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Antioxidant and anticancer activities of *Moringa oleifera* leaves

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***Moringa oleifera* leaves extracted with methanol and dichloromethane were screened for antioxidant activity. The *in vitro* cancer antiproliferative and chemopreventive properties were also investigated. Radical scavenging assays with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2´-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were used to determine the antioxidant activity. The antiproliferative assay was evaluated on three types of cancer cell lines: hepatocarcinoma (HepG2), colorectal adenocarcinoma (Caco-2) and breast adenocarcinoma (MCF-7), using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. The *in vitro* cancer chemoprevention was performed using quinone reductase (QR) induction assay on hepatoma (Hepa-1c1c7). The chemopreventive activity of the extracts was expressed as concentration to double QR activity (CD value). The methanol extract showed higher free radical scavenging activity than the dichloromethane extract (IC_{50} = 1.60±0.03 mg/ml in DPPH assay and IC_{50} = 1.02±0.06 mg/ml in ABTS assay). In the antiproliferative assay, the IC_{50} of dichloromethane extract varied from 112 to 133 µg/ml for HepG2, Caco-2 and MCF-7 cancer cells, but became more than 250 µg/ml for the methanol extract. In the chemopreventive assay, the dichloromethane extract had capacity to induce QR activity significantly (CD value = 91.36±1.26 µg/ml), while the methanol extract had no inductive effect. This study provides evidence that *M. oleifera* leaves possess antioxidant activity, as well as cytotoxic and chemopreventive properties. Therefore, it might be beneficial as a medicinal plant for alternative novel anticancer drugs and nutraceutical products.**

Key words: *Moringa oleifera*, antioxidant activity, quinone reductase, antiproliferation, cancer chemoprevention.

INTRODUCTION

Cancer is the leading cause of mortality worldwide. According to the cancer reports published by the World Health Organization (WHO) and the World Cancer Research Fund, the incidence of cancer is still increasing especially due to diet, environment and carcinogenic virus infections (WHO, 2008; World Cancer Research Fund, 2007). In hospitals, conventional drugs are commonly prescribed to cancer patients. However, due to less toxic and adverse effects of phytochemicals, the research on medicinal plants and cancer has been intensified (Johnson, 2007).

Moringa oleifera (*M. oleifera*) or drumstick is a member of Moringaceae, and it is grown extensively in many Southeast Asian countries particularly in Thailand, India, Philippines and Pakistan (Fuglie, 2001). It has long been known as a food plant in Thai cuisine and as an ingredient of Indian traditional medicine (Wutythamawech, 1997; Mishra et al., 2011). The leaves contain nutrients

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especially essential amino acids, vitamins, minerals and *β*-carotene (Sabale et al., 2008; Sharma et al., 2012). For this reason, it is used as an alternative source for nutritional supplements and growth promoters in some countries (Anwar et al., 2007). Apart from nutritional benefits, *M. oleifera* is reported to be used for the treatment of rheumatism, ascites, infection, hiccough in-fluenza and internal abscess (Anwar et al., 2007; Mishra et al., 2011). Many recent reports on disease prevention by *M. oleifera* have been reported. The leaf extract is capable of reducing hyperglycemia and dyslipidemia (Mbikay, 2012). The ethanol extract of the leaves preven-ted cyclophosphamide-induced micronucleus formation and DNA damage in mice (Sathya et al., 2010). The aqueous extract enhanced hepatic glutathione restoration (Fakurazi et al., 2008). Recently, Chadamas et al. (2010) reported that tender pods decreased the for-mation of erythrocyte micronucleus in mice injected with 7,12-dimethylbenz(a)anthracene. It also demonstrated inhibi-tory potential against azoxymethane-induced colon carcinogenesis (Budda et al., 2011). Moreover, it has been reported that the leaf extract had potent antiproliferative activity and apoptosis inducing capacity on tumor (KB) cell line (Sreelatha et al., 2011), and it also increased the cytotoxicity of chemotherapy on pancreatic cancer cells (Berkovich et al., 2013).

To date, a variety of biological activities of parts of *M. oleifera* have been reported. Nevertheless, there are limited evidences for *M. oleifera* leaf in terms of cancer therapy and prevention. Therefore, the aim of the present study was to investigate the *in vitro* antiproliferative activity of *M. oleifera* leaf extract on three types of human cancer cell lines (HepG2, Caco-2 and MCF-7). Further-more, the *in vitro* cancer chemoprevention was carried out using the established method, quinone reductase induction assay.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of analytical grade. The DPPH, ABTS and Folin-Ciocalteu's phenol reagent were purchased from Merck Co. (Darmstadt, Germany). The *β*-napthoflavone, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADP), menadione, dicoumarol, digitonin, glucose 6-phosphate, crystal violet, glucose 6-phosphate dehydrogenase (G6PD) and MTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The alpha-minimum essential medium (*α*-MEM), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic reagent for cell culture were purchased from Invitrogen Co. (California, USA).

Plant and extraction

The leaves of *M. oleifera* were collected during November to December, 2012 from Lampang Herb Conservation, Lampang, Thailand. This plant was identified and confirmed by comparing it with voucher specimens of known identity (ID: WP2614) deposited at the Queen Sirikit Botanical Garden, Chiang Mai, Thailand. The air-dried leaves of *M. oleifera* were ground into powder and stored at 4°C until extraction. Fifteen grams of leaf powder were extracted with 350 ml of methanol. Then, the liquid extract was filtered through Whatman no. 1 filter paper. The residue was subsequently extracted with 350 ml of dichloromethane. The filtrates were evaporated and lyophilized to obtain two crude extracts: methanol and dichloromethane extracts (ME and DE). Both extracts were kept in amber glass at -20°C until use.

Determination of total phenolic and flavonoid contents

The Folin-Ciocalteu method was used to determine the amount of total phenolic compound (Singleton et al., 1999). In brief, 100 µl of each diluted extract were mixed with 2.8 ml of deionized water and 2 ml of 50% Folin Ciocalteau's phenol reagent. It was incubated for 30 min at room temperature. The absorbance of the reaction mixture was measured at 765 nanometer (nm). The total phenolic content was expressed as milligram gallic acid equivalent per gram extract (mg GAE/g extract).

The total flavonoid content was determined using the aluminum chloride colorimetric method according to Chang et al. (2002). Briefly, 100 µl of each extract were mixed with 1.5 ml of 95% ethanol, 100 µl of 10% $AlCl_3$, 100 µl of 1 M potassium acetate and 2.8 ml of deionized water. Then, the absorbance of the reaction mixture was determined at 415 nm. The total flavonoid content was expressed as milligram quercetin equivalent per gram extract (mg QE/g extract).

DPPH radical scavenging assay

The free radical scavenging activity was tested according to Mensor et al. (2001). Various concentrations of the extracts were mixed with 80 mM of DPPH in methanol. Then, the solution was incubated for 30 min at room temperature. Quercetin was used as positive control. The optical density (OD) of the solution was measured at 517 nm by a double-beam spectrophotometer. The DPPH radical scavenging activity was calculated using the equation:

$$\text{Inhibition (\%)} = \frac{(OD_{control} - OD_{extract})}{OD_{control}} \times 100$$

The percentage of DPPH radical scavenging activity was calculated. The 50% inhibitory concentration (IC_{50}) was expressed as the quantity of the extract necessary to react with one half of DPPH radicals.

ABTS radical cation decolorization assay

Following the published method by Re et al. (1999), the protocol for ABTS assay was slightly modified. Briefly, the ABTS radical was prepared in 2.45 mM potassium persulfate. The solution was then left for 15 min in a dark place to obtain an ABTS radical solution. This solution was subsequently diluted with ethanol before use. To the diluted solution, various concentrations of each extract (300 µl) were added. Trolox was used as positive control. After incubating for 8 h in the absence of light, the absorbance was measured at 731 nm. The percentage of ABTS radical scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(OD_{control} - OD_{extract})}{OD_{control}} \times 100$$

The inhibitory percentage was calculated, and the IC_{50} was determined for each extract.

Cell culture

Human hepatocellular carcinoma (HepG2) (ATCC: 77400), colorectal adenocarcinoma (Caco-2) (ATCC: HBT-37) and breast adenocarcinoma (MCF-7) (ATCC: HTB-22) cell lines, and human dermal fibroblast (ATCC: PCS201012) were used in an antiproliferative assay. The cancer cell lines were cultured in 25 cm^2 culture flask using DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The human dermal fibroblast was cultured in fibroblast basal medium supplemented with FBS, glutamine, ascorbic acid, hydrocortisone and hemisuccinate. For a quinone reductase induction assay, the murine hepatoma cells designated as Hepa-1c1c7 (ATCC: CRL2026) were grown in supplemented α-MEM. These cells were maintained in a humidified incubator with an atmosphere comprising 5% CO_2 and 95% air at 37°C. The cells were harvested and plated either for cytotoxicity tests or subcultures when they reached 80% confluence.

Antiproliferative assay

The antiproliferation (cytotoxicity) of *M. oleifera* extracts on HepG2, Caco-2, MCF-7, Hepa-1c1c7 and fibroblast was evaluated by the MTT assay (Mosmann, 1983). The cells were plated at 1.0×10^3 cells per well in 96-well plates. Twenty four hours after plating, the cells were incubated with each extract (0 to 250 µg/ml) or cisplatin (anticancer drug) for 48 h at 37°C. The final DMSO (solvent) concentration did not exceed 0.2%. Then, 20 µl of MTT solution (5 mg/ml) were added to each well. The insoluble purple formazan crystal was dissolved in 100 µl of DMSO, and the absorbance was determined at 540 and 630 nm using a microplate reader. The percentage of cell viability was calculated. The concentration of the extract causing 50% inhibition of cancer cell growth was considered as IC_{50}.

Quinone reductase induction assay

The induction of QR activity was measured using the method established by Prochaska and Santamaria (1988), and Kang and Pezzuto (2004). The concentration of extract which allowed more than 70% Hepa-1c1c7 cell viability was used in this assay. Briefly, the cells were plated at 1×10^4 cells/well in 96-well plates, and allowed to adhere for 24 h. The cells were then treated with 0 to 100 µg/ml of each extract, β-naphthoflavone (QR inducer), or DMSO (solvent) for 48 h. The final concentration of DMSO did not exceed 0.2%. Duplicate plates were prepared: one for determining QR activity and one for cell density. For determining the QR activity, the cells were lysed and subsequently 200 µl of the reaction mixture was added. After 5 min, the reaction was stopped with 0.3 mM dicoumarol. The absorbance was measured at 610 nm, and the specific activity was calculated. The specific activity of QR is defined as nM/l MTT blue formazan reduced per min. The induction was calculated as the ratio of QR specific activity in the presence and absence of the extract. The concentration required to double the specific activity (CD) was determined by a curve of the ratio of QR specific activities versus concentration. In this assay, crystal violet staining was performed to determine cell density as the population of viable cells used to normalize the QR activity.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) of three-independent experiments. The analysis was performed using analysis of variance (ANOVA). The Bonferroni test with $P < 0.05$ was considered to test for a significant difference between control and treated groups.

RESULTS

Polyphenol and flavonoid contents of *M. oleifera* extracts

The polyphenol content of ME and DE were determined by Folin-Ciocalteau assay. As demonstrated in Table 1, the result showed that amounts of polyphenol were found in both extracts. ME had a higher content (216.45±4.64 mg GAE/g extract) than DE (100.12±3.70 mg GAE/g extract). In this study, the flavonoid content of *M. oleifera* extracts was determined using the aluminum chloride colorimetric method. The amount of flavonoid in ME was 65.38±2.37 mg QE/g extract and in DE 40.14±3.31 mg QE/g extract.

Free radical scavenging activity of *M. oleifera* extracts

The radical scavenging activity of *M. oleifera* extracts was evaluated using two methods including DPPH and ABTS assays. Table 2 presents the antioxidant activity of the extracts. It was found that ME exhibited the higher scavenging activity, with an IC_{50} of 1.60±0.03 mg/ml compared to DE (IC_{50} = 2.31±0.02 mg/ml). Apart from the DPPH assay, ABTS radical cation decolorization was carried out to confirm the antioxidant activity of the extracts. Both extracts showed ABTS free radical scavenging activity similar to the result of the DPPH assay. ME had a higher potential of radical scavenging (1.02±0.06 mg/ml) than DE.

Cancer cell antiproliferation of *M. oleifera* extracts

The antiproliferation was tested on HepG2, Caco-2, MCF-7 and human fibroblast cells. According to Figure 1, it was found that both extracts (0 to 250 µg/ml) contributed to similar cancer cell viability patterns. DE was more cytotoxic than ME. It showed a IC_{50} of 120.37±2.55, 112.46±3.74 and 133.58±2.47 µg/ml for HepG2, Caco-2 and MCF-7, respectively, while ME exhibited less cytotoxicity to all cancer cell lines (IC_{50} > 250 µg/ml). In addition, both extracts were tested in human fibroblast to observe their antiproliferation on normal cells. The results showed that 0 to 400 µg/ml of both extracts had no toxicity on human fibroblast (Figure 2). The cisplatin was used as positive control which was able to inhibit cancer cell proliferation. It had an IC_{50} of 13.34±1.44, 19.45±2.12 and 17.24±2.39 µM for HepG2, Caco-2 and MCF-7, respectively. According to the results, *M. oleifera* extracts not only exhibit antiproliferation on cancer cells, but also

Table 1. Total phenolic and flavonoid contents in *Moringa oleifera* extracts.

Moringa oleifera	**Total phenolics (mg GAE/g extract)**	**Total flavonoid (mg QE/g extract)**
Methanol extract	216.45±4.64	65.38±2.37
Dichloromethane extract	100.12±3.70	40.14±3.31

The values are the average of triplicate experiments, and are expressed as mean ± standard error of mean.

Table 2. Antioxidant activity of *Moringa oleifera* extracts.

Treatment	**50% Inhibitory concentration**	
	ABTS	**DPPH**
Methanol extract (mg/ml)	1.02±0.06	1.60±0.03
Dichloromethane extract (mg/ml)	3.06±0.11	2.31±0.02
Quercetin (µM)	-	0.06±0.01
Trolox (µM)	6.72±0.51	-

Antioxidant activity of *Moringa oleifera* extracts was determined by the radical scavenging activity of antioxidants against 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH). The values are the average of triplicate experiments, and are expressed as mean ± standard error of mean.

Table 3. Concentration of *Moringa oleifera* extracts to double quinone reductase activity.

Treatment	**CD value**
Methanol extract (µg/ml)	NI
Dichloromethane extract (µg/ml)	91.36±1.26
β-naphthoflavone (µM)	3.47±0.01

Concentration to double quinone reductase activity (CD value) is calculated and compared with negative control. The CD values are expressed as mean ± standard error of mean. *β*-naphthoflavone is used as an inducer of quinone reductase (positive control). NI = not induced

showed no cytotoxicity on normal cells.

In vitro cancer chemoprevention of *M. oleifera* extracts

Hepa-1c1c7 cells were used as *in vitro* model to measure QR activity. QR was recognized as chemopreventive enzyme. Its activity can be induced by specific inducer. In this assay, the concentration of extract, which did not interfere cell survival, was considered as tested dose. Table 3 shows the concentration of *M. oleifera* extracts to double QR activity. It was found that *β*-naphthoflavone had a CD value of 3.47±0.01 µM. Interestingly, only DE (0 to 100 µg/ml) was capable of inducing QR activity, whereas ME had no inductive effect. DE significantly increased the QR induction ratio in a dose-dependent manner ($P < 0.05$), with a CD value of 91.36±1.26 µg/ml (Figure 3).

DISCUSSION

Although *M. oleifera* has been reported for its benefits and biological activities, little is known scientifically about its antioxidant property and cancer prevention ability. Herein, *M. oleifera* extracts were evaluated for their *in vitro* antioxidant, antiproliferative and chemopreventive activities.

In the etiology of cancer, free radicals are one of the major factors necessary to cause DNA mutation, which in turn triggers the initiation stage of carcinogenesis (Johnson, 2007). Exogenous antioxidants from natural sources can improve the function of the endogenous antioxidant system which is responsible for preventing free radicals in the body (Johnson, 2004). Polyphenol is recognized as a potent antioxidant, and is found in *M. oleifera* extracts (Table 1). Recently, Charoensin and Wongpoomchai (2012) reported that the aqueous extract of *M. oleifera* leaves contained polyphenols and had DPPH radical scavenging activity. Furthermore, there are some reports which claim that *M. oleifera* leaves are rich in polyphenols and flavonoids and have antioxidant activity (Luqman et al., 2011; Santos et al., 2012). In accordance with the previous works, *M. oleifera* leaves extracted with methanol and dichloromethane also showed antioxidant activity (Table 2). The chemical analysis of *M. oleifera* extracted with methanol had shown that the major polyphenols comprised of gallic acid, quercetin and kaempferol (Sreelatha et al., 2011).

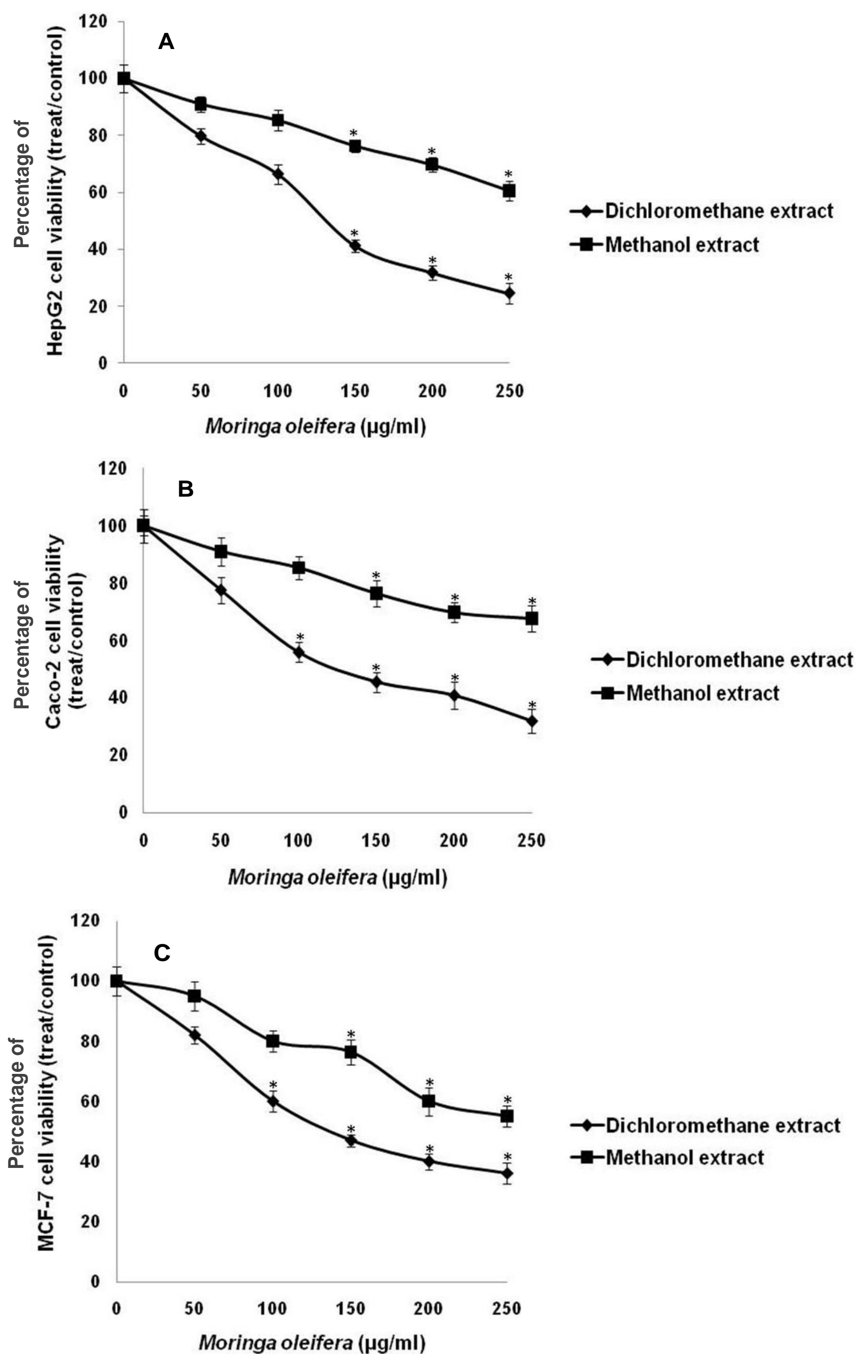

Figure 1. Effect of *Moringa oleifera* extracts on three kinds of cancer cell viability; HepG2 (A), Caco-2 (B) and MCF-7 (C). Each cancer cell type was incubated with various concentrations of extracts (0 – 250 µg/ml) for 48 h. Data are obtained from three independent experiments, and are shown as mean ± standard error of mean.
An asterisk (*) indicates significant difference ($p<0.05$) between negative control and treated groups.

Due to this reports, this might be the important evidence for ME having higher amounts of polyphenols than DE.

Accumulating reports have suggested that many naturally-occurring substances exhibit cancer chemotherapeutic effects (Khan et al., 2013). The main advantage advantage of using phytochemicals as anticancer agents is that they seem to have low adverse effects, and are more cost-effective than commercial drugs. Therefore, it is worth searching for new biologically-active phytochemicals. The present study is the first report of *M. oleifera*

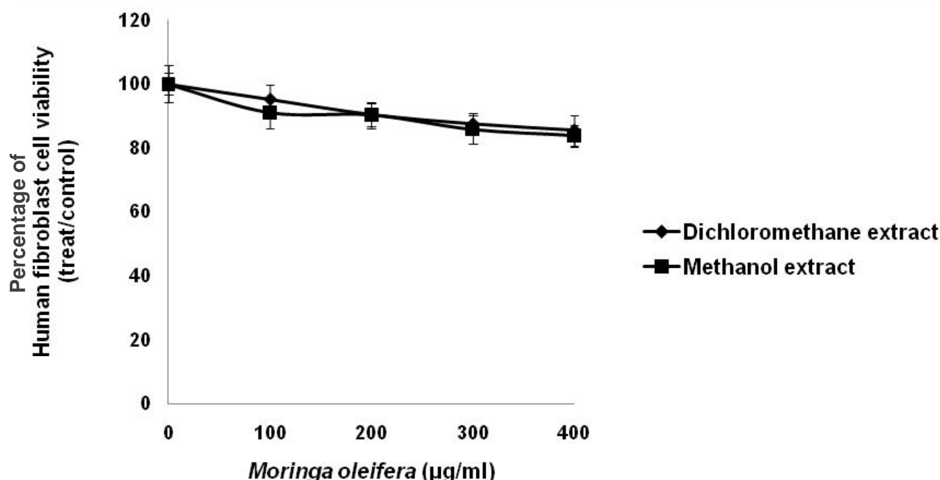

Figure 2. Cell viability of human fibroblast after treatment with *Moringa oleifera* extracts (0 – 400 µg/ml) for 48 h. Data are obtained from three independent experiments, and are shown as mean ± standard error of mean.

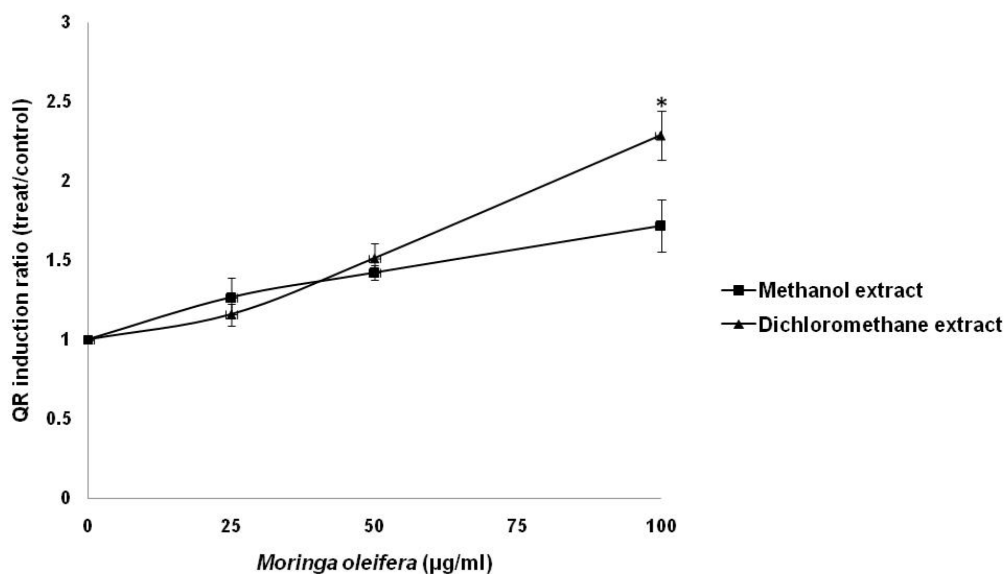

Figure 3. Quinone reductase (QR) induction by *Moringa oleifera* extracts. Hepa-1c1c7 cells were treated with 0 - 100 µg/ml of each extract, *β*-naphthoflavone (QR inducer), or DMSO (solvent) for 48 h Data are obtained from three independent experiments, and are shown as mean ± standard error of mean.

An asterisk (*) indicates significant difference of QR induction ($P < 0.05$) between negative control and treated groups.

extract regarding antiproliferations of HepG2, Caco-2 and MCF-7 cancer cells, which are not reported elsewhere. Moreover, it confirms the previous studies of cytotoxicity of *M. oleifera* extracts on human cancer cells such as pancreatic cancer cell (Panc-1) (Berkovich et al., 2013), colon cancer cells (SW480 and HCT18) (Pamok et al., 2011), and KB tumor cell (Sreelatha et al., 2011). It was found that DE had more potent cytotoxicity than ME on all cancer cell lines (Figure 1). According to the present work, each extract differently inhibited cell proliferation. This might be partly due to the differences in genotype and phenotype of cancerous cells and the active compounds in each extract. Different types of cancers have different mutational signatures (Alexandrov et al., 2013). The certain genes responsible for cell cycle and cell death are mutated in cancer cells, whereas all genes

in normal cells still remain original (Nik-Zainal et al., 2012). The mechanism underlying inhibition of cancer and normal cell proliferation is therefore determined by genetic differences which cause in both types of cells specificity and sensitivity to *M. oleifera* extract. However, the molecular mechanism by which the extract modulates cancer cell proliferation (cell cycle) and death (apoptosis) remains elusive and needs further investigation.

Although *M. oleifera* has variously biological activities, most of them rely on pod, seed and flower. As a result, it is needed to investigate more biological functions of its leaves. Since there are limited reports regarding cancer prevention, the present study aimed to evaluate chemo-preventive properties. In cancer research, there are many standard methods to evaluate whether a test sample is chemopreventive (Knasmuller et al., 2002). QR or NADPH:quinone oxidoreductase 1 (NQO1) is a phase II detoxifying enzyme and catalyzes the 2-electron reduction of a broad range of chemicals especially quinones. The 2-electron reduction of quinones to hydroquinones by QR is believed to be primarily a detoxifying reaction since it bypasses the formation of the carcinogenic semiquinone and other chemicals (Cuendet et al., 2006). It also protects cells against reactive oxygen species generated by quinones and related compounds (Gerhäuser et al., 2003). Elevated QR levels correlate with prevention of *in vivo* chemical-induced carcinogenesis in the stage of initiation and promotion (Cuendet et al., 2006). Furthermore, with advantages in terms of reliability, high throughput and less-time consumption, the *in vitro* assay, particularly cell-based testing system relevant for prevention of *in vivo* carcinogenesis, has been established and used in laboratories extensively (Gerhäuser et al., 2003). For these reasons, QR is widely used as the anticarcinogenic phase II marker enzyme for evaluating cancer chemopreventive agents rather than other enzymes (Kang and Pezzuto, 2004). In the present work, *M. oleifera* extracts were assayed for their ability to induce QR activity on Hepa1c1c7 cells. It was seen that DE could induce QR activity, whereas ME had no inductive effect (Figure 3). In recent reports, it was shown that the hot water extract of *M. oleifera* leaves had high polyphenols and antioxidant activity. It also showed potent QR induction (CD value = 99.70±10.44 µg/ml) and antimutagenicity against 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide induced mutagenesis (Charoensin and Wongpoomchai, 2010, 2012). The theory of QR induction in the context of structure-activity relationship is described. The flavonoids with 2 or 3 double bonds in the C ring are crucially essential for QR induction, while the hydroxylation of the B ring is not essential (Uda et al., 1997). The mechanism by which polyphenols and flavonoids induce QR gene expression is well understood. The induction of QR gene is regulated on the transcriptional level mediated by antioxidant response element (ARE), controlled by the nuclear factor E2-related factor 2 (Nrf2) (Nguyen et al., 2009). Activation of the Nrf2/ARE pathway by polyphenols with antioxidant activity (quercetin and kaempferol), or by non-flavonoid compounds (glucosinolate and sulforaphane) is the key step of QR gene up-regulation (Uda et al., 1997; Hwang and Jeffery, 2005). This molecular mechanism leads to the increased level of QR. From the present result, it was seen that *M. oleifera* extracted with dichloromethane induced a QR activity with higher potential than that extracted with methanol. Regarding polyphenols and flavonoids, both compounds were determined in DE, with relative amounts of that of ME. This could be the major factor for increased QR activity and cancer cell antiproliferation. Apart from flavonoids, *M. oleifera* leaves have been reported to release glucosinolate compounds, when extracted with less polar solvent. 4-(alpha-l-rhamnopyranosyloxy)-benzylglucosinolate and three monoacetyl isomers were isolated from the leaves (Bennett et al., 2003). In addition, the glycosides including niaziminin A and B, and isothiocyanates were reported (Faizi et al., 1995). This is the significant reason regarding the potent QR induction as well as the cancer cell antiproliferation of DE. Hence, both effects of *M. oleifera* might arise from the flavonoids alone, or from the synergy with other compounds.

Conclusion

Conclusively, the *M. oleifera* dichloromethane extract shows high antioxidant activity, potent cancer cell anti-proliferation, and induction of quinone reductase. These findings indicate the medicinal value of *M. oleifera* in terms of cancer chemotherapy and chemoprevention.

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