary biochemical analyses have suggested unique chemical compositions in six species, while others have similar properties as the cultivated species (Ariyarathne et al., 2018, Prathibhani et al., 2021). Nevertheless, there is no biochemical and antimicrobial analysis reported, including all the cinnamon species available in Sri Lanka. Since all wild cinnamon species except *C. dubium* are categorized in the endangered and vulnerable categories, it is important to assess the biochemical composition and antimicrobial activity for sustainable utilization and conservation of such genetic resources.

As such, we designed the current study to quantify key metabolites in the bark methanol extract of cinnamon and to assess the antimicrobial activity against two Gram-negative (-) human pathogenic bacterial strains.

MATERIALS AND METHODS

Sample collection

As described in Table 1, the cinnamon samples were collected by a field survey, as previously described in Bandusekara et al. (2020). Except for *C. ovalifolium, C. litseifolium*, and *C. verum* (wild), the remaining samples were collected from the same agroecological zone, as indicated in the Table 1.

Table 1. Details of the Cinnamomum species used in the study

Species	Voucher number	Location	Agroecological regions	GPS coordinates
Cinnamomum capparu-coronde (C1MA)	KGG.BS-2018-8-CC-M-1	NCRTC, *Matara*	IL_{1a}	6°01'34.7"N 80°33'41.8"E
Cinnamomum citriodorum (C2MA)	KGG.BS-2018-8-C-M-1	NCRTC, *Matara*	IL_{1a}	6°01'21.0"N 80°33'44.3"E
Cinnamomum dubium (C3MA)	KGG.BS-2018-8-D-M-3	NCRTC, *Matara*	IL_{1a}	6°01'42.6"N 80°33'39.6"E
Cinnamomum litseifolium (C4MA)	RAAK.BS-2018-9-L-D-1	MRS, *Dalpitiya*	WM_{2a}	7°07'57.0"N 80°35'16.4"E
Cinnamomum ovalifolium (C5HP)	DSA.PCG.BS-2018-5-O-HP-1	HPNP, *N'eliya*	WU_3	6°48'12.6"N 80°48'11.2"E
Cinnamomum rivulorum (C6MA)	KGG.BS-2018-8-R-M-2	NCRTC, *Matara*	IL_{1a}	6°01'30.9"N 80°33'41.0"E
Cinnamomum sinharajaense (C7MA)	KGG.BS-2018-8-S-M-1	NCRTC, *Matara*	IL_{1a}	6°01'41.9"N 80°33'42.8"E
Cinnamomum verum (*wild*) (C8BI)	DSA.PCG.BS-2018-3-Z-B-2	GONP, *Bibila*	DL_{2a}	7°11'18.6"N 81°22'23.5"E
Cinnamomum verum (*Sri Gemunu)* (C10MA)	-	NCRTC, *Matara*	IL_{1a}	

Note: Agroecological regions are expressed as previously described by Punyawardena (2007).

Preparation of bark extracts

Bark samples were collected separately from the first secondary branch of cinnamon plants as described in Table 1. All the branches were around 9 – 11 cm in circumference and were free of pest and disease attacks. Since traditional peeling was not possible with wild species, the bark was hand-peeled as chips and air-dried for ten days following the conventional drying process. Dried bark was ground into a powder and sieved through US sieves with mesh number eight (typical nominal wire diameter = 1.00 mm) to obtain fine, even-sized particles. Thirty grams of powder were subjected to extraction with 100 mL of methanol for 1 hour in a shaking water bath at 40 °C. The same step was repeated twice, and the collected methanol extract was centrifuged at 4,000 rpm for 10 minutes. Half of the supernatant of each sample was filtered through Whatman's No. 1 filter paper and stored at 4 °C for HPLC analysis. From the other half, methanol was evaporated by a rotary evaporator at 50 °C under a vacuum to obtain crude extract and stored in a refrigerator (4 °C) until used for an antimicrobial assay test.

HPLC analysis

The commercial standards of trans-cinnamaldehyde (PubChem CID: 637,511), eugenol (PubChem CID: 3314), coumarin (PubChem CID: 323), coumaric acid (PubChem CID: 637,542), cinnamyl alcohol (PubChem CID: 5,315,892), cinnamyl acetate (PubChem CID: 5,282,110), and all HPLC grade reagents for extractions and analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HPLC analysis was conducted as previously described by Liyanage et al. (2021) with some modifications. The separation and quantitative determination of selected compounds of cinnamon bark methanol extracts were performed using an Agilent 1260 chromatography system (Agilent Technologies) with a diode array detector (DAD). A Zorbax Eclipse Plus C18 column (150 mm x 4.6 mm, particle size 5µm) was used, and the column temperature was set at 20 °C. Ten microliters of methanol extracts were injected and separated in a solvent gradient between 0.1 (v/v) orthophosphoric acid and 50 - 100 % (v/v) acetonitrile (ACN) at a 1 mL per minute flow rate. The ACN-time combinations were 80 % (v/v) ACN for 0 - 20 min, 50 % (v/v) ACN for 20 - 30 min, and 100 % (v/v) ACN for 30 - 45 min. The highest peaks for trans-cinnamaldehyde, cinnamyl alcohol, cinnamyl acetate, eugenol, coumarin, and coumaric acid were detected at 290 nm, 265 nm, 265 nm, 285 nm, 280 nm, and 290

nm, respectively, in three technical replicates from each cinnamon sample.

Determination of antimicrobial properties

The cinnamon bark crude extracts were tested against two Gram-negative bacteria species, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. We used the disc diffusion assay of the Clinical and Laboratory Standards Institute (CLSI) Performance Standards in a 4 mm thick Muller-Hinton Agar (MHA) medium (Wanger, 2007). Each bacterium was first subcultured in nutrient broth at 37°C for 24 hrs. A cinnamon bark-crude extract of 10.0 mg, dry weight, was dissolved in 50 µL of 10% Dimethyl Sulfoxide (DMSO). Sterile filter paper disks (Whatman's No.1, 6 mm in diameter) were impregnated with cinnamon extracts. Sterile solutions of 50 µL of DMSO (negative control) and 10 µL gentamicin (positive control) were used to determine the sensitivity of one strain in each experiment. Along with control disks, the antimicrobial properties of commercially available sodium metabisulphite (10.00 mg/ 50 µL of DMSO) were also tested.

A sterile cotton swab was dipped into the suspension within 15 minutes after adjusting the turbidity of the inoculum suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube to remove excess fluid from the swab. The dried surface of the plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated twice, rotating the plate approximately 60° to ensure an even distribution of inoculum. The partially opened, inoculated Petri dishes were left for 5 minutes to absorb any excess surface moisture before applying the drug-impregnated disks. Extracts of impregnated antimicrobial disks were placed on the surface of the inoculated agar plates using sterile forceps. The plates were placed in an inverted position and incubated at 37 °C for 20 hrs. The antimicrobial activity was evaluated by measuring the inhibition zones expressed in millimeters of inhibition against the tested organisms. Three impregnated antimicrobial disks were used for each sample as technical replicates for the analysis.

Data analysis

Limits of detection (LOD) and limits of quantification (LOQ) values of trans-cinnamaldehyde, eugenol, cinnamyl alcohol, cinnamyl acetate, coumarin, and coumaric acid were calculated as in Liyanage et al. (2021). Only the accessions consisting of a detectable level of each chemical (above the LOD value) were considered in the HPLC analysis. Species collected in the same agroecological zones were fitted into a randomized complete block (CRD), and the mean separation was done using Turkey's Studentized Range (HSD) test at a significant level of $\alpha = 0.05$. Statistical analysis was conducted using the SAS 9.1.1 (University Edition) software package. Microsoft Excel 2019 was used to derive descriptive statistics, including means and standard deviations.

RESULTS & DISCUSSION

All the species evaluated had a considerable number of peaks in their bark HPLC profiles, suggesting the presence of many compounds in methanol extracts (Figure 1). However, due to the limitations of the standards, we could only identify and quantify trans-cinnamaldehyde, eugenol, coumarin, coumaric acid, cinnamyl alcohol, and cinnamyl acetate. While the methanol extracts of the barks of *C. verum, C. sinharajaense,* and *C. capparu-coronde* had similar HPLC profiles (Figure 1), *C. litseifolium* and *C. citriodorum* had unique profiles. Interestingly, cinnamaldehyde was not present in *C. dubium, C. rivulorum,* and *C. ovalifolium*.

None of the species contained detectable levels of coumarin or coumaric acid. Liyanage et al. (2021) also recorded that over 70% of the studied samples of *C. verum* did not consist of detectable levels of coumarin or coumaric acid. Coumarin is considered a carcinogenic substance when present in high concentrations (Lake, 1999). Consequently, the level of coumarin substantially impacts the cinnamon sector. The maximum amount

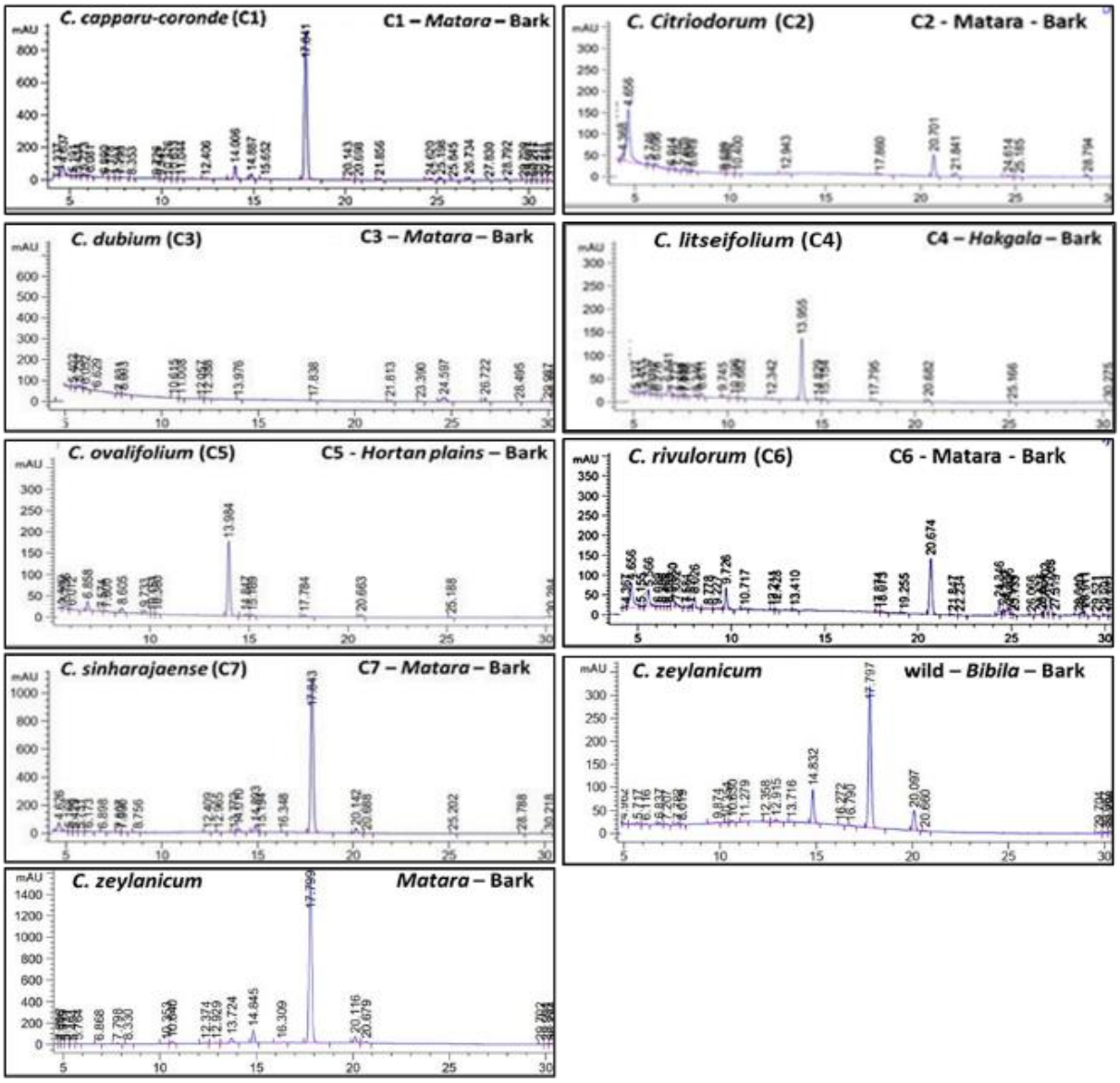

Figure 1 Bark HPLC profiles of Cinnamomum species in Sri Lanka

Note: Axis **-** Retention time (RT) (min), Peak area (mAU). Retention time and absorption wavelength (sig) (nm) for each chemical component: cinnamaldehyde (RT 17.9, sig 290), cinnamyl alcohol (RT 14.1, sig 265), cinnamyl acetate (RT 25.3, sig 265), eugenol (RT 20.7, sig 285), coumarin)RT 12.9, sig 280), coumaric acid (RT 7.3, sig 290).

of coumarin permitted by the European Parliament and Council of the European Union (EPCEU) varies from 0.05 g/kg in traditional and/or seasonal bread items to 0.005 g/kg in desserts (Regulation (EC) No 1334/2008) (Sproll et al., 2008). The current LOD value of coumarin (0.0014 g/Kg) indicates that both cultivated and wild species of the genus *Cinnamomum* in Sri Lanka are healthier than cassia cinnamon.

The highest cinnamaldehyde content was observed in Sri *Gemunu,* followed by *C. sinharajaense*, *C. capparu-coronde*, and *C. verum* wild accessions. Interestingly, *C. sinharajaense* and *C. capparu-coronde* had a considerable cinnamaldehyde content compared to *C. verum* (wild); those figures were compatible with recent findings by Ariyarathne et al. (2018). *C. litseifolium* had a high eugenol content compared to other samples. Eugenol is the main compound in the leaves of *C. verum* but is low in its bark

(Liyanage et al., 2021). The highest number of HPLC peaks was observed for *C. capparu-coronde* (15) and the lowest for *Sri Gemunu* (08). Samples collected from the same agroecological zone showed significant differences in cinnamaldehyde, eugenol, cinnamyl acetate, and cinnamyl alcohol (Table 2). This result indicates that the wild *Cinnamomum* morphological diversity previously studied by Bandusekara et al. (2020) and the genetic diversity previously studied by Chandrasekara et al. (2021) are also represented in the biochemical profiles..

The antimicrobial disc susceptibility assay results revealed significant differences among tested genus *Cinnamomum* species against two pathogenic strains, *E. coli*, and *P. aeruginosa. P. aeruginosa and E. coli* have been recognized as opportunistic human pathogens that cause high morbidity and mortality in immune-compromised and hospitalized individuals (Lee and Gallagher 2015). Gentamicin showed the highest inhibition zones as a positive control for both pathogenic strains. Variety *Sri Gemunu* showed significantly the highest inhibition zones for both pathogenic strains, followed by *C. sinharajaense* (Figure 2 and Table 2). The results obtained in the present study for *C. verum* were compatible with the reports of Keskin and Toroglu (2011), Mandal et al. (2011), and Rasheed and Thajuddin (2011).

Table 2 Antimicrobial inhibition percentages and biochemical composition of genus Cinnamomum in Sri Lanka

Species	*Antimicrobial activity*		*HPLC analysis*					
	Average Inhibition Percentage (%) compared to the negative control		*Bark concentrates (mg/g)*					*Total number of peaks*
	E. coli	*P. aeruginosa*	*Cinnamyl alcohol*	*Cinnamyl Acetate*	*Eugenol*	*Cinnamaldehyde**	*Coumarin/Cumaric acis*	
C. capparu-coronde	4.6±0.4^{d}	4.4±0.5^{d}	0.01±0.05^{b}	0.02±0.03^{c}	0.67±0.09^{b}	6.34±0.31^{c}	nd	15
C. citriodorum	2.6±0.5^{e}	4.4±0.4^{d}	nd	nd	nd	0.32±0.12^{d}	nd	13
C. dubium	0.0	0.0	nd	nd	0.04±0.01^{c}	nd	nd	13
C. litseifolium	1.5±0.6	2.9±0.7	0.08±0.03^{b}	1.06±0.023^{a}	5.11±0.06^{a}	0.17±0.33	nd	10
C. ovalifolium	0.0	0.0	1.57±0.23	nd	0.85±0.13	nd	nd	06
C. rivulorum	0.0	0.0	nd	nd	0.25±0.09	nd	nd	12
C. sinharajaense	35.4±2.5^{c}	23.5±2.4^{c}	0.27±0.18^{a}	0.05±0.01^{c}	0.67±0.23^{b}	8.20±1.41^{b}	nd	07
C. verum (wild)	6.2±0.8	13.2±1.8	0.02±0.07	nd	0.72±0.11^{b}	6.20±1.24	nd	09
Sri Gemunu	56.9±3.4^{b}	69.1±4.1^{b}	0.07±0.03^{b}	0.16±0.02^{b}	0.82±0.21^{b}	18.31±2.13^{a}	nd	08
Positive control Gentamicine	184.6±4.5^{a}	110.3±5.1^{a}	-	-	-	-	-	-
Negative control (DMSO)	0.0	0.0	-	-	-	-	-	-

Note: Inhibition percentage = [(Average sample inhibition zone – Average inhibition zone of DMSO)/ Average inhibition zone of DMSO] × 100.

Retention time (RT) (min), absorption wavelength (sig) (nm) and LOD (mg/g) value for each chemical component: cinnamaldehyde (rt17.9, sig 290) (LOD = 0.072), cinnamyl alcohol (rt14.1, sig 265) (LOD = 0.011), cinnamyl acetate (rt25.3, sig 265) (LOD = 0.009), eugenol (rt20.7, sig 285) (LOD = 0.018), coumarin rt12.9, sig 280) (LOD = 0.0014), coumaric acid (rt 7.3, sig 290) (LOD = 0.040). nd = not detectable. The total number of peaks was counted at an absorption wavelength of 290 nm and above 30 (AmU.min) peak area.

Interestingly, *C. sinharajaense* showed a higher inhibition zone against both pathogens than the wild *C. verum* species (Figure 2 and Table 2). This suggests that domestication has selected higher concentrations of beneficial chemicals and superior qualities. *C. capparu-coronde, C. dubium, C. ovalifolium, C. litseifolium*, and *C. rivulorum* did not show significant differences in inhibition zones and did not show positive inhibition percentage compared to the negative control (DMSO) (Figure 2 and Table 2). The rest of the wild species showed a positive inhibition effects as follows; *C. sinharajaense* (average inhibition percentage for *E. coli* - 35.4% and *P. aeruginosa* 23.5%), *C. verum (wild)* (6.2% and 13.2%), *C. capparu-coronde* (4.6% and 4.4%), *C. citriodorum* (2.6% and 4.4%), and *C. litseifolium* (1.5% and 2.9%) (Table 2).

Saleem et al. (2015) reported that cinnamon bark oil, cinnamaldehyde, and eugenol at 0.01% (*v/v*) significantly decreased the biofilm formation of enterohemorrhagic *E.coli* O157:H7 (EHEC). Further, the literature reveals that eugenol and cinnamaldehyde have antimicrobial effects against Gram-positive and Gram-negative bacteria (Kumar and Kumari, 2019). The current results show that the species with cinnamaldehyde showed a positive inhibition zone (Table 2). Further, the study revealed a positive correlation between cinnamaldehyde content and the inhibition zone (Figure 3). Besides that, *C. litseifolium* also showed a positive inhibition zone, which could be a result of the high bark eugenol content. Further, the present study suggests investigating the antimicrobial properties of *C. sinharajaense*, *C. capparu-coronde, C. citriodorum,* and *C. litseifolium* at different concentration levels and against a wide range of pathogenic strains.

Overall, there is evident for inter-species diversity. While some species, such as *C. sinharajaense*, *C. capparu-coronde,* and *C. verum,* share a similar chemical composition, others, such as *C. citriodorum*, *C. dubium*, *C. ovalifolium, C. rivulorum,* and *C. litseifolium* exhibit unique HPLC profiles. The sample collected from the same location also had a clear difference, suggesting the species' genetic diversity. However, the wild species did not show considerable antimicrobial activity excepts for *C. sinharajaense.* It was shown that *C. sinharajaense* exhibited significant performances in HPLC profiles and antibacterial activity, which suggests that the Ceylon cinnamon sector has future promise. Further, *C. capparu-coronde* also had more or less performance than the rest of the wild species. However, since the genus is capable of cross-pollination, HPLC and the antibacterial activity of *C. sinharajaense* and *C. capparu-coronde* must be researched at the intra-species level so that they may be utilized more effectively.

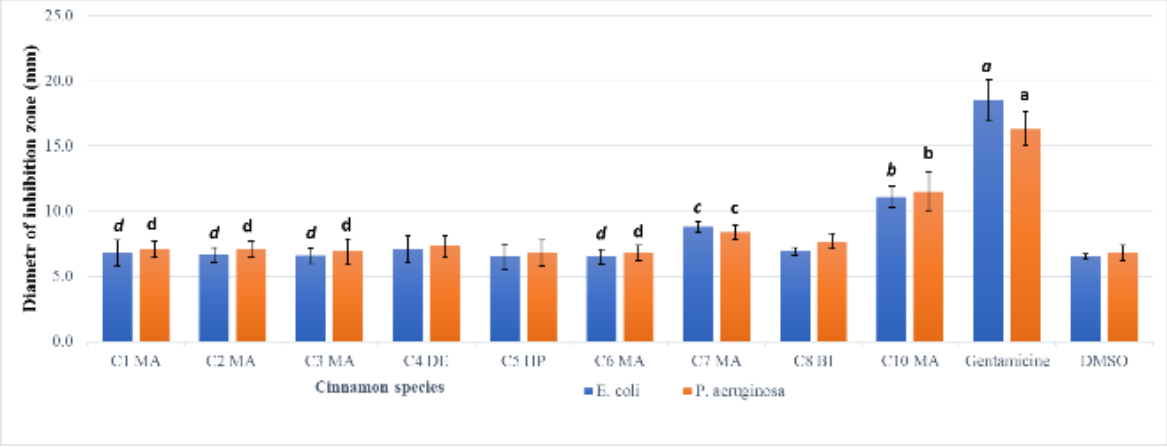

Figure 2. The antimicrobial properties of methanol extracts of genus Cinnamomum species against E. coli (A) and P. aeruginosa (B).

Note: *C. capparu-coronde (C1), C. citriodorum (C2), C. dubium (C3), C. litseaefolium (C4), C. ovalifolium (C5), C. rivulorum (C6) C. sinharajaense (C7), C. verum (C8) and C. verum (wild and cultivated) (C9*). Negative control (DMSO), Positive control Gentamicin. Different letters indicate significant differences in the sample collected from the same location.

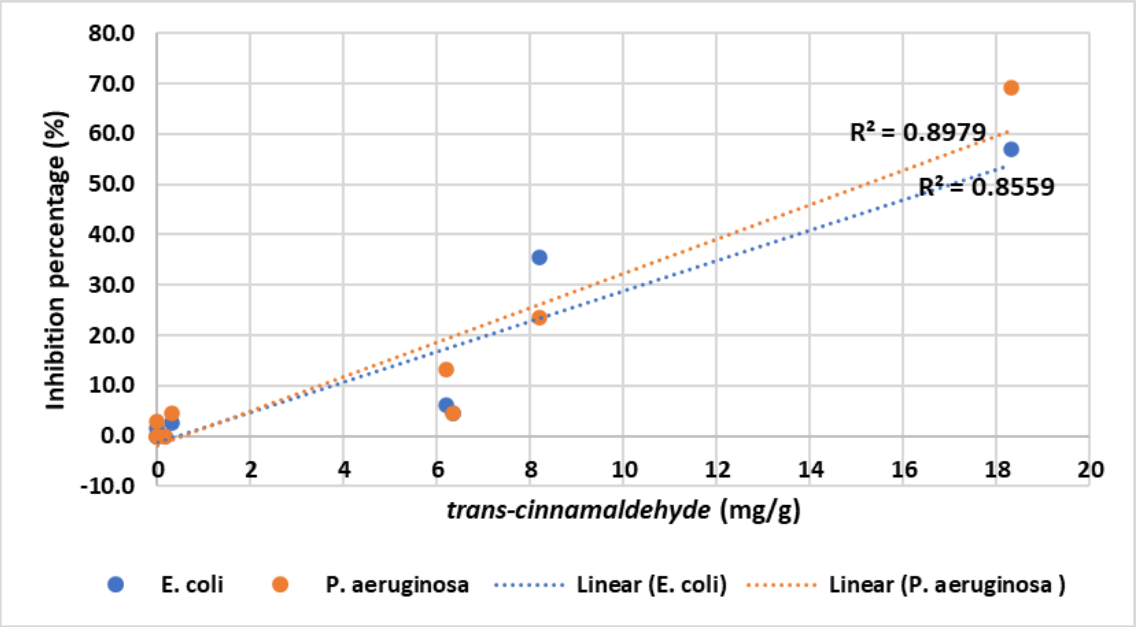

Figure 3 Relationship between *trans*- Cinnamaldehyde and antimicrobial properties of methanol extracts of genus *Cinnamomum* species.

CONCLUSIONS

According to the findings of this study, none of the species showed a detectable level of coumarin or coumaric acid. There was a significant difference among *Cinnamomum* species for cinnamaldehyde, eugenol, cinnamyl acetate, and cinnamyl alcohol. Variety *Sri Gemunu* showed the significantly highest cinnamaldehyde content and antimicrobial activity. From wild species, *C. sinharajaense* showed significant performances in cinnamaldehyde content and antimicrobial activity against considered pathogens. *C. dubium, C. ovalifolium,* and *C. rivulorum* showed no antibacterial action and no detectable cinnamaldehyde. The study also suggested a positive correlation between cinnamaldehyde content and antimicrobial activity. Accordingly, *C. sinharajaense* can potentially be a valuable candidate for application in the pharmaceutical industry and future breeding programs.

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