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Antibacterial Activities of Essential Oils Extracted from Leaves of *Murraya koenigii* by Solvent-Free Microwave Extraction and Hydro-Distillation

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Solvent-free microwave extraction (SFME) for the isolation of essential oil from leaves of *Murraya koenigii* L. (Rutaceae) has been compared with the conventional hydro-distilled oil (HD) in terms of yield, composition, antioxidant activity, and antibacterial activity against *Listeria innocua*. The yield of essential oil obtained from 30 min of SFME was similar to that of HD for 180 min. By GC-MS analysis, the major compounds of the essential oil extracted by SFME, which were obtained in somewhat lower amounts than in the essential oil obtained by HD, were α-copaene (44.3%), β-gurjunene (25.5%), isocaryophyllene (12.1%), β-caryophyllene (8.7%) and germacrene D (2.9%). The content of oxygenated terpenes, slightly higher for the SFME-essential oil (2.3%) than the HD-essential oil (1.4%), were much lower than that of nonoxygenated terpenes in both oils. DPPH radical scavenging activities of both essential oils were relatively low (10%-24%). Complete inhibition of growth of *L. innocua* was observed with both SFME and HD essential oils, at 400 and 600 µg/mL (minimum inhibitory concentration), respectively. The SFME-essential oil at 300 µg/mL provided 92% inhibition, indicating its potential as a natural antimicrobial agent.

Keywords: *Murraya koenigii* L., Curry leaf, Antibacterial activity, *Listeria innocua*, Microwave extraction, Hydro-distillation.

Curry leaf (*Murraya koenigii* L.), family Rutaceae, is a perennial plant that is native to India, Sri Lanka and other South Asian countries. The leaves, which are rich in minerals, and vitamins A and B, are a good source of amino acids, proteins, carbohydrates and alkaloids [1]. They have a slightly pungent, bitter and feebly acidic taste, characteristics which are retained after drying [2]. The aqueous fraction of the plant was found to be nontoxic and showed no side effects when evaluated in hypoglycemic activity assays [3]. *Murraya* species have been reported to be widely used in traditional medicine in eastern Asia, Australia and South Africa, where they are used as tonics, stomachics, stimulants, carminative agents to treat influenza, for treatment of fever, bronchial asthma, dysentery and the bites of poisonous animals, as well as a source for perfumery and flavor [4].

Bacterial resistance to antimicrobial agents has become a serious problem worldwide with resistance mechanisms that have been identified and described for all the known antibiotics currently available for clinical use [5]. The volatile oil of certain *Murraya* species obtained by conventional techniques has been shown to possess antibacterial and antifungal activities [4]. Different solvent extracts from leaves of the plant exhibited very strong antioxidant [6] and antimicrobial [7] activities. With the increasing demand for volatile oil use by the pharmaceutical and food industries, efficient methods of essential oil extraction for antimicrobial and other applications have become important.

Steam distillation, hydro-distillation (HD) and simultaneous distillation-extraction methods are the most commonly used methods for extraction of essential oils. However, losses of some volatile compounds, long extraction times, low extraction efficiency, degradation of ester compounds through thermal or hydrolytic effects, and toxic solvent residues in the extract have been encountered in conventional extraction methods [8]. Recently, microwave-assisted extraction methods appeared to be particularly attractive due to fast heating of aqueous samples [9]. Solvent-free microwave extraction (SFME) is a new technique which combines microwave heating with dry distillation at atmospheric pressure for isolation and concentration of essential oils in fresh plant materials. Without any added solvent or water, microwave heating of *in situ* water within the plant tissues releases essential oil, which is evaporated by the *in situ* water of the fresh plant material [10]. If dry plant material is used, the sample is commonly moistened by soaking in water for some time and then the excess water is drained off [11]. A condenser connected on top of the extraction flask condenses the distillate continuously. Several previous studies have compared the efficiency of SFME with HD for the isolation of volatile oil fractions from various plant species including *Rosmarinus officinalis* [12], *Laurus nobilis*, *Melissa officinalis* [13], and *Thymus vulgaris* L. [14]

To the best of our knowledge, this is the first report of the application of SFME for extracting essential oil from *M. koenigii*. The objective of the study was to compare the SFME procedure with the conventional HD method in terms of volatile compounds content using gas chromatography-mass spectrometry (GC-MS), antioxidant activity by DPPH$^{\bullet}$ radical scavenging assay, and antibacterial activity against a marker organism, *Listeria innocua*.

Specific gravity and refractive index values of the essential oils extracted from leaf powder of *M. koenigii* using SFME and HD methods were the same (p>0.05) (Table 1). Figure 1 shows the

changes in the yields (%) of essential oils from leaf powder with extraction time for both the HD and SFME methods. There was no significant difference between the yield after 30 min (0.82% yield) and 180 min (0.84% yield) of extraction for SFME and HD, respectively ($p>0.05$), indicating the requirement of a much shorter extraction time with SFME compared to that of HD. Changes in the yields were not observed beyond these periods. Similar to the current study, 0.83% of yield was reported after 4 h of hydro-distillation in a previous study [15]. Interestingly, half the total essential oil yield (0.42%) could be obtained at an extraction time of 10 min using SFME in the current study. Results indicated that considerably shorter extraction times were adequate for the SFME procedure compared with that of HD. This might be associated with the high pressure gradient formed inside the plant material and the fact that microwave absorption results in significant internal heating, thus creating significantly higher internal pressures, which enhance oil extraction from the leaf powder [16].

Table 1: Specific gravity and refractive index values of the essential oils from *Murraya koenigii* obtained by HD and SFME.

	HD-essential oil	SFME-essential oil
Specific gravity[a] (22±0.2°C)	0.72±0.01	0.73±0.01
Refractive index[a] (25±0.2°C)	1.438±0.002	1.456±0.001

[a]: means ±standard deviation (SD) (n=3).
HD: Hydro-distillation, SFME: Solvent-free microwave extraction

Twenty-five major constituents were identified using GC-MS analysis of essential oils obtained from SFME and HD at 30 and 180 min of extraction, respectively (Table 2). Volatile component profiles of both essential oils indicated that use of microwave irradiation did not adversely affect the composition of the SFME-essential oil. The majority of the 25 compounds detected were common to the essential oils extracted by both methods. The major volatile compounds detected for both oils were α-copaene (42.0%-44.3%), β-gurjunene (23.3%-25.5%), isocaryophyllene (11.2%-12.1%), β-caryophyllene (8.6%-8.7%) and germacrene D (2.8-2.9) (Table 2). In the study of Raina *et al.*, β-caryophyllene, β-gurjunene, β-elemene and β-phellandrene were reported to be the most important contributors to the intense characteristic aroma of *M. koenigii* [17]. The percentage of oxygenated compounds was found to be slightly higher for the SFME-essential oil (2.3%) than that for the HD-essential oil (1.4%) (Table 1). In spite of the minor differences in the contents of volatile compounds between

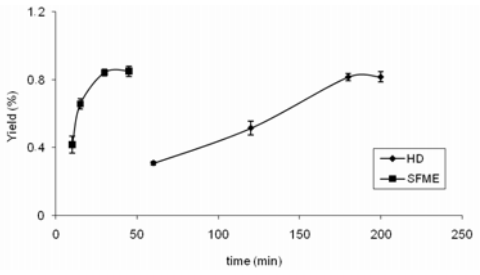


Figure 1: The change in the yields of essential oils with extraction time, obtained by SFME (solvent-free microwave extraction) and HD (hydro-distillation).

the SFME- and HD-essential oils for the majority of the compounds, the contents of many compounds were statistically different ($p<0.05$). Although the amounts of these individual compounds were low in the SFME-oil, all were oxygenated (α-terpineol, bornyl acetate, (*E*-)-nerolidol, spathulenol and caryophyllene oxide), but present in higher contents in the SFME-essential oil ($p<0.05$). β-Thujene, α-pinene, β-pinene, (*E*-)-β-ocimene and δ-elemene were the compounds that existed in fairly

Table 2: Contents of volatile compounds in *Murraya koenigii* essential oils obtained by SFME and HD, analyzed by GC-MS.

	Compounds[x]	LRI[y]	TI	QI	Content (%)[z]	
					SFME	HD
1	α-Pinene	936	93	92,91,77	0.2±0.01kjB	1.8±0.03fA
2	β-Pinene	945	93	41,69,39	0.2±0.01lkB	1.2±0.01gA
3	α-Phellandrene	960	93	77,91,136	0.1±0.02nmB	0.2±0.00lA
4	β-Thujene	975	93	41,91,77	0.3±0.01ikjB	2.9±0.03eA
5	(*E*)-β-Ocimene	1060	93	41,79,91	0.3±0.03hB	0.8±0.05iA
6	α-Terpineol	1158	59	93,121,136	0.1±0.01lm	t
7	Bornyl acetate	1230	95	93,121,43	0.1±0.01nm	ND
8	δ-Elemene	1310	121	93,136, 41	0.3±0.04ihjB	0.9±0.00hA
9	α-Cubebene	1350	161	105,91,120	0.1±0.02nA	0.1±0.01mA
10	α-Copaene	1370	161	119,105,93	44.3±0.03aA	42.0±0.04aB
11	β-Cubebene	1388	161	105,91,120	t	0.1±0.01m
12	α-Cedrene	1430	41	119,161,91	t	0.1±0.02m
13	β-Caryophyllene	1435	93	133,91,41	8.7±0.00dA	8.6±0.00dB
14	β-Gurjunene	1445	161	41, 105, 91	25. 5±0.01bA	23.3±0.02bB
15	Isocaryophyllene	1452	93	69,41,133	12.1±0.03cA	11.2±0.02cB
16	α-Humulene	1464	93	80,41,121	t	0.1±0.01m
17	γ-Muurolene	1477	161	105,119,933	0.3±0.02ihB	0.4±0.00kA
18	Germacrene D	1470	161	105,91,41	2.9±0.03eA	2.8±0.02eB
19	α-Selinene	1488	41	105,107,204	0.3±0.04ikj	t
20	(*E*)-Nerolidol	1552	41	69,43,71	0.1±0.01nm	t
21	Spathulenol	1565	43	41,205,119	0.9±0.03gA	0.8±0.00ihB
22	Caryophyllene	1575	43	41,79,93	0.9±0.03gA	0.4±0.02kB
23	α-Cadinol	1640	43	95,121,41	t	ND
24	Valencene	1648	161	204,105,93	1.3±0.05fA	0.5±0.01jB
25	γ-Eudesmol	1676	189	161, 204, 133	0.1±0.00nmA	0.1±0.02mA
	Total identified				99.0	98.3
	Oxygenated				2.3	1.4
	Extraction time				30	180

[x]: Compounds are listed in order of elution from the GC column; [y]: Linear retention indices relative to C_9-C_{23} *n*-alkanes on the VF-5MS column
[z]: Contents given in percentages obtained using relative GC peak areas; means of contents±standard deviation (SD); means of contents followed by the same lowercase letter within each column and the same uppercase letter within each row are not significantly different [Tukey's Studentized range test ($p<0.05$)]; HD: hydro-distillation; SFME: solvent-free microwave extraction; QI: qualifying ions; TI: target ion; t: trace (<0.01%); ND: not detectable..

high amounts in the HD-essential oil compared with that of the SFME-essential oil ($p<0.001$). Malwal and Sarin, who studied the composition of the volatile oil from *M. koenigii* obtained by hydro-distillation, identified α-pinene, β-pinene, β-caryophyllene, sabinene and limonene as the major components [18]. In previous studies that were related to the current work, β-caryophyllene, β-phellandrene, α- and β-pinene, β-gurjunene and β-thujene were among the major components detected in the volatile fraction of the same plant material [17] These variations in the composition of the various components could be related to geo-environmental factors, local soil characteristics, different genotypes and harvesting time of the plant.

A concentration-dependent scavenging action of DPPH$^{\bullet}$ radical was observed for the essential oils extracted by both SFME and HD (Table 3). However, the free radical scavenging ability of the oils was very low as only about 25% inhibition could be achieved at high concentration (1500 µg/mL). Similar findings were reported by Rao *et al*. [19]. The low antioxidant activity of these essential oils may be due to the presence of non-phenolic terpene compounds (Table 2). It is well-known that the existence of a functional hydroxyl group bonded to an aromatic benzene ring is an essential key factor for scavenging radical species.

The essential oils of *M. koenigii* were also assessed for their antibacterial activity using *Listeria innocua*, a marker human pathogenic bacterium. Essential oils obtained from both extraction procedures applied at concentrations from 250 to 600 µg/mL showed strong inhibitory activity against the growth of *L. innocua* (Table 4). However, it was important to note that low concentrations

Table 3: Percentage (%) of DPPH$^{\bullet}$ radicals scavenged by *Murraya koenigii* essential oils obtained by SFME and HD at varying concentrations.

Concentration (µg/mL)	% of DPPH scavenged [x,y]		
	HD-essential oil	SFME-essential oil	Average
1500	20.2±0.4 c A	28.2±0.3 a A	24.2 A
800	17.1±0.2 d B	24.4±0.3 b A	20.8 B
500	15.4±0.4 de B	20.7±0.3 c B	18.1 C
200	10.4±0.3 f C	14.8±0.3 e C	12.6 D
100	8.7±0.3 g D	11.9±0.7 f D	10.3 E
Average	14.4 b	20.0 a	

[x] : Means ± standard error (n = 3), HD: hydro-distillation; SFME: solvent-free microwave extraction, [y] : Data were transformed (log) before analysis to achieve normality. Untransformed values are shown in the Table; a–g: Means followed by the same lowercase letter within each row are not significantly different; A–E: Means followed by the same uppercase letter within each column are not significantly different [Tukey's Studentized Range test ($p < 0.05$)]. Effects of concentratıon [C] and extraction method [T] were significant ($p < 0.05$); the C X T interaction was not significant ($p < 0.05$).

Table 4: Percentage (%) growth inhibition of *Listeria innocua* by various concentrations of *Murraya koenigii* essential oils obtained by SFME and HD.

Concentration (µg/mL)	Antibacterial activity (% inhibition) [x]		
	HD-essential oil	SFME-essential oil	Average
600	100.0±0.00 a A	100.0±0.00 a A	100.0 A
400	91.0±1.15 b B	99.0±0.58 a AB	95.0 B
300	84.7±1.86 b B	91.7±1.45 b B	88.2 C
250	66.7±1.45 c C	72.7±2.03 c C	69.7 D
200	23.3±0.88 ef D	35.3±2.96 d D	29.3 E
100	17.7±1.76 f DE	27.7±1.76 de D	22.7 F
50	13.3±1.45 gh E	18.3±1.45 fg E	15.8 G
25	5.0±1.15 i F	6.7±1.45 hi F	5.8 H
Average	50.2 b	56.4 a	

[x] : Means ± standard error (n = 3), HD: hydro-distillation; SFME: solvent-free microwave extraction; a–i: Means followed by the same lowercase letter within each row are not significantly different; A–F: Means followed by the same uppercase letter within each column are not significantly different [Tukey's Studentized Range test ($p < 0.05$)]. Effects of concentration [C], extraction method [T] and C X T interaction were significant ($p < 0.05$).

of the oils possessed good antibacterial activity. The SFME-essential oil at 250 µg/mL inhibited bacterial growth by 73% and is thus a promising antibacterial agent. The comparable value for the HD-essential oil was 67%. It is also of interest that the % inhibition values for both oils increased almost two and three times as their concentration increased from 200 µg/mL to 250 µg/mL.

The sesquiterpene and oxygenated sesquiterpene contents of the SFME-essential oil are slightly higher than those of the HD-essential oil and this is probably responsible for the observed difference between the activities of the two essential oils in inhibiting the growth of *L. innocua.* Recently, carbazole alkaloids with pancreatic lipase inhibitory activity have been identified from *M. koenigii* [20-22], and their potential contribution to antimicrobial activity needs to be investigated.

Application of SFME for the isolation of essential oil from leaves of *M. koenigii* was compared with the conventional HD method, using the Clevenger apparatus. SFME seems to have some advantages over HD due to reduced extraction times, no solvent use and higher antibacterial activity. It can be concluded that the antibacterial activities of *M. koenigii* essential oil obtained by SFME may serve as a source for antibacterial agents. Further investigations are needed for assessing the sensory attributes of the essential oil obtained from *M. koenigii* before any recommendations are made for applications in food preservation.

Experimental

Materials: 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) was obtained from Sigma-Aldrich, Canada. The 96-well microplates and HPLC grade methanol were purchased from Fisher Scientific (Ottawa, ON, Canada). *Listeria innocua* (ATCC33090) was obtained from Microbiologies Inc., Saint Cloud, MN, USA. Fresh branches of *M. koenigii* L. were obtained from Jamaica through Pete's Frootique, Bedford, NS, Canada during April 2009. The branches were air dried at 30±2°C for 48 h using a convection oven with air circulation (Milner Agincourt, ON, Canada). The detached leaves (without stems) were ground into a fine powder using a Wiley mill with 1 mm sieve screen (Model Laboratory Heavy Duty, Arthur Thomas Co., Philadelphia, PA, USA) and kept in a freezer (-80°C) until use for extraction.

Hydro-distillation (HD): Dried leaf powder (250 g) was mixed with 2 L of water and subjected to hydro-distillation for different times up to 200 min in a Clevenger apparatus (Glassco Laboratory Equipment, Dorval, QC, Canada). The essential oil collected was dried with anhydrous sodium sulfate at a concentration of 2% w/v oil. The oil was kept at 4°C until used.

Solvent-free microwave extraction (SFME): A household microwave oven (model DMW1153W; 1100 W, 2450 MHz, Danby Products, Guelph, ON, Canada) at 90% power level was used for the extractions. The microwave oven was modified by drilling a hole at the top for connection of a 100 mL capacity flat bottom flask that was placed in the oven and a Clevenger apparatus set up outside the oven through the hole. The hole around the neck of the flask was covered with polytetrafloroethylene (teflon) to prevent leakage of microwaves. The plant material was moistened before the SFME procedure. In this regard, 60 g of dried leaf powder was soaked in 1000 mL of water for 30 min and the excess water drained off. The moistened material was placed in the flask as described above without adding any additional water. As the microwave heating started, the vapor was condensed that passed through the condenser outside the oven. The process was performed for different periods up to 45 min until no more oil was collected. The essential oil was collected in an amber vial, dehydrated over anhydrous sodium sulfate and kept at 4°C until used. The yield of essential oil was calculated as (g essential oil/g dry weight) x 100.

Refractive index measurements: Refractive index measurements were made at 25±0.2°C using a refractometer (Bellingham Stanley Ltd. RFM 330, England).

Antioxidant activity by DPPH$^{\bullet}$ radical scavenging assay: The assay was carried out as described by Rupasinghe *et al*. [23]. A control sample was also tested applying the same procedure to a solution with no antioxidant material and its absorbance was recorded as $A_{control}$. The free radical scavenging activity of each solution was calculated as percent inhibition according to the following equation:

$$\% \text{ inhibition} = 100 (A_{control} - A_{sample}) / A_{control} \quad (1)$$

Antibacterial activity: *L. innocua* was used as a marker for *L. monocytogenes* (24). The bacteria were maintained on tryptic soy agar slants at 4°C. For experimental purposes, a loopful (~20 µL) of the bacterium was transferred to a tube containing 10 mL of tryptic soy broth (TSB) and incubated overnight at 32°C. This overnight culture was centrifuged at 3,000 × *g* for 10 min and the pellet was dissolved in 0.1% peptone water. The turbidity was adjusted to McFarlands standards (Med-Ox Diagnostics Inc., Ottawa, Ontario, Canada) to obtain an initial inoculum of approximately 1×10^7 cfu/mL. To determine the antibacterial activity, the absorbance was read at 600 nm using a spectrophotometer (model DU70, Beckman Instruments Inc., Fullerton, CA, USA) after adjusting the absorbance to the blank with the respective growth medium. The antimicrobial activity was expressed as % inhibition of the growth of *L. innocua* in the presence of essential oil with respect to control

containing no additive. Minimum inhibitory concentrations (MIC) of essential oils were determined by serial dilution in the nutrient agar, having concentrations of 10, 25, 50, 75, 100, 200, 400, 600 µg/mL. The inoculum was prepared from fresh overnight broth culture in nutrient broth. Plates were incubated for 24 h at 32°C. MIC was recorded as the lowest extract concentration demonstrating no visible growth in the broth [14].

GC/MS analysis of essential oil: GC/MS analyses were performed on a Varian CP 3800 GC equipped with Varian Saturn 2200 MS detector and a capillary column (30 m length and 0.25 mm I.D., 0.25 µm film thickness) (Walnut Creek, CA, USA). The stationary phase was 5% phenyl-methylpolysiloxane. The instrument was operated in EI mode at 70 eV. The transfer line and injector temperature were at 250°C and 200°C, respectively. High purity helium was used as carrier gas (2 mL/min). GC oven temperature was at 100°C for 7 min, programmed to 250°C at a rate of 5°C/min, and kept constant at 250°C for 1 min. The split ratio was 1:10.

Statistical analysis: All made in triplicate and measurements were the values were reported as means of the measurements with standard deviations (SD) or standard errors in tables, and SD bars in figures. Effect of concentration and type of essential oil on $DPPH^{\cdot}$ radical scavenging (% inhibition) and antibacterial activity were evaluated. A completely randomized design (CRD) was used. The assumptions of normality of residuals were tested using the Anderson-Darling test. Assumptions of constant variance were tested by plotting residual versus fits scatter diagram [23]. The data were analyzed using ANOVA methods of the general linear model (GLM) procedure by the SAS Institute, Inc. [24]. Significant differences among means were determined by the Tukey's Studentized Range test at $\alpha = 0.05$.

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