Cinnamomum iners as Mitogen-Activated Protein Kinase Kinase (MKK1) Inhibitor

Kam-Lee Pang, Wei-Li Thong and Siew-Eng How

Abstract- **Novel inhibitors targeting signal transductions have emerged for cancer therapies. Cinnamomum species have been reported to inhibit the proliferation of various cell lines. In this study, the methanol and acetone extracts of Cinnamomum iner's leaves demonstrated significant anti-kinase activity against MKK1 in the signal transduction pathway at quantity as low as 1 and 6 g respectively. Both of the extracts were found to contain polyphenol and flavonoid with potent anti-oxidation activity against DPPH free radicals (IC50=0.2 and 0.3 mg/mL respectively). In conclusion, the extracts may serve as potential MKK1 inhibitors which can be developed into anti-cancer drug.**

Index Terms— **Anti-cancer drug, anti-kinases, Cinnamomum iners, MKK1, signal transduction**

I. INTRODUCTION

Cancer is a leading cause of death worldwide. In many ways, cancer is due to mis-regulated signal transductions [1]. Protein kinase is an enzyme that catalyses protein phosphorylation which involves in the regulation of many different cellular processes, such as glycogen metabolism, calcium transport, muscle contraction, gene expression, protein synthesis, intracellular transport, phototransduction, cell cycle progression and apoptosis [2,3]. Most of the 30 known tumour suppressor genes and more than 100 dominant oncogenes are protein kinases [4]. Mitogen-activated protein kinases (MAPK) are activated by MAP kinase kinases (MAPK kinases) and MAPK kinase kinases (MAPKK kinases) that mediate a succession of discrete signaling cascades [5]. These cascades are activated by a multitude of extra-cellular stimuli, including a variety of tumor promoters. Therefore, novel inhibitors targeting protein kinases have become the focus of development of new cancer therapies [6].

Food phytochemicals such as polyphenols are associated with potential preventive activity against cancer [7]. The genus Cinnamomum comprises of about 250 different species. Cinnamon polyphenols such as cinnamaldehyde, 2-hydroxycinnamaldehyde, and eugenol are the main bioactive compounds that contribute to most of the biological activities of cinnamon. Chemical analysis of cinnamon leaves

has revealed the presence of essential oils, tannins, phenolic acids, cinnamaldehyde, eugenol, cinnamophilin, hydroxychalcone and coumarin [8]. However, to our knowledge, there are limited publications on the biological activities of Cinnamomum iners. Hence, the need to study their biological effects becomes imperative.

Manuscript received September 10, 2009.

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II. METHODOLOGY

A. Analysis of Chemical Contents and Anti-oxidation Activity

The fresh leaves of cinnamon were collected from Sabah Tea Plantation, Malaysia, air dried, ground and extracted using 70 % methanol or 50% acetone at room temperature for 24 hours. The extract was filtered, dried in vacuo and freeze dried. The total polyphenol content of the extract was determined by modification of the classical Folin and Ciocalteu method [9] using gallic acid as a reference. The total flavonoid content was determined according to Aluminium Chloride colorimetric method [10] using quercetin as a standard. The free radical scavenging activity of C. iners was determined using DPPH assay [11]. The chemical profiles of the extracts were analyzed using reverse phase high performance liquid chromatography (RP-HPLC) (Agilent 1200 series equipped with DAD detector). The column used was Zorbax Eclipse SDB – C18. The flow rate was set at 1.0 mL per minute and the injected volume was 50 µL. The column temperature was kept at 35°C and the pressure was kept between 0 – 400 bar. The mobile phase solvents were water/acetonitrile (0min: 91% : 9%, 20min 50%: 50%, 25min 91%:9%).

B. MKK1^{P386} and MKK1^{P386}-MSG5 Screening Systems

The MKK1^{P386} and MKK1^{P386}-MSG5 screening systems based on mutant yeast strains (*S. cerevisiae* MKK1^{P386} , *S. cerevisiae* MKK1^{P386}-MSG5) developed by Watanabe *et al.* [12] were employed to screen for protein kinases inhibitors. The series number of strain MKK1^{P386} used was H10068 which is a transformant of wild type cells, strain 1788 (*MATa/MATa ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1*) with pNV7-MKK1^{P386} (*GAL1p-MKK1^{P386}*) [12]. The genotype of strain H10068 is *MATa/MATa ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1* [pNV7-MKK1^{P386}].

The cultivation medium of yeast strain H10068 consists of yeast nitrogen base without amino acids and ammonium sulphate (0.67 %, w/v), casein acid hydrolysate (0.5 %, w/v), ammonium sulphate (0.5 %, w/v), D (+) glucose monohydrate (2.0 %, w/v), bacteriological agar no.1 (1.8 %, w/v), adenine (hemisulphate salt), L-Histidine HCl, L-Tryptophan, and L-Leucine . For preparing 100 mL of the medium, 0.67 g of yeast nitrogen base without amino acids

and ammonium sulphate, 0.5 g of casein acid hydrolysate, 0.5 g of ammonium sulphate and 2 g of D (+) glucose monohydrate were weighed and dissolved with 70 mL distilled water in a 150 mL-Erlenmeyer flask. Then, 100 µL of adenine (hemisulphate salt) stock solution (0.075 gmL^{-1}), 100 µL of L-Histidine HCl stock solution (0.030 gmL^{-1}), 100 µL of L-Tryptophan stock solution (0.040 gmL^{-1}) and 100 µL of L-Leucine stock solution (0.030 gmL^{-1}) were added into the medium. The pH of the medium was adjusted to 7.2. After adjusting the medium's pH, 1.8 g of bacteriological agar no.1 was added into the medium. Then, the medium volume was topped up to 100 mL with distilled water. After that, the medium was autoclaved for 15 minutes at 121 °C and poured into petri dishes. After the medium had solidified, a single colony of yeast strain H10068 was selected from the stock and streaked onto the cultivation medium. The plate was incubated at 28 °C and the growth of yeast was closely observed.

The fermentation medium of yeast strain H10068 consists of yeast nitrogen base without amino acids and ammonium sulphate (0.67 %, w/v), casein acid hydrolysate (0.5 %, w/v), ammonium sulphate (0.5 %, w/v), D (+) glucose monohydrate (2 %, w/v), adenine (hemisulphate salt), L-Histidine HCl, L-Tryptophan, and L-Leucine. For preparing 100 mL of the medium, 0.67 g of yeast nitrogen base without amino acids and ammonium sulphate, 0.5 g of casein acid hydrolysate, 0.5 g of ammonium sulphate and 2 g of D (+) glucose monohydrate were weighed and dissolved with 70 mL distilled water in a 150 mL-Erlenmeyer flask. Then, 100 µL of adenine (hemisulphate salt) stock solution (0.075 gmL^{-1}), 100 µl of L-Histidine HCl stock solution (0.030 gmL^{-1}), 100 µl of L-Tryptophan stock solution (0.040 gmL^{-1}) and 100 µL of L-Leucine stock solution (0.030 gmL^{-1}) were added into the medium. The pH of the medium was adjusted to 7.2. After adjusting the medium's pH, the medium volume was topped up to 100 ml with distilled water. After that, the medium was autoclaved for 15 minutes at 121 °C. After autoclaving the medium, a single colony of yeast was selected from cultivation medium plate and inoculated into the sterile fermentation medium. The broth culture was then incubated at 28 °C and 220 rpm for 2 days in an orbital shaker.

The fermented mutant yeast strain *S. cerevisiae* $MKK1^{P386}$ from the broth was centrifuged to obtain pellet. The pellet was washed twice Phosphate Buffer Solution (PBS). Then, 2 mL of the yeast culture was pipetted into every 100 mL of the screening medium. Pre-prepared agar was poured into petri dishes (25 mL per plate) and left to solidify at room temperature. Sterile 6-mm diameter paper discs (Whatman No.3) were arranged on a sterile glass petri dish. Then 20 µL of extract (100 mg/mL) was applied onto each disc. The discs were then left to dry in a laminar flow cabinet. Dried paper discs were put on the screening medium with a pair of sterile forceps. Each extract was tested on the two different plates, glucose plate and galactose plate. The plates were then incubated at 28 °C for 5 days.

The concentration dependency test was carried out in the same manner by varying the quantity of extract. The observations were recorded based on the growth/ no growth on the plates and around the paper discs. All the screenings were done in triplicates.

The $MKK1^{P386}$-MSG5 assay was done in quite a similar way using the mutant yeast strain *S. cerevisiae* $MKK1^{P386}$-MSG5.

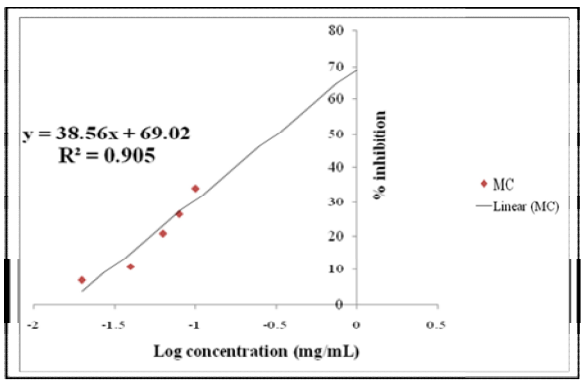
III. RESULT AND DISCUSSION

A. Chemical contents and anti-oxidation activity

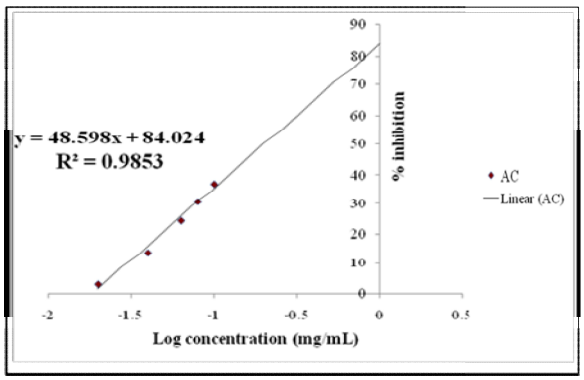
The percentage yield of methanol (MC) and acetone (AC) extracts was 6.57% and 8.26% respectively. The polyphenol and flavonoid contents of the extracts and its anti-oxidation activity are listed in Table I. The strong inhibitory activities of MC and AC [IC_{50}= 0.3 and 02. mg/mL respectively, Fig.1(a) and (b)) against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals suggest that the extracts are potent radical scavengers, therefore act as antioxidants, this is possibly due to the polyphenol constituents. AC had higher polyphenol and flavonoid contents compared to MC. Wang reported that there is a positive linear correlation between phenolic compounds and the antioxidant capacity of herbs and spices [13].

Table I Chemical contents and anti-oxidation activity of the cinnamon extracts

Chemical Content & Activity	**MC**	**AC**
Polyphenol Content (mg GAE/g)	248.6	284.4
Flavonoid Content (mg QE/g)	12.0	21.6
Anti-oxidation Activity, IC_{50} (mg/mL)	0.3	0.2



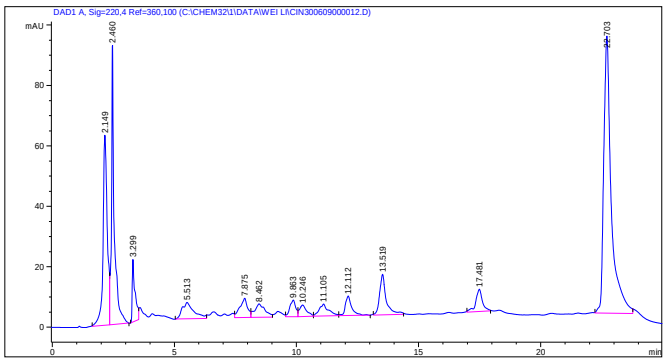
(a)



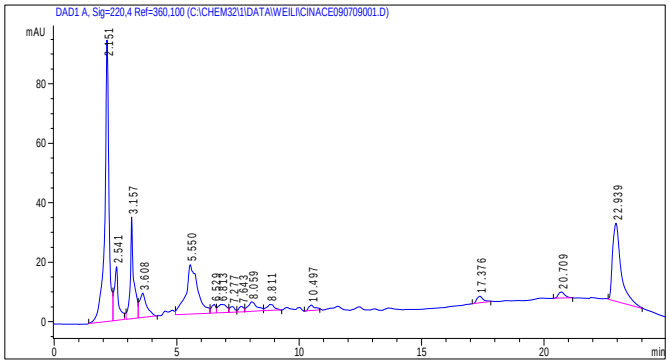
(b)

Fig. 1 IC_{50} of anti-oxidation activity of (a) MC and (b) AC which were 0.3 and 0.2 mg/mL respectively as analyzed using linear plots.

The chemical profiles of MC and AC are shown in Fig. 2 (a) and (b) which were different in number and quantity of compounds. There were at least 13 compounds present in the MC with 3 major compounds at retention time 2.1, 2.5 and 22.7 min. Liquid chromatography-mass spectrometry LCMS analysis shows that the 3 major components were not eugenol or cinnamaldehyde which are commonly found in cinnamon species (data not shown).



(a)



(b)

Fig. 2 The chemical profile of (a) MC and (b) AC as analyzed using RP-HPLC under similar conditions. The MC contained more compounds compared to AC.

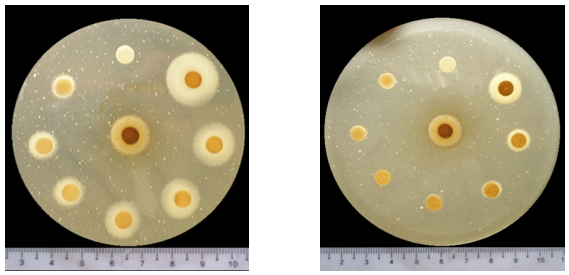
B. Anti-kinase activity

The anti-kinase properties of the extract are summarized in Table II. MKK1^{P386} is a hyperactive mutation of MKK1 which changes serine to praline at the position 386. The MKK1^{P386} mutation is able to suppress both the Pkc1 and Bck1 deletion and inhibit the growth when over expressed by fusion to the strong GAL1 promoter. Thus, over expression of this gene can be achieved by inducing it with an addition of galactose in the media. As a result, if there is a potent enzyme inhibitor, there would be yeast growth on the galactose plate only [12]. The cinnamon extracts showed no inhibitory zone on the glucose plate but growth around the discs on galactose plate (16 & 15 mm for MC and AC respectively), thus these extracts can be considered as inhibitors of MKK1^{P386} gene. However, the exact mechanisms by which these extracts affect the MAPK pathway still remain unknown and additional studies are needed. The extracts were able to display anti-MKK1 activity at low quantity (1 and 6 µg for MC and AC respectively) in concentration dependency tests as shown in Fig. 3 and Table 3. The results show that MC was more potent than AC as MKK1 inhibitor, though the polyphenol and flavonoid contents as well as the anti-oxidation activity of MC were lower. This suggests that the MC might contain higher quantity of bioactive compounds targeting MKK1 as supported by the chemical profiles shown in Fig. 2.

Table II Bioactivities of the cinnamon extract against MKK1 & MSG 5

Sample		Glucose		Galactose		Activity
	Assay	Plate	Inhibition µ ± σ (mm)	Plate	Growth µ ± σ (mm)	
Negative Control	MKK &MSG5	ü	0.0± 0.0	û	0.0 ± 0.0	No Activity
Comparative Control	MKK1	ü	0.0± 0.0	û	11.0 ± 0.0	Moderate
MC	MKK1	ü	0.0± 0.0	û	16.0 ± 1.0	Potent
AC	MKK1	ü	0.0± 0.0	û	15.0 ± 1.0	Potent
MC	MSG5	ü	15.0± 0.8	ü	0.0± 0.0 (Inhibition)	Potent
AC	MSG5	ü	15.0± 0.6	ü	0.0± 0.0 (Inhibition)	Potent

Abbreviation:
ü : Growth of yeast on whole plate
û : No growth of yeast on whole plate
Note:
Concentration of samples : 100 mgmL^{-1}
Volume of each sample : 20 µL
Diameter of paper disc : 6 mm
Negative control : 70 % MeOH & 50% acetone
Comparative control : tea extract
Replicate : triplicates



(a) (b)

Fig. 3 Concentration dependency activity of the extract (a) MC and (b) AC against MKK1^{P386} (Galactose plate). Starting from upper disc, clockwise, negative control, samples (20, 10, 8, 6, 4, 2 and 1 µL were applied); Middle disc, comparative control (tea extract). Concentrations of samples: 100 mg/mL. The results show that MC was more potent than AC against MKK1^{P386}.

Table III Concentration dependency activity of the extract against MKK1^{P386}

Sample (µL)	Galactose plate						
	Growth of yeast µ ± σ (mm)						
	1	2	4	6	8	10	20
Comparative Control	-	-	-	-	-	-	12.0 ± 0.0
MC	7.0 ± 0.0	10.0 ± 0.5	11.0 ± 0.8	13.0 ± 0.0	14.0 ± 1.2	14.0 ± 1.0	18.0 ± 0.5
AC				9.0 ± 0.8	10.0 ±1.5	10.0 ±1.4	14.0 ±1.0

The MKK1^{P386} inhibitor can target on MKK1 or MPK1 in the PKC1 pathway [12]. Hence, in order to specify the inhibitor targets on MKK1, MSG5 screening system must be carried out. MSG5 is a dual-specificity protein phosphatase (DSP), which involves in the negative regulation of MAPKs by dephosphorylating both threonin- and tyrosine-conserved residues located at the activation loop [12]. In the MSG5 screening system, MKK1^{P386}-MSG is grown in both glucose and galactose plates. The glucose plate has a yeast growth because the GAL1 promoter is not induced. On the other hand, the galactose plate also has yeast growth due to over expression of MSG5 phosphatase that suppresses the growth

inhibitory effect of over expression of MKK1^{P386} gene. The inhibitory activity of an extract against MKK1 is confirmed if there is a growth inhibition zone around the disc on glucose plate but no growth inhibition on the galactose plate. Both of the MC and AC extracts showed inhibition zones (15 mm) around the discs on glucose plate, hence, these extracts were confirmed to contain MKK1^{P386} inhibitor targeting on MKK1 and not MPK1.

IV. CONCLUSION

In conclusion, the results suggest that the cinnamon extracts (especially the methanol extract) possess potent activity against MKK1 in the MAPK signaling pathway. In addition, the extracts demonstrate significant anti-oxidation activity possibly due to the present of polyphenols. Further study can be carried out to isolate the bioactive compounds towards the development of therapeutic MKK1 inhibitors for cancer diseases treatment.

ACKNOWLEDGMENT

Siew-Eng How thanks to Universiti Malaysia Sabah for the financial support to present this paper at the 2009 International Conference on Food Engineering and Biotechnology on September 25 - 27, 2009 at Cairo, Egypt.

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