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Anti-inflammatory activity of cinnamon (*C. zeylanicum* and *C. cassia*) extracts – identification of *E*-cinnamaldehyde and *o*-methoxy cinnamaldehyde as the most potent bioactive compounds

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Chronic inflammation is a contributing factor in many age-related diseases. In a previous study, we have shown that Sri Lankan cinnamon (*C. zeylanicum*) was one of the most potent anti-inflammatory foods out of 115 foods tested. However, knowledge about the exact nature of the anti-inflammatory compounds and their distribution in the two major cinnamon species used for human consumption is limited. The aim of this investigation was to determine the anti-inflammatory activity of *C. zeylanicum* and *C. cassia* and elucidate their main phytochemical compounds. When extracts were tested in LPS and IFN-γ activated RAW 264.7 macrophages, most of the anti-inflammatory activity, measured by down-regulation of nitric oxide and TNF-α production, was observed in the organic extracts. The most abundant compounds in these extracts were *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde. The highest concentration of *E*-cinnamaldehyde was found in the DCM extract of *C. zeylanicum* or *C. cassia* (31 and 34 mg g^{-1} of cinnamon, respectively). When these and other constituents were tested for their anti-inflammatory activity in RAW 264.7 and J774A.1 macrophages, the most potent compounds were *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde, which exhibited IC_{50} values for NO with RAW 264.7 cells of 55 ± 9 μM (7.3 ± 1.2 μg mL^{-1}) and 35 ± 9 μM (5.7 ± 1.5 μg mL^{-1}), respectively; and IC_{50} values for TNF-α of 63 ± 9 μM (8.3 ± 1.2 μg mL^{-1}) and 78 ± 16 μM (12.6 ± 2.6 μg mL^{-1}), respectively. If therapeutic concentrations can be achieved in target tissues, cinnamon and its components may be useful in the treatment of age-related inflammatory conditions.

1. Introduction

Chronic inflammation plays an important role in the pathogenesis of a wide variety of acute and chronic diseases including cardiovascular and neurodegenerative diseases, obesity, type 2 diabetes and even ageing in general.[1–5] To date, pharmacotherapy of inflammatory conditions is predominantly based on the use of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are commonly used to manage pain and inflammation (swelling and redness) associated with some types of arthritis (such as rheumatoid arthritis) and other musculoskeletal disorders. While NSAIDs are effective in relieving pain, fever and inflammation, they can cause unwanted side effects including serious gastrointestinal toxicity such as the formation of stomach ulcers and gastric bleeding.[6,7] Some NSAIDs, particularly COX-2 inhibitors, have been linked to increased blood pressure, greatly increased risk of congestive heart failure, occurrence of thrombosis and myocardial infarction.[8] Together, these findings provide the motivation for the development of anti-inflammatory treatments with fewer adverse effects.

Cinnamon has been used in Asia as a food additive and has a long history in traditional Indian and Chinese medicine.[9]

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Cinnamon bark (*Cinnamomum zeylanicum Syn C. zeylanicum*, family: Lauraceae) is one of the oldest herbal medicines for treating inflammation and pain.[10] In traditional Chinese medicine, for example, cinnamon is used as an analgesic and antipyretic for colds, fever, headache, myalgia, arthralgia, and amenorrhea.[11]

A variety of cinnamon species have been studied for their anti-diabetic and anti-microbial properties as well as for their anti-cancer and anti-arthritis properties in cell and animal models.[12] However, the major focus of the medical use of cinnamon has been the treatment of diabetes.[13] A significant number of clinical trials indicate that cinnamon appears to be an effective option for lowering blood sugar in uncontrolled type 2 diabetics. The consumption of cinnamon at doses of 120 mg per day to 6 g per day was reported to be associated with a statistically significant decrease in levels of fasting plasma glucose, total cholesterol, LDL-C, and triglyceride levels, and an increase in HDL-C level.[13]

We have previously shown that, out of 115 foods tested, *Cinnamomum zeylanicum* displayed among the highest anti-inflammatory activity.[14] However, so far, only a few studies have been conducted examining the inflammatory effects of cinnamon. Moreover, most studies on anti-inflammatory properties of cinnamon were conducted with the species *Cinnamomum osmophloem* kaneh (Lauraceae) from Taiwan.[15,16] This species was chosen generally for analysis because the chemical constituents of its oil are similar to those of *Cinnamomum cassia* bark oil,[15] which is the common additive to foods and beverages referred to simply as 'cinnamon'. However, so far, only a few studies have investigated the anti-inflammatory activity of the most common cinnamon species used as food, *C. zeylanicum* and *C. cassia*. In one study, Kanuri *et al*. showed that pre-challenge with an alcohol extract of cinnamon bark suppressed lipopolysaccharide (LPS)-induced MyD88, iNOS, and TNF-α expression as well as NO formation almost completely. The authors further showed that this cinnamon extract might protect the liver from acute alcohol-induced steatosis.[17] In a further study, the efficacy of the polyphenol fraction from *C. zeylanicum* bark (CPP) was evaluated in animal models of inflammation and rheumatoid arthritis. Dose–response studies of CPP (50, 100, and 200 mg kg^{-1}) were conducted in acute (carrageenan-induced rat paw edema), subacute (cotton pellet-induced granuloma), and sub-chronic (AIA, adjuvant-induced established polyarthritis) models of inflammation in rats. CPP showed a significant reduction in elevated serum TNF-α concentration in the AIA model in rats. CPP also demonstrated mild analgesic effects during acute treatment as evidenced by the reduction in the writhing and paw withdrawal threshold of the inflamed rat paw during the acetic acid-induced writhing model and Randall–Selitto test. In conclusion, the authors suggest, that CPP has a beneficial action in animal models of inflammation and arthritis and therefore can be considered as a potential anti-rheumatic agent with disease-modifying action.[18]

However, it is not widely known, which compounds except cinnamaldehyde exert these anti-inflammatory properties in cinnamon. Therefore, in this study, we have examined the anti-inflammatory activities of *C. zeylanicum* and *C. cassia* extracts and identified all the chemical constituents contributing to this activity using RAW 264.7 and J774A.1 macrophages, and determined their potency.

2. Results

2.1. Comparison of the yields of different extraction procedures

Four grams of *C. zeylanicum* or *C. cassia* powder was extracted using a sequential extraction procedure with solvents of increasing polarity, starting with DCM followed by EtOAc, EtOH, MeOH and water in order to separate the lipophilic (for GC-MS analysis) from the hydrophilic compounds. In a second experiment, a direct extraction using a single polar solvent, either EtOH or water, was conducted to allow comparison with the sequential extraction procedure. The sequential extraction provided a higher overall yield compared to the direct extraction procedure for *C. zeylanicum* and *C. cassia* (1.48 ± 0.70 g and 1.64 ± 0.54 g, respectively), compared to 1.09 ± 0.35 g and 1.07 ± 0.44 g (for EtOH), and 0.338 ± 0.032 g and 0.530 ± 0.170 g (for water, Table 1).

2.2. Determination of the anti-inflammatory activity of the cinnamon extracts

Nitric oxide (NO) and tumour necrosis factor are key mediators of the inflammatory response. After exposure to bacterial lipopolysaccharide (LPS) and Interferon gamma (IFN-γ), macrophages respond with release of NO and TNF-α,[19,20] which can trigger a number of pathophysiological consequences including tissue damage. Hence, measuring inhibition of NO and TNF-α production in LPS or LPS and IFN-γ stimulated cells represents a widely used experimental model for examining the anti-inflammatory effects of chemical compounds.[20]

Sequential and direct extracts of both cinnamon species were tested in LPS + IFN-γ activated 264.7 macrophages using NO and TNF-α production as readouts to determine their anti-inflammatory activity. Due to insolubility of the low polarity

Table 1 Yield of cinnamon extracts after extraction and freeze-drying derived from 4 g cinnamon powder

	Yield of extracts (g)	
Extraction solvent	*C. zeylanicum*	*C. cassia*
Sequential		
Dichloromethane	0.162 ± 0.026	0.165 ± 0.008
Ethyl acetate	0.151 ± 0.091	0.149 ± 0.065
Ethanol	0.361 ± 0.164	0.396 ± 0.141
Methanol	0.450 ± 0.190	0.521 ± 0.126
Water	0.364 ± 0.228	0.404 ± 0.195
Total	**1.48 ± 0.70**	**1.64 ± 0.54**
Direct		
Ethanol	1.09 ± 0.35	1.07 ± 0.44
Water	0.338 ± 0.032	0.530 ± 0.170

Table 2 Summary of anti-inflammatory activity and toxicity of *C. zeylanicum* extracts

C. zeylanicum Extraction solvent	Inhibition of NO production (IC_{50} in μg mL^{-1} ± SD)	Inhibition of TNF-α production (IC_{50} in μg mL^{-1} ± SD)	Cytotoxicity (LC_{50} in μg mL^{-1} ± SD)
Sequential extraction			
Dichloromethane	24.3 ± 1.3	76.4 ± 12.8	194.8 ± 26.0
Ethyl acetate	14.0 ± 4.0	66.2 ± 5.6	120.2 ± 13.3
Ethanol	32.6 ± 4.3	87.4 ± 15.8	646 ± 46
Methanol	482 ± 10	310 ± 14	>500
Water	20.8 ± 2.2	91.2 ± 13.3	606 ± 16
Direct extraction			
Ethanol	122 ± 21	36.4 ± 1.6	866 ± 56
Water	245 ± 12	270 ± 29	607 ± 7

Table 3 Summary of anti-inflammatory activity and toxicity of *C. cassia* extracts

C. cassia extraction solvent	Inhibition of NO production (IC_{50} in μg mL^{-1} ± SD)	Inhibition of TNF-α production (IC_{50} in μg mL^{-1} ± SD)	Cytotoxicity (LC_{50} in μg mL^{-1} ± SD)
Sequential extraction			
Dichloromethane	22.8 ± 1.4	121 ± 3.0	204 ± 30
Ethyl acetate	19.7 ± 6.0	78.4 ± 1.5	140 ± 9.0
Ethanol	47.4 ± 0.4	117 ± 3	620 ± 115
Methanol	322 ± 1	358 ± 19	>500
Water	103 ± 4	83.6 ± 25.3	600 ± 30
Direct extraction			
Ethanol	157 ± 39	51.2 ± 0.7	501 ± 147
Water	180 ± 56	354 ± 24	629 ± 31

extracts in the cell culture media, they were diluted in DMSO or EtOH, leading to final concentrations of up to 0.5% DMSO or EtOH in cell culture medium. This concentration of organic solvents did not affect cell viability by more than 10% (data not shown).

In the sequential extracts of *C. zeylanicum*, the DCM, EtOAc and EtOH extracts contained the majority of the anti-inflammatory activity compared to the methanol and water extracts (Table 2). The ethyl acetate extract was the most potent, as it inhibited both NO and TNF-α production with IC_{50} values of 14.0 ± 4.0 μg mL^{-1} and 66.2 ± 5.6 μg mL^{-1} respectively (Fig. 1A, Table 2). Interestingly, the ethyl acetate extract was also relatively toxic with an LC_{50} value of 120.2 ± 13.3 μg ml^{-1}. The sequential organic extracts of *C. cassia* showed similar results, the DCM, EtOAc and EtOH fractions also contained most of the anti-inflammatory activity (IC_{50} values for NO: 22.8 ± 1.4 μg mL^{-1}, 19.7 ± 6.0 μg mL^{-1} and 47.4 ± 0.4 μg mL^{-1}, respectively) (Fig. 1B, Table 3). The sequential ethyl acetate extract showed the highest activity in regards to inhibition of TNF-α production with an IC_{50} value of 78.4 ± 1.5 μg mL^{-1} (Fig. 1B, Table 3).

The direct EtOH and water extracts of both cinnamon species inhibited the LPS + IFN-γ induced production of NO and TNF-α but mostly with lower potency (Tables 2 and 3). For example, the direct EtOH extract of *C. zeylanicum* demonstrated stronger inhibition of NO and TNF-α production (with the IC_{50} values of 122 ± 21 μg mL^{-1} and 36.4 ± 1.6 μg mL^{-1}, respectively) than the water extract (Table 2). Interestingly, direct ethanolic extracts of both cinnamon species showed potent activity in regards to inhibition of TNF-α production (IC_{50}: 36.4 ± 1.6 and 51.2 ± 0.7 μg mL^{-1}, respectively) (Tables 2 and 3).

2.3. Identification of the major constituents of the cinnamon extracts by GC-MS

Our data showed that the lipophilic extracts, *i.e.* the DCM, EtOAc and EtOH fractions of the sequential extracts of both cinnamon species showed considerably higher anti-inflammatory activity than the hydrophilic extracts, indicating that the most potent anti-inflammatory compounds were lipophilic and/or volatile. We therefore used GC-MS to identify the major constituents in these extracts.

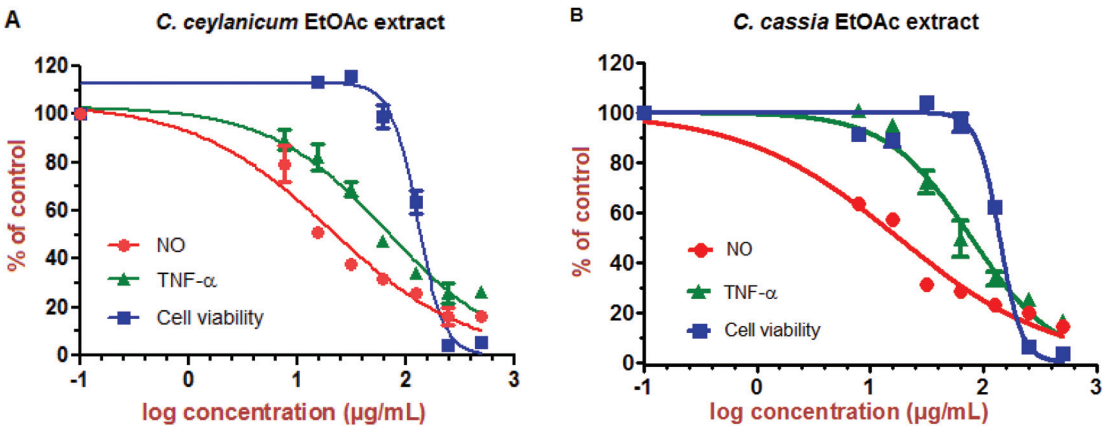

Fig. 1 Down regulation of LPS and IFN-γ induced production of pro-inflammatory markers by a sequential EtOAC extract of *C. zeylanicum* and *C. cassia*. RAW264.7 macrophages were activated with LPS and IFN-γ in the presence of increasing concentrations of *C. zeylanicum* (A) or *C. cassia* (B) EtOAc extracts derived from the sequential extraction. Nitric oxide and TNF-α production as well as cell viability were determined after 24 h. Results represent the mean ± SD of 3 experiments (in triplicate).

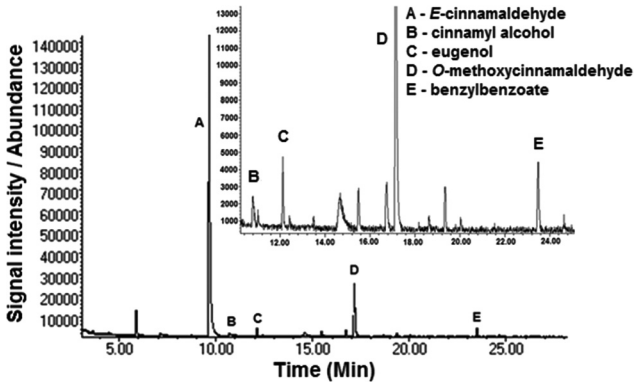

Fig. 2 Gas chromatogram of the *C. zeylanicum* EtOAc extract derived from the sequential extraction. *E*-cinnamaldehyde, cinnamyl alcohol, eugenol, *o*-methoxycinnamaldehyde and benzyl benzoate could be identified and quantified. The magnified insert (top right) shows the four minor peaks.

Five major compounds were identified and quantified from *C. zeylanicum* (as an example, the EtOAc extract of *C. ceylanicum* is shown in Fig. 2) and 4 major compounds were identified and quantified from *C. cassia*. These compounds were *E*-cinnamaldehyde, *o*-methoxycinnamaldehyde, eugenol, benzyl benzoate, coumarin and cinnamyl alcohol (Table 4). The highest concentration of *E*-cinnamaldehyde was found in the DCM extract of either *C. zeylanicum* or *C. cassia* (125 and 135 mg per 4 g of cinnamon, respectively; Table 4).

2.4. Identification of the major constituents of the cinnamon extracts by UPLC-PDA/MS

In the GC-MS analysis of methanol and water extracts, only *E*-cinnamaldehyde could be identified in methanolic extracts and no compounds in water extracts. Therefore, UPLC-PDA/MS was additionally employed to identify the constituents in the MeOH and water extracts of both *C. zeylanicum* and *C. cassia.* Five major compounds were identified by UPLC-MS and then quantified by UPLC-PDA (Table 4).

2.5. Anti-inflammatory activities of cinnamon constituents

In order to identify which of the constituents was responsible for the anti-inflammatory activity of the extracts, individual compounds (Fig. 4) were tested for their anti-inflammatory activity in the same manner as the extracts using two different cell lines, RAW 264.7 and J774A.1 macrophages as described previously.[21] All compounds except coumarin demonstrated considerable anti-inflammatory activity, determined as inhibition of LPS + IFN-γ induced NO and TNF-α production (Fig. 3, Tables 5 and 6). The most potent compounds were *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde, which exhibited IC_{50} values for NO with RAW 264.7 cells of 55 ± 9 μM (7.3 ± 1.2 μg mL^{-1}) and 35 ± 9 μM (5.7 ± 1.5 μg mL^{-1}), respectively; and IC_{50} values for TNF-α of 63 ± 9 μM (8.3 ± 1.2 μg mL^{-1}) and 78 ± 16 μM (12.6 ± 2.6 μg mL^{-1}), respectively (Table 5). Similar results were obtained with the J774A.1 cell line. Again, the most potent compounds were *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde, which exhibited IC_{50} values for NO of 51 ± 2 μM (6.7 ± 0.3 μg mL^{-1}) and 38 ± 2 μM (6.2 ± 0.3 μg mL^{-1}), respectively and IC_{50} values for TNF-α of 51 ± 5 μM (6.7 ± 0.7 μg mL^{-1}) and 79 ± 7 μM (12.8 ± 1.1 μg mL^{-1}), respectively (Table 6).

3. Discussion and experimental

Cinnamon has been reported to be beneficial for the amelioration of many inflammatory diseases including control of blood glucose levels in diabetes arthritic pain.[22] In spite of its widespread use, research on its anti-inflammatory properties has been limited. The pioneering work by the group of Chang *et al*. has demonstrated anti-inflammatory activity from the essential oil of *Cinnamomum osmophloeum* Kaneh. (Lauraceae).[15] However, less is known about the compounds responsible for the anti-inflammatory activity of the 'true' cinnamon of Sri Lanka, *Cinnamomum zeylanicum* and the 'Chinese' cinnamon, *C. cassia*, and our study was aimed at identifying the amount and potency of the major anti-inflammatory compounds in these foods.

The highest level of anti-inflammatory bioactivity was observed in the organic fractions which were more potent than the methanol and water extracts (Tables 2 and 3), suggesting that the majority of anti-inflammatory activity is exerted by lipophilic compounds. The organic fractions (DCM, EtOAc) of both *C. zeylanicum* and *C. cassia* showed anti-inflammatory activity comparable to the data reported by Tung *et al.*,[15] who reported an IC_{50} value for NO inhibition for the essential oil isolated from *C. osmophloeum* twigs of 11.2 μg mL^{-1}. For TNF-α, our results are comparable to data reported by Chao *et al.*,[16] who showed that the essential oil from the leaves of *C. osmophloeum* had inhibitory effects on LPS-induced TNF-α production. They showed that 52 ng mL^{-1} TNF-α was released from LPS-stimulated cells and that this TNF-α secretion was reduced to 35 ng mL^{-1} (67% of control) by 60 μg mL^{-1} essential oil from *C. osmophloeum* leaves. When cytotoxicity was investigated, the sequential DCM and EtOAc extracts of both cinnamon species caused some degree of cell death, with an LC_{50} value of 120–200 μg mL^{-1}, values much lower than the extracts from the polar solvents EtOH, MeOH and water (Tables 2 and 3). However, none of the major compounds identified appear to be the responsible for cytotoxicity, indicating that minor constituents might be responsible for the cytotoxicity in this fraction.

Cinnamon bark extracts are complex mixtures and therefore we identified and quantified all major compounds in the two cinnamon species using GC-MS and UPLC-PDA/MS. *E*-cinnamaldehyde, *o*-methoxycinnamaldehyde, cinnamyl alcohol, benzyl benzoate, eugenol, and cinnamic acid demonstrated considerable anti-inflammatory activity in terms of inhibition of NO production, whereas only cinnamaldehyde, *o*-methoxycinnamaldehyde and benzyl benzoate were potent inhibitors of TNF-α production. In detail, the most potent compounds were *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde which

Table 4 Compounds identified from *C. zeylanicum* and *C. cassia* by GC and UPLC-PDA analysis (mean ± SD, n = 3)

Solvent	*C. zeylanicum*		*C. cassia*	
	Concentrations of the compounds in extract[b] (mM)	Weight of the compounds in extract[c] (mg)	Concentrations of the compounds in extract[b] (mM)	Weight of the compounds in extract[c] (mg)
Dichloromethane (GC)				
E-Cinnamaldehyde	948 ± 74	125 ± 9.8	1019 ± 89	135 ± 12
Cinnamyl alcohol	7.5 ± 1.2	1.0 ± 0.2	9.7 ± 0.8	1.3 ± 0.1
Eugenol	25.8 ± 3.0	4.2 ± 0.5	n.d.	n.d.
Coumarin	n.d.[a]	n.d.	1.7 ± 0.3	0.25 ± 0.04
o-Methoxy cinnamaldehyde	130 ± 17	21.1 ± 2.8	171 ± 22	27.8 ± 3.4
Benzyl benzoate	10.3 ± 1.7	2.2 ± 0.4	n.d	n.d.
Ethyl acetate (GC)				
E-Cinnamaldehyde	601 ± 62	79.4 ± 8.2	425 ± 42	56.2 ± 5.5
Cinnamyl alcohol	Trace	Trace	2.4 ± 0.3	0.35 ±
Eugenol	9.5 ± 1.5	1.6 ± 0.3	n.d.	n.d
Coumarin	n.d.	n.d.	2.4 ± 0.2	0.35 ± 0.03
o-Methoxy cinnamaldehyde	210 ± 12	34.1 ± 1.9	467 ± 35	75.7 ± 5.7
Benzyl benzoate	8.0 ± 1.0	1.7 ± 0.2	n.d.	n.d.
Ethanol (GC)				
E-Cinnamaldehyde	493 ± 98	65.1 ± 12.9	356 ± 33	47.1 ± 4.3
o-Methoxy cinnamaldehyde	Trace	Trace	44.9 ± 4.2	7.3 ± 0.7
Methanol (UPLC)				
E-Cinnamaldehyde	8.66 ± 0.03	1.14 ± 0.01	1.78 ± 0.02	1.78 ± 0.02
o-Methoxy cinnamaldehyde	1.24 ± 0.02	0.20 ± 0.01	n.d.	n.d.
Cinnamic acid	0.94 ± 0.01	0.14 ± 0.01	0.68 ± 0.01	0.68 ± 0.01
Eugenol	1.25 ± 0.18	0.20 ± 0.03	1.09 ± 0.44	1.09 ± 0.44
Coumarin	2.64 ± 0.03	0.39 ± 0.01	0.90 ± 0.25	0.90 ± 0.25
Water (UPLC)				
E-Cinnamaldehyde	0.35 ± 0.01	0.05 ± 0.01	0.28 ± 0.02	0.04 ± 0.01
o-Methoxy cinnamaldehyde	0.26 ± 0.01	0.04 ± 0.01	0.50 ± 0.01	0.08 ± 0.01
Cinnamic acid	3.24 ± 0.01	0.48 ± 0.01	2.91 ± 0.01	0.43 ± 0.01
Coumarin	1.50 ± 0.03	0.22 ± 0.01	0.92 ± 0.11	0.14 ± 0.02
Direct extracts				
Ethanol				
E-Cinnamaldehyde	706	93.3	741	97.9
Eugenol	Trace	Trace	239	38.8
Coumarin			0.94	0.14
o-Methoxy cinnamaldehyde	Trace	Trace	n.d.	n.d.
Water				
E-Cinnamaldehyde	7.46	0.99	6.79	0.90

[a] n.d. = not detectable. [b] Concentrations of the compounds in each extract extracted from 4 g of cinnamon powder redissolved at a concentration of 1 mg ml^{-1}. [c] Weights of the compounds in each extract extracted from 4 g of cinnamon powder.

exhibited IC_{50} values for NO (RAW 264.7 cells) at concentrations of 55 μM and 35 μM respectively and IC_{50} for TNF-α of 63 μM and 78 μM, respectively (Table 5). The data were confirmed in J774A.1 cells, where *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde emerged as the most potent compounds (Table 6).

These results are comparable to results reported by Tung *et al*. who reported an IC_{50} value of 88.4 μM for NO inhibition for *E*-cinnamaldehyde in LPS-activated RAW 264.7 macrophages.[15] Our results indicate that *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde are the principal anti-inflammatory compounds in *C. zeylanicum* and *C. cassia*. Our *in vitro data* suggest that cinnamon and its components may be used as for the amelioration of age-related inflammatory conditions, if therapeutic concentrations can be achieved in target tissues. As most active cinnamon compounds are aldehydes, it was suggested safety might pose some limitations for the use of cinnamon and its components as therapeutic drugs. In chronic toxicity studies, oral doses of *E*-cinnamaldehyde >2620 mg kg^{-1} per day in mice and >940 mg kg^{-1} per day in rats produced nearly 100% mortality.[23] However, these doses appear to be much higher than used in human consumption. For humans, the World Health Organization (WHO 1984) suggested an acceptable daily intake level of cinnamaldehyde of 0.7 mg kg^{-1} body weight, and cinnamon doses ranged from 1 to 6 grams (corresponding to approximately 400 mg *E*-cinnamaldehyde) per day have been used in human clinical trials without major side effects.[13,24] For coumarin, the German

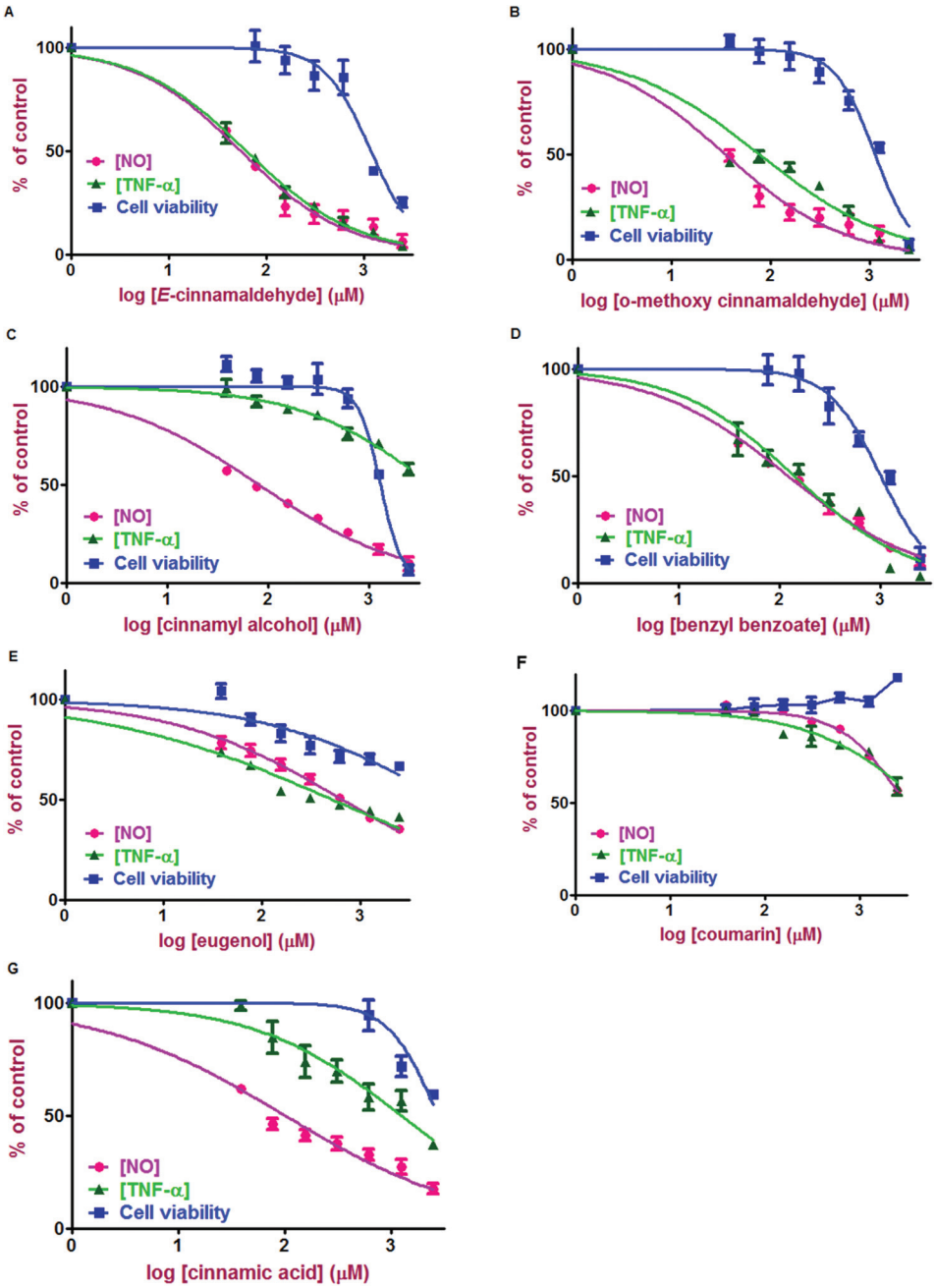

Fig. 3 Dose-dependent effects of cinnamon compounds on LPS and IFN-γ induced production of nitric oxide and TNF-α. RAW264.7 macrophages were activated with LPS and IFN-γ in the presence of increasing concentrations of cinnamon compounds (A) *E*-cinnamaldehyde, (B) *o*-methoxy-cinnamaldehyde, (C) cinnamyl alcohol, (D) benzyl benzoate, (E) eugenol, (F) coumarin and (G) *E*-cinnamic acid. Nitric oxide and TNF-α production as well as cell viability were determined after 24 h. Results represent the mean ± SD of 3 experiments (in triplicate).

Federal Institute for Risk Assessment has established a tolerable daily intake (TDI) of 0.1 mg coumarin per kg body weight. Furthermore, European health agencies and researchers have warned against consuming high amounts of *C. cassia*, because of its coumarin content.[25] However, without detailed *in vivo* studies with an inflammatory readout in animals or humans, it is difficult to calculate if the TDI of coumarin would be exceeded if a therapeutic dose of cinnamon extract is consumed. However, our data demonstrate that coumarin is not one of the major anti–inflammatory compounds, and therefore *C. zeylanicum* which contains little coumarin would be a safer option in this regard.

An important issue for clinical efficacy of cinnamon compounds is also their bioavailability, as the compound needs to be present in therapeutic concentrations in the target tissue. Orally applied (radiolabelled) cinnamaldehyde (up to 500 mg kg^{-1} bw) undergoes nearly complete absorption as demonstrated in rats and mice. One of the studies demonstrated that

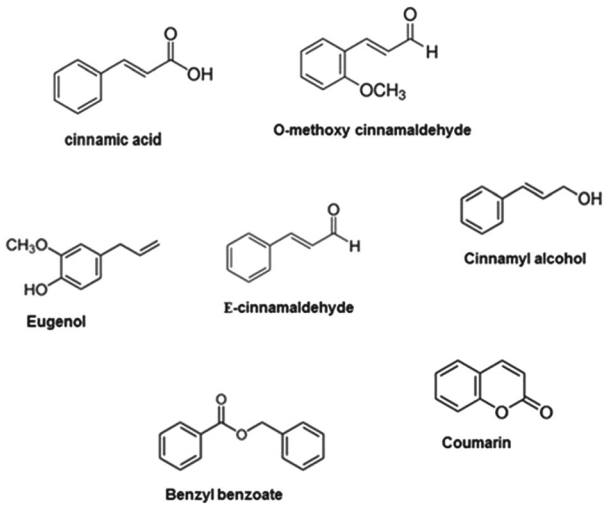

Fig. 4 Structures of the identified cinnamon compounds. A total of seven compounds were identified in cinnamon by GC-MS and UPLC-MS analysis, including *E*-cinnamaldehyde, *o*-methoxycinnamaldehyde, cinnamyl alcohol, benzyl benzoate, eugenol, coumarin and *E*-cinnamic acid.

94% of the administered dose could be recovered in the excreta in 72 h in both species, with most (75–81%) present in urine after 24 h. Surprisingly, plasma concentrations of cinnamaldehyde are quite low (0.2 μg ml^{-1}), when rats are given an oral dose of 500 mg kg^{-1}.[26] This apparent discrepancy can be explained by the large volume of distribution (2392 ± 52 l kg^{-1}) which indicates that *E*-cinnamaldehyde is trapped in certain compartment, such as adipose tissue.[26]

In summary, due to the unique pharmacokinetic parameters of cinnamon aldehydes, it is difficult to predict directly from pharmacokinetic data if their tissue concentrations are high enough to lead to an anti-inflammatory effects *in vivo*, and further animal and human studies will be required to prove their clinical efficacy.

3.1. Materials

3.1.1. Plant material. *Cinnamomum zeylanicum* Breyn (Lauraceae) (Sri Lankan cinnamon) and *Cinnamomum cassia* Nees & T. Nees J. Presl (Lauraceae) (Chinese cinnamon) were supplied in powder form by Sunrise Botanicals, Uralla, NSW, Australia.

3.1.2. Chemicals and reagents. DMSO, 95% ethanol, bovine serum albumin, lipopolysaccharide (LPS) (*E. coli* serotype 0127:B8), EDTA, *N*-(1-1-napthyl) ethylenediamine dihydrochloride, penicillin G sodium benzyl, resazurin sodium 10%, streptomycin, sulfanilamide, tetra methyl benzidine (TMB), trypan blue 0.4%, benzyl benzoate, furfural, *E*-cinnamaldehyde, *p*-cymene, β-caryophyllene, *o*-methoxycinnamaldehyde, eugenol, cinnamyl alcohol, citral, cinnamic acid, estragole, coumarin and cinnamyl acetate were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and glutamine were GIBCO brands purchased from Life Technologies (Mulgrave, VIC, Australia). IFN-γ (murine) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Diatomaceous earth was the Dionex brand purchased from Thermo Fisher Scientific Australia (Scoresby, VIC, Australia).

Table 5 Anti-inflammatory activity and toxicity of cinnamon compounds determined in RAW 264.7 macrophages

Cinnamon compound	Inhibition of NO production (IC_{50} in μM ± SD)	Inhibition of TNF-α production (IC_{50} in μM ± SD)	Cytotoxicity (LC_{50} in μM ± SD)
E-Cinnamaldehyde	55 ± 9	63 ± 9	1191 ± 226
o-Methoxycinnamaldehyde	35 ± 9	78 ± 16	1142 ± 134
Cinnamyl alcohol	82 ± 12	>2500	1311 ± 103
Benzyl benzoate	123 ± 17	142 ± 27	117 ± 186
Eugenol	670 ± 95	529 ± 89	>2500
Coumarin	>2500	>2500	>2500
E-Cinnamic acid	102 ± 21	1256 ± 369	>2500

Table 6 Anti-inflammatory activity and toxicity of cinnamon compounds determined in J774A.1 macrophages

Cinnamon compound	Inhibition of NO production (IC_{50} in μM ± SD)	Inhibition of TNF-α production (IC_{50} in μM ± SD)	Cytotoxicity (LC_{50} in μM ± SD)
E-Cinnamaldehyde	51 ± 2	51 ± 5	546 ± 89
o-Methoxycinnamaldehyde	38 ± 2	79 ± 7	539 ± 86
Cinnamyl alcohol	61 ± 5	1933 ± 487	1564 ± 160
Benzyl benzoate	195 ± 31	209 ± 24	1646 ± 157
Eugenol	475 ± 82	450 ± 37	>2500
Coumarin	2010 ± 245	>2500	>2500
E-Cinnamic acid	147 ± 19	1204 ± 158	>2500

4. Methods

4.1. Sequential extraction of cinnamon samples

Four grams of each cinnamon sample were mixed in a 4 : 1 ratio with diatomaceous earth and sequentially extracted using the following solvents of increasing polarity: dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH), methanol (MeOH) and water. Extractions were performed at a specific temperature for each solvent on a Dionex Accelerated Solvent Extractor 350 using 5 min static time and 2 cycles. Temperatures and extraction times were varied according to the solvent of extraction. DCM was heated to 100° C for 5 min, EtOAc to 100° C for 5 min. EtOH to 120° C for 6 min, MeOH to 120° C for 6 min, water to 180° C for 9 min. The extracts were subsequently evaporated using a Büchi Syncore Polyvap R6 evaporator at 60 °C using 130 rpm speed, until all the solvents were removed. The pressure was varied according to the solvent of evaporation. DCM and EtOAc were evaporated at 350 mbar, EtOH and MeOH at 200 mbar and water at 50 mbar. The resulting extracts were then freeze dried using a Telstar vacuum freeze drier with Edwards XDS10 Scroll pump for 24 to 48 hours. Dried extracts were stored in a freezer at −20 °C. For GC-MS and UPLC-MS analysis, dried extracts (1 mg) were redissolved in 1 mL of EtOH, MeOH or water. For cell culture applications, dried extracts (1 mg) were dissolved in 1 mL DMEM media (DCM and EtOAC extracts in 1% DMSO in 99% DMEM, EtOH and MeOH extracts in 1% EtOH in 99% DMEM and water extracts in 100% DMEM water), respectively.

4.2. Direct extraction of cinnamon with EtOH or water

Each cinnamon species sample (4 g) was extracted in a 4 : 1 ratio with diatomaceous earth with EtOH and water. EtOH was heated to 120° C for 6 min and water was heated to 180° C for 9 min. The extracts were freeze dried using a vacuum freeze dryer as described previously. The dried extracts (1 mg) were resuspended in 1 mL of EtOH, MeOH or water for chemical analysis using GC-MS and 1 mg of dried extracts were dissolved in 1 mL of DMEM media (EtOH extract in 1% EtOH in 99% DMEM and water extract in 100% DMEM water) for cell culture applications.

4.3. Analysis of cinnamon extracts by GC-MS

GC-MS analysis was performed on an Agilent 7890A gas chromatograph with 5975C inert XL EI/CI mass selective detector (MS) and CombiPral autosampler. Gas chromatography separation was performed on J&W scientific HP-5MS column (30 m × 0.25 mm ID, 0.25 μm). Stock solutions of each extract (1 mg ml^{-1}) were used for the injection. The injection volume of sample was 1 μL, using a split ratio of 10 : 1, at a temperature of 250 °C. The syringe was then rinsed with ethanol five times after each injection. Separation was performed at a constant carrier gas flow rate of 1 mL min^{-1}. The oven temperature was initially 80 °C for 1 min and then increased at a rate of 4 °C min^{-1} until 200 °C was reached. The MS transfer line was set to a temperature of 250 °C, the EI source to 230 °C and the quadrupole at 150 °C. We employed a solvent delay of 3 min and the acquisition mode was set to scan 40–500 *m*/*z*.

The components in each fraction were identified using comparison of their GC retention times, interpretation of their mass spectra and confirmation by mass spectral library search using the National Institute of Standards and Technology (NIST) database. The relative concentration of each compound in cinnamon extracts were quantified based on the peak area integrated by the analysis program. Hence stock solutions of the standards containing *trans*-cinnamaldehyde; eugenol, benzyl benzoate, *o*-methoxy cinnamaldehyde, cinnamyl alcohol and coumarin (500 μM) were prepared in EtOH. The mass spectrometer detector response was calibrated by injection of a solution of a mixture of the standards in ethanol. Sample solutions were then analysed at concentrations of 1 mg mL^{-1} in the solvent of extraction. Concentrations of each analyte were calculated using respective peak areas and detector response factors.

4.4. Analysis of cinnamon extracts by UPLC-PDA/MS

Chromatography was performed using a Waters Acquity UPLC instrument. A Waters Acquity UPLC BEH C18 column was used, of dimensions 2.1 × 150 mm, with stationary phase particle diameter of 1.7 μm. A column temperature of 35 °C was used. Injections of 10 μL were made in full loop mode (loop overfill factor × 4) using a method run time of 20 min and solvent flow rate of 0.2 mL min^{-1}. Solvent A consisted of 0.1% (v/v) formic acid in ultrapure water and solvent B was 0.1% (v/v) formic acid in LC-MS grade methanol. The chromatographic method consisted initially of 5% solvent B, ramped linearly to 100% solvent B over 15 min. At 17 min, the solvent composition returned to 5% B and maintained this composition until completion of the run. Mass spectrometry was performed in order to positively confirm the identity of each of the chromatographic peaks in question by mass spectrum. Detection of analytes was accomplished with photodiode array detector (PDA) and mass spectrometry. 3D spectra were obtained over the wavelength range 190–500 nm with resolution of 1.2 nm and sampling rate of 20 points s^{-1}. 2D spectra were measured at 254 nm with resolution of 4.8 nm.

Mass spectrometry was performed using a Waters Xevo TQ-MS triple quadrupole mass spectrometer with the following settings: desolvation gas temperature 300 °C; desolvation gas flow 500 L h^{-1}; cone gas 0 L h^{-1}; and source temperature of 150 °C. In positive mode the capillary voltage was 3.5 kV and the cone voltage 30 V. In negative ion mode the capillary voltage was 2.5 kV and cone voltage 30 V. Negative and positive spectra were recorded simultaneously over the *m*/*z* range 100–500 using a scan time of 1 second. Solutions of the standard analytes were prepared at a concentration of 50 mM in 30% (v/v) aqueous methanol.

Samples were run using triplicate injections and peak integration of the compounds separated by UPLC was performed by UV-Vis spectrophotometry in two different ways: a) at 254 nm and b) in the wavelength range 100–500 nm. Agreement between the two methods was excellent. Quantification

of each compound was accomplished by division of the peak area of a compound of unknown concentration by the peak area of the same compound of known concentration (standard), multiplied by the concentration of the standard.

4.5. Maintenance of RAW 264.7 and J774A.1 macrophages

RAW 264.7 and J774A.1 macrophages were grown in 175 cm^2 flasks on DMEM containing 5% fetal bovine serum (FBS) that was supplemented with Penicillin (100 u ml^{-1}), Streptomycin (100 μg ml^{-1}) and L-Glutamine (2 mM). The cell line was maintained in 5% CO_2 at 37 °C, with media being replaced every 3–4 days. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman, as opposed to using trypsin, which can remove membrane-bound receptors.[27] The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS), cell density was estimated using a Neubauer counting chamber. The cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60 000 cells/100 μl cell suspension. 100 μl of this cell suspension was dispensed into the wells of 96-well plates. Plates were incubated at 37 °C; 5% CO_2 for 24 h before the activation experiments were carried out.

4.6. Activation of macrophages

From each well, the medium was removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, 50 μL volumes of the dilutions in DMEM were added an hour prior to addition of activator. A combination of 10 μg ml^{-1} LPS and 10 U ml^{-1} (1 unit = 0.1 ng mL^{-1}) IFN-γ, both in DMEM, was used for activation. Maximum concentrations of 1.25 mg mL^{-1} (direct extracts) and 0.5 mg mL^{-1} (sequential extracts) were used and a minimum of 6 doses (made by serial dilution) were employed. After activation, the cells were incubated for 24 h at 37 °C and then NO, TNF-α and cell viabilities were determined. Unactivated cells (exposed to media alone) were used as negative control and activated cells as positive control. The effects of solvents on readouts were initially determined, but as the anti-inflammatory or cytotoxic effects of the solvents were <10% even at the highest concentration used, parameters were compared to the "no solvent" controls.

4.7. Determination of nitric oxide by the Griess assay

Nitric oxide was determined by the quantification of nitrite using the Griess reagent as described previously.[28] In detail, Griess reagent was freshly made up of equal volumes of 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% HCl. From each well, 50 μl of supernatant was transferred to a fresh 96-well plate and mixed with 50 μl of Griess reagent and measured at 540 nm in a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The concentration of nitrite was calculated using a standard curve with sodium nitrate (0–250 μM), and linear regression analysis.

4.8. Determination of TNF-α by ELISA

The diluted supernatants were used for determination of TNF-α using a sandwich ELISA according to manufacturer's instructions as described previously (Peprotech Asia, Rehovot, Israel) with small modifications.[27] In detail, the capture antibody was used at a concentration of 0.5 μg ml^{-1} in PBS (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl) (pH 7.4). Serial dilutions of TNF-α standard from 0 to 10 000 pg mL^{-1} in diluent (0.05% Tween-20, 0.1% BSA in PBS) were used as internal standard. TNF-α was detected with a biotinylated second antibody and an Avidin peroxidase conjugate with TMB as detection reagent. The colour development was monitored at 655 nm, taking readings every 5 min. After about 30 min the reaction was stopped using 0.5 M sulphuric acid and the absorbance was measured at 450 nm using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia) and expressed as a percentage of that of control cells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

4.9. Determination of cell viability by the Alamar Blue assay

100 μl of Alamar Blue solution (10% Alamar Blue (Resazurin) in DMEM media) was added to each well, incubated at 37 °C for 1–2 h. After incubation, fluorescence intensity was measured with the microplate reader (excitation at 530 nm and emission at 590 nm) and results were expressed as a percentage of the intensity of that in control cells, after background fluorescence was subtracted.

4.10. Data presentation and analysis

Six experiments were combined to determine the IC_{50} (for NO and TNF-α inhibition) and LC_{50} (for cell viability) values using the four parameter sigmoidal dose–response function in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

5. Conclusion

In summary, our findings indicate that in both cinnamon species, *E*-cinnamaldehyde and *o*-methoxycinnamaldehyde are responsible for most of the inflammatory activity of cinnamon. If therapeutic concentrations (*e.g.* by using advanced delivery methods such as microencapsulation) can be achieved in target tissues without toxicity, cinnamon and its components may be of use as a treatment for the amelioration of age-related inflammatory conditions.

Abbreviations

NO	Nitric oxide
TNF-α	Tumour necrosis factor alpha
LPS	Lipopolysaccharide

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
iNOS Inducible nitric oxide synthase
DCM Dichloromethane
EtOAc Ethyl acetate
EtOH Ethanol
MeOH Methanol
NOS Nitric oxide synthase
ASE Accelerated solvent extraction

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