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Chemical Composition, Anti-neuraminidase, and Anti-atherogenic Activities of the Essential Oil from two Varieties of *Alpinia zerumbet* Leaves

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The present study describes the chemical composition and neuraminidase (NA) enzyme inhibition and anti-atherogenic properties of the essential oils (EO) of two varieties of Alpinia zerumbet (alpinia) leaves. A total of forty eight compounds were identified using GC-MS studies, which showed that tairin variety contained more compounds than shima variety. The EO inhibited NA in a dose-dependent manner with IC_{50} values of 41.5 ± 2.7 and 62.3 ± 3.1 µg/ml for tairin and shima varieties, respectively. Furthermore, the kinetic studies of NA inhibitions revealed that tairin EO exhibited slow, time-dependent and mixed type of inhibitions. It seems that the minor components of tairin EO have better inhibitory properties. In order to assess the anti-atherogenic activities of EO, inhibitions of 15-lipoxygenase (15-LOX) and low density lipoprotein (LDL) oxidation were investigated. The results also showed that tairin EO had better activities against 15-LOX and LDL oxidation (IC_{50} = 235 ± 7, and 133 ± 3 µg/ml), than shima EO (IC_{50} = 465 ± 12, and 195 ± 5 µg/ml). Finally, the acute toxicity analysis using Caenorhabditis elegans showed that LC_{50} values were more than 2500 µg/ml. These findings suggest that tairin EO could be a possible candidate for further investigations in search for bioactive compounds against neuraminidase.

Keywords: *Alpinia zerumbet*, Essential oil, Neuraminidase inhibition, Anti-atherogenic activity, Toxicity

Introduction

Alpinia [*Alpinia zerumbet* (family *Zingiberaceae*)] is a perennial ginger growing widely in the subtropics and tropics. It is used in folk medicine for its anti-inflammatory, bacteriostatic, and fungistatic properties (Zhoghbi *et al*., 1999*)*. The aqueous extract of its leaves has demonstrated hypotensive activity (Laranja *et al*., 1991) mainly due to flavonoids and kava pyrones (Mpalantinos *et al*., 1998). In Okinawa, two varieties of alpinia are widely found: *tairin* and *shima*. *Tairin* [*A. zerumbet* (Pers.) B. L. Burtt & R. M. Sm. var. *excelsa* Funak & T. Y. Ito] is a taller plant with long stems whereas *shima* [*A. zerumbet* (Pers.) B. L. Burtt & R. M. Sm.] is short and has bushy structures. Both kinds of leaves are used to prepare a traditional food, *mu-chi*, and it is believed that it prevents from catching cold.

In our previous studies with alpinia leaf, we have demonstrated antioxidant and antimicrobial activities (Elzaawely *et al*., 2007a-c). We have also reported the advanced glycation end product and HIV-1 integrase and neuraminidase (NA) inhibitions by alpinia leaf (Chompoo *et al*., 2011; Upadhyay *et al*., 2011a). Furthermore, we identified anti-atherogenic properties and skin diseased related enzyme inhibition activities of alpinia leaf (Chompoo *et al*., 2012a, b). Recently, we showed that alpinia leaf extract can increase the lifespan of *Caenorhabditis elegans* (Upadhyay *et al*., 2013). In this study, we investigated the anti-neuraminidase and anti-atherogenic properties of essential oil (EO) obtained from two varieties of alpinia leaf.

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The pharmacological properties of alpinia EO have been reported by several researchers (Laranja *et al*., 1991; Bezerra *et al*., 2000; Leal-Cardoso *et al*., 2004). Besides, alpinia EO has been associated with the antimicrobial and larvicidal activities (Cavalcanti *et al*., 2004; Victoria *et al*., 2009). However, this is the first report on the NA inhibitions and anti-atherogenic activities of alpinia EO. We further investigated the kinetic mechanism of NA inhibition by the EO of *tairin* variety and also studied its acute toxicity using *C. elegans*.

Materials and Methods

Extraction of EO from alpinia leaf- The EO was obtained from 500 g of fresh alpinia leaf by steam distillation for 4 hr. The distillate was extracted with diethyl ether and the solvent was carefully removed under vacuum at 35^{0}C. The obtained EO was dissolved in methanol and kept under refrigeration until use.

Neuraminidase inhibition assay- The enzyme assay was performed as reported previously (Upadhyay *et al*., 2011a,b) by measuring the amount of 4-methylumbelliferone that is cleaved by the influenza virus NA (Sigma, M8639) from the flurogenic substrate, 4-methylumbellifery-l-α-$_D$-N-acetylneuramic acid sodium salt hydrate (NANA) (Sigma, M8639). In all assays, samples were dissolved in methanol at appropriate concentrations, and methanol was used as control. Briefly, the reaction was started by addition of NANA to previously added enzyme and sample, and fluorescence was measured in spectrofluorometer (Corona Electric, Japan). The excitation wavelength was set at 360 nm, and the emission wavelength was set at 450 nm. For kinetic studies, we used a time-driven protocol with initial velocity recorded over

a range of substrate concentrations for different inhibitor concentrations (0, 30, 40, 50, and 60 µg/ml). Secondary plot was drawn by plotting the slopes of the obtained lines (Km/Vm) against the substrate concentrations. For time dependent studies, we obtained progress curves for 600 s at several pre-incubation times using 40 µg/ml of EO and the slopes of lines were plotted against pre-incubation time. To study the effect of enzyme concentrations on the inhibitory activity by EO, we used different concentrations of NA at EO concentration of 40µg/ml. The inhibition was calculated using the following equation.

$$\% \text{ Inhibition} = [1-(S - S_0)/(C-C_0)] \times 100$$

Where, S and C represent relative fluorescence units (RFU) for sample and control after reaction time and S_0 and C_0 are RFU at zero time.

Anti-atherogenic activities

15-LOX inhibition assay- Enzyme inhibition was determined as described previously in borate buffer (0.2 M, pH 9.0) by measuring the increase in absorbance at 234 nm in 5 min after addition of 15-LOX, using linoleic acid (134 µM) as substrate (Lyckander and Malterud, 1996). The final enzyme concentration was 500 units/ml and the test samples were dissolved in DMSO solutions. All the measurements were carried out at least twice using ferulic acid as positive control. Calculations of enzyme activity were carried out as previously described, and IC_{50} values were determined by linear interpolation between the measuring points closest to 50% activity.

LDL cholesterol oxidation assay- The oxidation of LDL was investigated as described by Rattan & Arad, 1998. $CuSO_4$-induced oxidized-LDL generation were performed using 100 µL of 220 µg/ml LDL incubated at 37 ^oC in dark with 10 µL of 55 µM $CuSO_4$ and 10 µL of EO for 24 h. The reaction was stopped by adding 50 µL of 1 M EDTA and placing the sample at -20 ^oC for TBA reactive substance (TBARS) assay. The generation of malonyldialdehye (MDA) equivalents during LDL oxidation was estimated by the TBARS assay using the method described elsewhere (Steinbrecher *et al*., 1994). LDL oxidation was carried out as described above. After oxidation, LDL was mixed with 1.5 ml of 0.67% TBA and 1.5 ml of 20% TCA. After placing samples in boiling water (98^0C) for 30 min, the reaction product was kept for 30 min at 25^0C and centrifuged for 15 min at 4^0C. The supernatants were read on a spectrophotometer at 532 nm, using the blank containing 220 µg/ml LDL only. The yields of MDA were used as a standard and the results were expressed as nanomoles of MDA equivalents.

GC-MS analysis

The compounds of EO were identified using DB-5MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm; Agilent Technologies, J and W Scientific Products, Folsom, CA, USA). The carrier gas was helium and the GC oven temperature program was as follows: 80 ^0C hold for 1 min, raised at 10 ^0C/min to 220 ^0C, followed by raised to 330 ^0C at 20 ^0C/min and hold for 6 min. The injector and detector temperatures were set at 250 ^0C and 280 ^0C, respectively and the injection volume was 1.0 µL in the splitless mode. Mass spectra were scanned form *m/z* 50-600 amu and the electron impact ionization energy was 70 eV. Quantitative determinations of EO components were done based on the peak area measurements. Retention indices were determined relative to the retention times of a series of *n*-alkane standards (C7 to C31, Restek Corporation, PA, USA), and compared with published values [Elzaawely 2007a-c; Shellie *et al*., 2002; Sibanda *et al*., 2004).

Acute toxicity assessment of EO using *C. elegans*

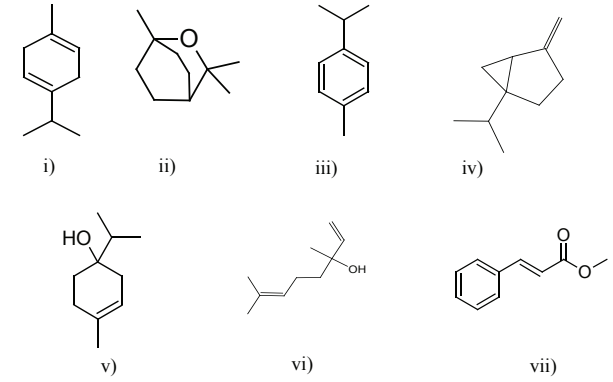
The acute toxicity of *tairin* EO was investigated according to a procedure described previously with slight modifications (Ura *et al*., 2002). The assay used wild type N2 strains, obtained from Caenorhabditis Genetic Center, University of Minnesota, MN, USA. Synchronization of worm culture was achieved by treating gravid hermaphrodites with bleach (50% sodium hypochlorite; 2.5 M sodium hydroxide) and recovering the hatched L1 larvae on nematode growth medium (NGM) plates. Day four larva (L4) were collected from the NGM plates and washed several times using S-basal medium. About 30-40 larvae in 500 µl S-basal were transferred to 1.5 ml of eppendorf tubes and were exposed to various concentration of *tairin* EO at 20 ^0C. After incubating for 24 h, the S-basal medium containing both living and dead nematodes were transferred to fresh NGM plates and the numbers of nematodes living and dead were counted. Survival was scored as the number of animals responsive to the gentle touch by platinum tip under dissecting microscope. The median lethal concentration (LC_{50}) was calculated using the PROBIT method.

Statistical analysis

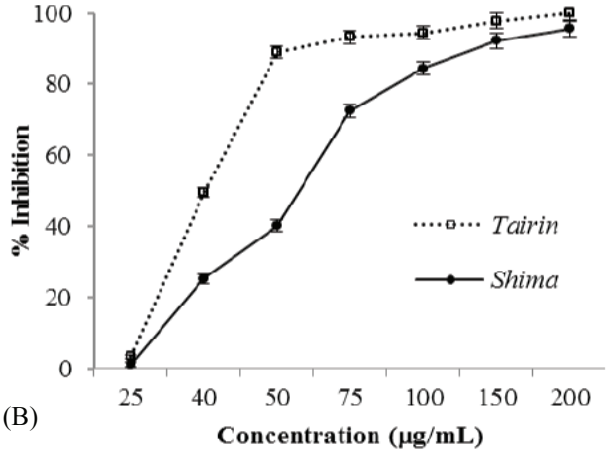
IC_{50} values were expressed as mean ± standard error by plotting the curve with percentage of inhibition versus concentration of the individual experiments measured (n = 3). Statistical analysis was performed by one-way ANOVA and upon significant difference, means were separated using Tukey's HSD range test at $P = 0.01$. All statistical analyses were performed using SPSS version 16.0 for Windows.

Results and Discussion

Chemical composition of the EO- The GC-MS analyses identified 39 major compounds in *tairin* variety while only 14 compounds could be identified in *Shima* variety. The EO was a complex mixture mainly consisting of monoterpenes and sesquiterpenes. The chemical structures of major compounds identified in EO of two varieties of alpinia leaf are shown in Figure 1A.



(A)



(B)

Figure 1. (A) Chemical structures of major compounds present in the EO of *tairin* and *shima* leaf i) γ-terpinene, ii) cineole, iii) *p*-cymene, iv) sabinene, v) 4-cravamenthenol, vi) β-linalool, and vii) methyl cinnamate. (B). Concentration dependent inhibition of NA activity by EO of *tairin* and *shima* varieties.

The major compounds in the EO were γ-terpinene (14.59%), cineole (13.82%), *p*-cymene (13.50%), sabinene (12.51%), and *p*-cravamenthenol (11.92%) for *tairin* variety. Other compounds with significant presence were terpinolene (4.19%), ?-thugene (4.12%), caryophyllene oxide (3.02%), caryophyllene (2.4%), α-pinene (2.02%), and α-terpineol (1.28%). In the case of *shima* variety, cineole (37.8 %) was the major compound followed by β-linalool (17.12%). The other major compounds identified were methyl cinnamate (6.34%), benzylacetone (4.21%), and α-terpineol (3.36%).

The amount and number of compounds varied greatly in two different varieties with only five compounds identified in both varieties. The identified compounds with their retention index and amount in percentage for two different varieties are listed in Table 1. All the compounds are arranged in the order of their retention times.

Table 1. The main chemical components of the essential oil from two varieties of alpinia leaf

	Compounds	Retention Index	Peak area (%)	
			Tairin	***Shima***
1	α-Thugene	929	4.12	-
2	α-Pinene	934	2.02	-
3	Norborndadiene	943	0.08	-
4	Camphene	947	0.22	-
5	Benzaldehyde	963	-	1.59
6	Sabinene	974	12.51	-
7	β-Pinene	976	3.15	-
8	Myrcene	989	0.69	-
9	α-Phellandrene	1002	0.31	-
10	Terpinolene	1014	4.19	-
11	*p*-Cymene	1024	13.50	-
12	1,8-Cineole	1031	13.82	37.80
13	γ-Terpinene	1059	14.59	-

14	cis-β-Terpineol	1068	0.55	-
15	β-Linalool	1100	0.50	17.12
16	2,5-Norbornadiene	1111	-	0.71
17	cis-*p*-Menth-2-en-1-ol	1121	0.59	-
18	Terpinen-4-ol	1173	11.92	-
19	α-Terpineol	1193	1.28	3.36
20	*trans*-*p*-Menth-1-en-3-ol	1206	0.42	-
21	Benzylacetone	1237	0.06	4.21
22	Piperitone	1248	0.03	0.1
23	Bornyl acetate	1280	0.37	-
24	Cumic alcohol	1287	0.18	-
25	Carvacrol	1296	0.13	-
26	2-*tert*-Butylphenyl pivalate	1299	-	1.23
27	Ethyl-3-hydroxy-3-methylbutanote	1302	-	0.09
28	Isopiperitenon	1306	-	0.18
29	Thymol	1317	0.05	-
30	2-Hydroxy-3,5-dimethylcyclopent-2-en-1-one	1319	-	0.13
31	*p*-Menth-1,4-dien-7-ol	1324	0.07	-
32	Methyl cinnamate	1381	-	6.34
33	Caryophyllene	1411	2.40	-
34	2,6-Diethylnitrosobenzene	1426	-	0.69
35	α-*trans*-Bergamoene	1428	0.09	-
36	Aristole-9-ene	1434	0.15	-
37	α-Humulene	1446	0.38	-
38	Butylcyclopentane	1477	-	0.63
39	α-Selinene	1479	0.15	-
40	γ-Cadinene	1505	0.42	-
41	α-Bulnesene	1514	0.25	-
42	Nerolidol	1559	0.38	-
43	Caryophyllene oxide	1573	3.02	-
44	Carotol	1593	0.13	-
45	Humulene epoxide	1599	0.29	-
46	β-Eudesmol	1646	0.65	-
47	*iso*-Aromdendrene epoxide	1662	0.21	-
48	α-Zingiberene	1701	0.02	-
	Total	**-**	**93.89**	**74.18**

Neuraminidase inhibitory activities of EO- With the ever-present threat of a pandemic derived from influenza virus and the emergence of resistant strain to synthetic drugs, the importance of searching for novel compounds from plant-based source intensifies. In this regard, we investigated the NA inhibitory activities of EO of two varieties of alpinia leaves. The results showed a dose-dependent inhibition of NA with a sharp increase at the lower concentrations (Figure 1B). The IC_{50} value of NA inhibition by *tairin* was found to be 41.5 ± 2.7 µg/ml, while that of *shima* variety was 62.3 ± 3.1 µg/ml (Table 2). Our previous study reported that alpinia leaves and rhizomes inhibited NA with IC_{50} of 43 and 57 µg/ml, respectively. In this study, the activity of *tairin* was similar to the leaves while it had better results than the rhizomal extracts, however *shima* EO showed a lesser activity than our previous study (Upadhyay *et al*., 2011a).

Table 2. IC_{50} (in µg/mL) values of different inhibitory activities of EO.

Sample	*Tairin*	*Shima*
Neuraminidase	41.5 ± 2.7	62.3 ± 3.1
15-LOX	235 ± 7	465 ± 12
LDL oxidation	133 ± 3	195 ± 5

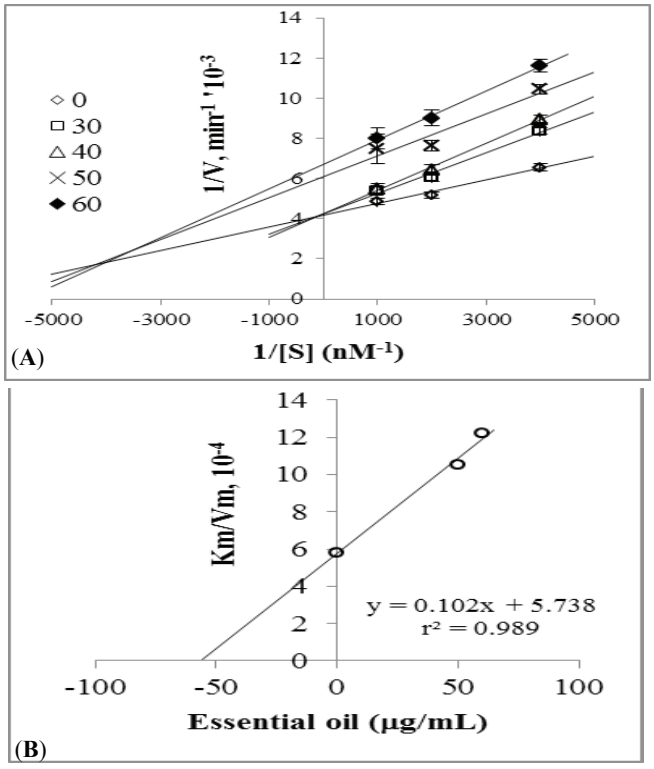


Figure 2. Effect of tairin EO on neuraminidase inhibition. (A) Lineweaver-Burk plot in the presence of EO at concentrations of 0, 30, 40, 50, and 60 µg/ml. (B) Secondary plot of Lineweaver-Burk plot. The slopes were plotted against the respective concentrations.

We conducted kinetic studies of enzyme inhibition using *tairin* EO (Figure 2A). At EO concentrations less than its IC_{50} values (30 and 40 µg/ml), the enzyme was competitively inhibited with Km value of 0.04 min^{-1}, while at higher concentrations (50 and 60 µg/ml), EO had a mixed type of inhibition against NA. This suggests that at lower concentrations, EO binds with the free enzyme or the enzyme-substrate complex at its active site, however, at higher concentrations; it binds to a site different from the active site where the substrate binds. The estimated K_i value of 56.25µg/ml was obtained from the secondary plot of Lineweaver-Burk plot (Figure 2B).

We further investigated the inhibitory mechanism of *tairin* EO at its IC_{50} concentration. We began with exploring the effect of pre-incubation time on the inhibition of the hydrolysis of neuramic acid. The results did not indicate any specific relationship between the pre-incubation time and enzyme activity (Figure 3A). Furthermore, when the slopes of the lines were plotted against the time, it was found that there is a decrease in the slope till a pre-incubation time of 20 min, making it a slow, time-dependent inhibitor (dotted line, Figure 3B).

However, when the time of pre-incubation was increased, it was found that there is a linear rise in the slope indicating that the residual activity increased with pre-incubation time (dashed lines, Figure 3B). These results suggest that for a short pre-incubation time, it follows kinetics of a slow inhibitor, while at prolonged incubation; the EO behaves in a different manner. The reasons for this behavior are yet to be understood, however, we assume that it may be due to the presence of a mixture of components in EO. On carrying out the statistical analysis, it was found that the inhibitory activities at pre-incubation times of 10 and 20 min are significantly higher than that of the control, thereby indicating the optimal pre-incubation times.

Furthermore, when the effect of enzyme concentration on NA activity was probed, it was found that with increasing enzyme concentration, the residual activity of the enzyme also increased (Figure 4), which is a natural phenomenon with most of the enzyme inhibitions, and agrees with our previous results (Upadhyay *et al.*, 2011 a,b).

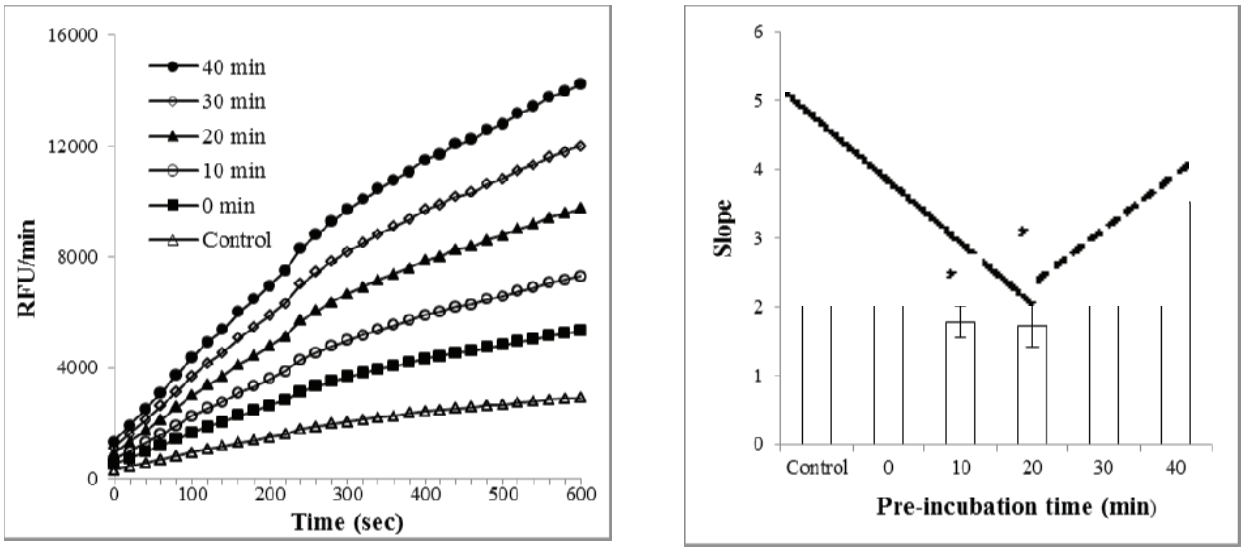


Figure 3. Effect of pre-incubation time on hydrolysis of substrate by NA: (A) time-dependent inhibition of NA in the presence of 40 µg/ml tairin EO; (B) slopes of the lines of panel A at different incubation time

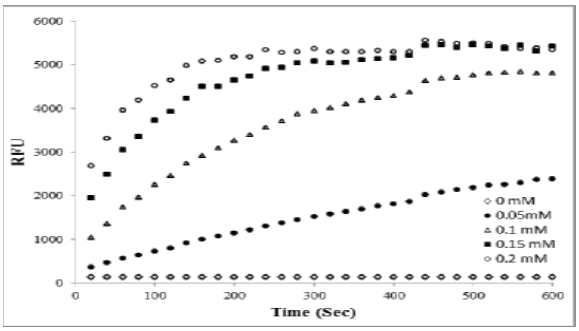


Figure 4. Effect of enzyme concentration on NA inhibition. A typical plot of residual activity of NA at various enzyme concentrations (0-0.2 U/ml)in the presence of mg/ml *tairin* EO

NA inhibitions by major compounds identified in tairin EO- In order to identify the EO components with NA inhibitory activity, we investigated the NA inhibitions by four major compounds of *tairin* EO. It was found that *p*-cymene, ?-terpinine, and terpinen-4-ol had higher inhibitory activity against NA with IC_{50} of 140 ± 7, 280 ± 9, and 400 ± 12 µg/ml, respectively (Figure 5). However, 1,8-cineole a major compound in both the varieties had poor inhibition properties (IC_{50} > 3000 µg/ml). Since the *tairin* EO had better inhibitory properties than the individual compounds, it seems that the minor components in the EO may have greater roles in the inhibition of NA.

There are reports on inhibitory activities of EO of botanical families against the influenza virus (Zai-Chang *et al*., 2005; Hayashi *et al*., 2007). In one study, compounds like terpinen-4-ol, terpinolene and α-terpineol have shown the inhibitory effect of influenza A/PR/8 virus at doses below the cytotoxic dose (Garozzo *et al*., 2009). However, in a recent report, it was found that although tea tree oil has anti-influenza virus activity, it did not have anti-NA activity (Garozzo *et al*., 2011). Although the effectiveness of alpinia EO against the influenza virus is yet to be confirmed, our results are quite promising since we found NA inhibitions at low micro molar range and may have better activities against the virus.

Anti-atherogenic activities of EO- It is widely accepted that the oxidative modification of plasma lipoproteins, particularly LDL, plays an important role in the initiation of atherosclerosis. The process of atherosclerosis begins with the accumulation of lipids within the artery wall (Shepherd *et al*., 1995). 15-LOX is a lipid-oxidizing enzyme that is considered to contribute to the formation of oxidized lipids in the atherosclerotic lesions (Bocan *et al*., 1998). Furthermore, when the levels of plasma triglycerides are low, high density lipoprotein (HDL) cholesterol levels tend to be high. HDL opposes atherosclerosis directly, by removing cholesterol from foam cells by inhibiting the oxidation of LDL (Barter, 2005). Beside LDL, the 15-LOX form hydroperoxy derivatives of linoleic acid and arachidonic acid and is induced in atherosclerotic plaques (Harats *et al*., 2000). Hence, if LDL cholesterol oxidation and/or 15-LOX are inhibited, the formation of atherosclerosis may be prevented.

Our results showed that EO of *tairin* had significantly better activities than *shima* EO. In both EOs, there was linear inhibition of LDL cholesterol oxidation (Figure 6A) while 15-LOX was inhibited logarithmically (Figure 6B). In both cases, increase in inhibitory activity was seen in a dose-dependent manner. The IC_{50} values for the 15-LOX and LDL oxidation inhibitions for *tairin* EO were found to be 235 ± 7 µg/ml and 133 ± 3 µg/ml, respectively, while for *shima* EO the values were 465 ± 12 µg/ml and 195 ± 5 µg/ml, respectively (Table 2). These results were also similar to NA inhibitions results where *tairin* EO was found superior to *shima* EO.

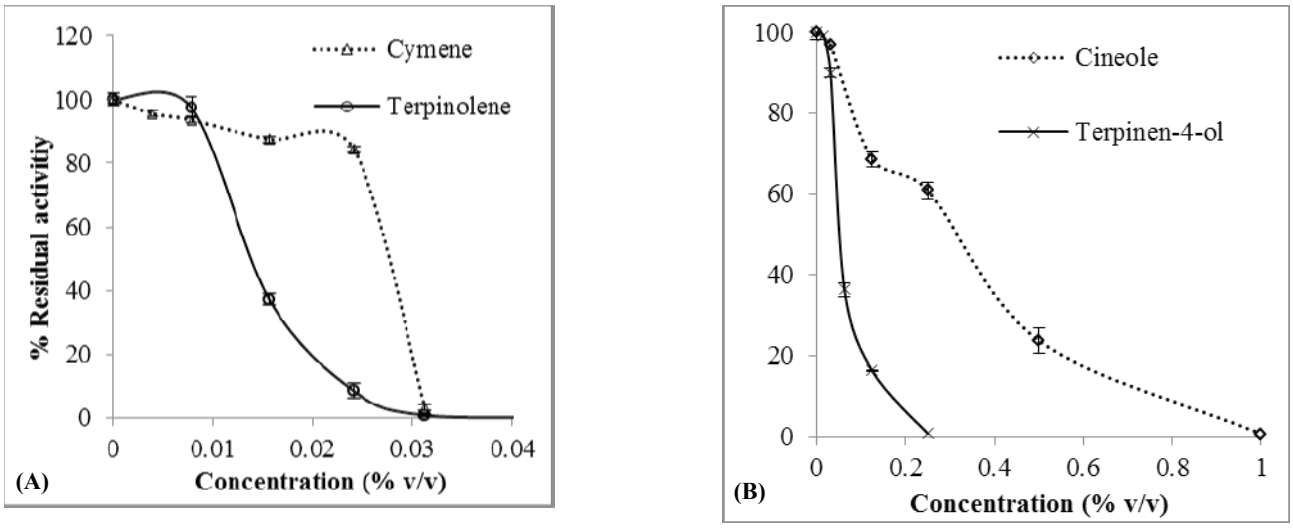


Figure 5. Concentration-dependent NA inhibitions by the major compounds identified in *tairin* EO

A number of reports on the inhibitory activities of terpenoids against 15-LOX (Amagata *et al*., 2003) and LDL oxidation (Laranjinha *et al*., 1995; Dugas *et al*., 1998) are available. Our study also identified several terpenoidal compounds in EO of alpinia and we assume that these compounds might have a role in inhibiting the formation of atherosclerosis by mediating inhibitions of 15-LOX and LDL oxidation. However, the differences in the activities between the two varieties seem to be due to a large variation in the number and amount of the compounds present in the EO of two different varieties.

Acute toxicity analysis of tairin EO using C. elegans- The determination of median lethal concentration (LC_{50}) is recognized as first step for risk assessment of synthetic and natural chemicals. Therefore, in order to consider *tairin* EO as possible candidate for drug designing, toxicity evaluation was very essential. In this regard, we measured the acute toxicity of *tairin* EO using *C. elegans* as animal model. Two independent trials were performed with three replications in each trial. It was found that increasing concentration of EO had significant effect on the survival of *C. elegans*, however, almost 50% survival was seen at a concentration of 2500 µg/mL (Figure 7). The 24 h-LC_{50} values of 2730 and 2885 µg/ml in two different assays were obtained from PROBIT analysis. These results show that the toxicity of *tairin* EO is very low when compared with the concentrations used in inhibiting NA.

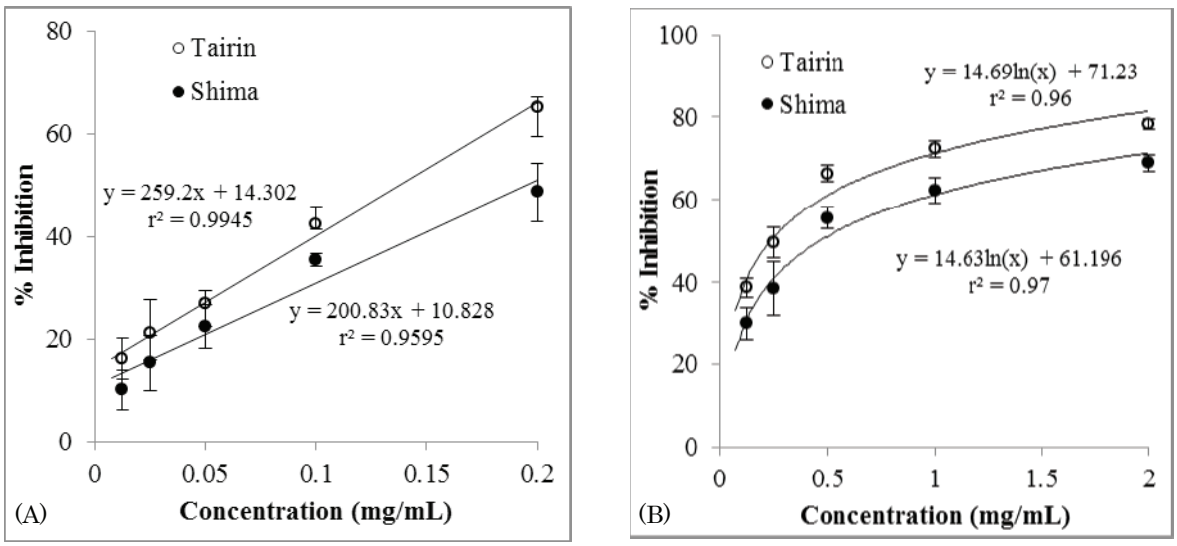


Figure 6. Concentration-dependent inhibition of LDL (A) and 15-LOX (B) enzymes by EO of *tairin* and *shima* varieties.

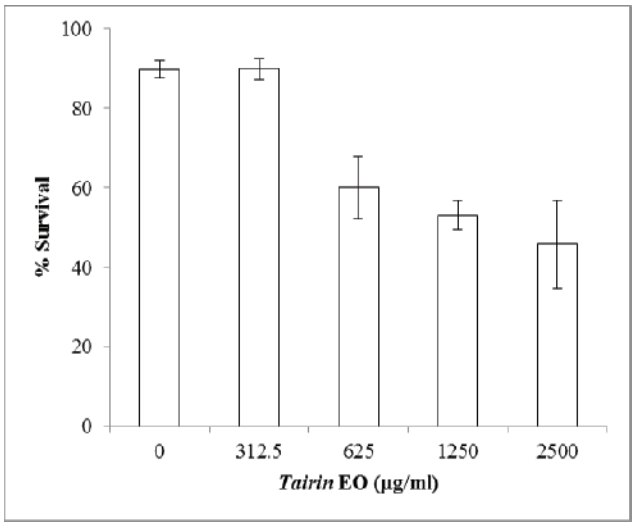


Figure 7. Concentration-response survival rate of *C. elegans* after 24 h exposure to tairin EO. Values represent mean ± SE for six different results

Conclusion

Essential oils have always been a subject of great interests in anti-oxidant and antimicrobial activities. However, in this study we identified novel properties of alpinia leaf EO against NA and atherogenic activities. Our results indicate that EO of alpinia leaf could be used as sources in inhibiting these diseases. The low IC_{50} value for neuraminidase inhibitions by alpinia EO will encourage the researchers to further investigate viral inhibition at cellular level and/or *in vivo* studies.

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