ORIGINAL ARTICLE

Effects of Ultrasound Assisted Sequential Extraction (UASE) of *Moringa oleifera* Leaves Extract on MCF 7 Human Breast Cell Line

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ABSTRACT

Introduction: Breast cancer is the leading factor of mortality among women globally. According to the World Health Organization (2015), breast cancer is the second most common cancer after lung cancer; and contributes to nearly 15% of all cancer death among women in 2015. *Moringa oleifera* (*M. oleifera*) is a highly nutritious vegetable with various therapeutic benefits including anticancer. The therapeutic benefits are attributed to its bioactive compounds. Thus, study on the bioactive compounds of *M. oleifera* using various extraction methods with different extracting solvents have been the main focus of many researchers. **Methods:** The current study was carried out using Ultrasound Assisted Sequential Extraction (UASE) method and three extracting solvents (99.7% ethanol, 50% ethanol and deionised water) with ascending polarity. The yielded extracts were tested for possible anticancer effects against human breast adenocarcinoma cell line, MCF-7 and non-tumorigenic cell line, MCF-10A using microtitrate tetrazolium (MTT) assay. **Results:** The IC_{50} values of the 99.7% ethanol, 50% ethanol and deionised water extracts were 25, 200 and 180 µg/mL, respectively. **Conclusion:** *M. oleifera* could be a potential preventative and/or therapeutic agent for breast cancer, either used alone or as an adjunct to the standard chemotherapeutic drugs.

Keywords: *Moringa oleifera*, MCF-7, Ultrasound assisted extraction, Sequential extraction, MTT assay

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INTRODUCTION

According to the World Health Organization (WHO) (2016), cancer is defined as the uncontrolled growth of cells that can invade and spread to nearby sites of the body in a process known as metastasis. Approximately 7.6 million people are being diagnosed with various forms of cancer every year (1). Breast cancer is the most common cancer that affect women, followed by colorectal, lung, uterine cervix and stomach cancers; with nearly 1.7 million new cancer cases diagnosed in 2012. Statistical estimation suggests almost every woman has the risk of developing this cancer. Therapies for breast cancer, such as chemotherapy could cause side effects, which will worsen the patient's condition (2) while long term therapies exposed cancer patients to the development of drug resistance. Therefore, safer alternative treatments have been introduced by incorporating integrative medicine namely herbal plants into the treatment plan (3, 4). In fact, it has been proven that natural products impressively have anticancer effects on many types of cancer.

Comprehensive literature search on the medicinal plants with anticancer properties indicates *Moringa oleifera* (*M. oleifera*) as one of the most extensively studied medicinal plants due to its promising therapeutic values. *M. oleifera* is a perennial angiosperm plants belongs to the Moringaceae family. Several studies have shown the phytochemical activities of *M. oleifera* depend upon the type of extracting solvents and extraction method applied. Moreover, extraction using non conventional method and extracting solvents with ascending polarity has been reported to extract high quantity of total phenolic contents (5, 6).

As shown in *in vitro* anticancer studies, *M. oleifera* possessed anticancer effects due to its anti-proliferative and antioxidant traits. Several studies have revealed the radical scavenging activities of *M. oleifera* leaf (MOL) extract that may contribute to its observed anticancer effects against various cancer cell lines (3, 4, 7, 8). In present days, treatments for cancer are expensive with many side effects. Hence, there is a need for a plant extract which is tolerable at higher dose and doesn't cause any adverse effects while being desirable for cancer therapeutic uses. Based on the previous studies,

M. oleifera leaf extracts have shown strong anticancer properties (9). Therefore, MOL extract was chosen in order to study the possible anticancer effects on breast cancer cell line, MCF-7. By considering the effect of extraction method and extracting solvent on the therapeutic potential of the plant extract, Ultrasound Assisted Sequential Extraction (UASE) method using solvents with the ascending polarity were applied in the present study with the aim to optimize the anticancer activities of MOL extract.

MATERIALS AND METHODS

Ultrasound assisted sequential extraction

The dried leaf powder samples of *M. oleifera* were bought from Herbagus, Kepala Batas. Sequential extraction was carried out as follows: 25 grams of powder was weighed and mixed with 250 mL of absolute ethanol. The resultant mixture was sonicated for 30 minutes, transferred to 50 mL centrifuge tubes and centrifuged. The supernatant (extract) was collected an transferred into a 1000 mL Schott Bottle, vacuum filtered using filter paper size 601 to get a crystal clear extract and stored in 4°C. The remaining pellets in 50 mL centrifuge tubes were further extracted using the subsequent solvents which were ethanol 50% and deionised water by applying the same method. The absolute ethanol extract of *M. oleifera* leaves was concentrated using rotary eva porator. Meanwhile, the 50% ethanol extract of *M. oleifera* was dried using freeze dry after rotary evaporator due to its high water content. Deionized water extract of *M. oleifera* was also freeze dried to obtain the final yield. To prepare *M. oleifera* extract (MOE) stock solution, 10 mg of each MOE was dissolved in 1 mL of DMSO and filtered. The stocks were kept at -20°C. The extractive values (%) was calculated based on the formula below:

$$\text{Extractive Value (\%)} = \frac{\text{Final Yield (g)}}{\text{Initial powder used (g)}} \times 100$$

Growth inhibition of human breast adenocarcinoma cells

The assay was performed in triplicate and dimethyl sulfoxide (DMSO) was used as the negative control. 3-4-5-Dimethylthiazol-2- yl-2, 5-diphenyltetrazolium bromide (MTT) assay was used to estimate cell proliferation according to the previously described method. The cells (1×10^4 Cellular morphology) of passages 4 to 7 were treated with *M. oleifera* at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/mL.

RESULTS

Final yield of sequential extraction

The sequential extraction of the *M. oleifera* leaf produced three different types of extracts: ethanol 99.7%, ethanol 50% and aqueous extracts. The ethanol 99.7% extract is deep green in colour and is in the form of a thick sticky paste (Figure 1). The ethanol 50% extract is in powder form with a light brown colour (Figure 2) and the aqueous extract which was extracted with deionised water was also in powdered form with an off-white colour (Figure 3).

The extractive yield of the three different extracts ranged from the highest of 68.76% for the ethanol 50% extract to 64% for the ethanol 99.7% extract and the lowest yield which was the aqueous extract which had a yield of 56.76%. The results for the extractive yield together with the respective weight of starting material and final weight of the dried extract is shown in Table 1.

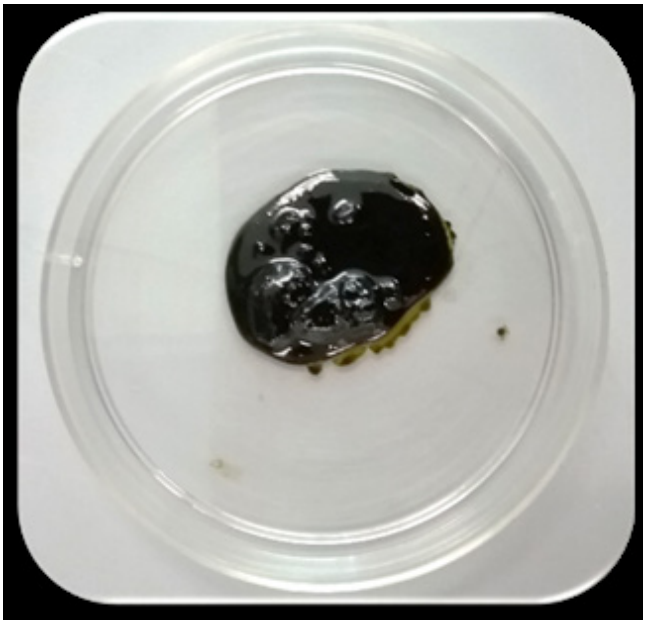

Figure 1: *M.oleifera* Leaf Extract of Ethanol 99.7%. The 99.7% ethanol extract of the *M.oleifera* leaf after drying shows a deep green colour and has a thick sticky texture.

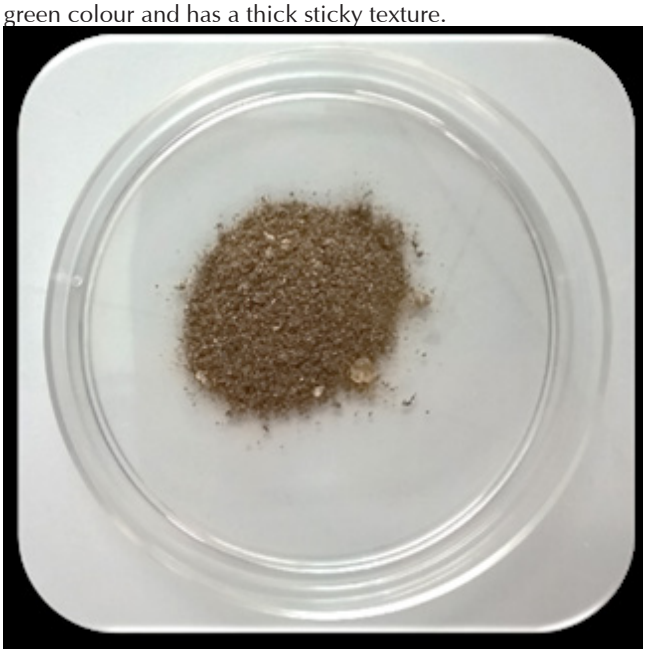

Figure 2: *M.oleifera* Leaf Extract of Ethanol 50%. The 50% ethanol extract of the *M.oleifera* leaf after freeze-drying shows a light brown coloured powder

Figure 3: *M.oleifera* Leaf Extract of Deionised water. The aqueous extract of the *M.oleifera* leaf after freeze drying appears as an off-white coloured powder.

Table 1: Extractive Value (%) of three types of extracts

Solvent	Before Extraction (powder/pellets) (g)	After Extraction + Freeze Dry (Final Yield)	Extractive Value (%)
99.7% Ethanol	25.00	16.00	64.00
50% Ethanol	22.17	17.19	68.76
Deionised Water	20.91	14.19	56.76

Extractive value was calculated in comparison to the initial weight, 25g

Anti-proliferative activities of *M. oleifera* sequential leaf extracts

In vitro effects of *M. oleifera* leaf extracts were evaluated towards Human Breast Adenocarcinoma cell (MCF-7) to investigate the presence of cytotoxic activities against this cancer cell lines. The extracts were also tested on normal breast cancer cells (MCF-10A) to determine its cytotoxicity on normal cells. The results of the MTT assay as presented in Figure 4 shows that the IC_{50} of the different extracts were significantly lower for the MCF-7 cells compared to the MCF-10A cells. Interestingly, the IC_{50} of the ethanol 99.7% extract for the MCF-7 cells was 25ug/mL whereas for MCF-10A was five times higher with an IC_{50} of 150ug/mL (Figure 5).

DISCUSSION

The significance of phytochemical work increases rapidly as certain phytocompounds have shown promising biological activities. Phytochemical investigation provides data on the chemical constituents of the plant itself; however many isolated natural compounds have not undergone any biological testing.

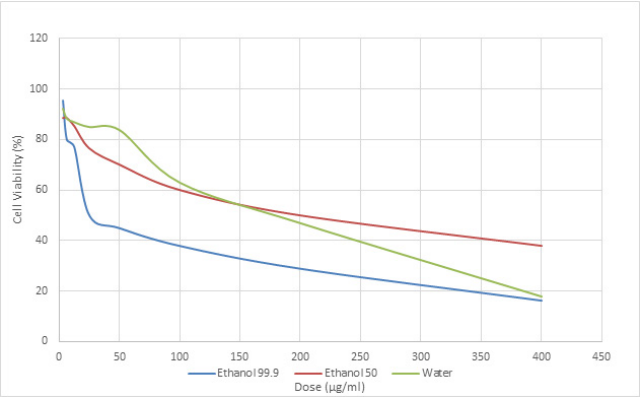

Figure 4: Toxicity Effects of the *M.oleifera* (Ethanol 99.7%, Ethanol 50%, Deionised Water) extracts against breast cancer cell line (MCF-7) with 72 hours of incubation.

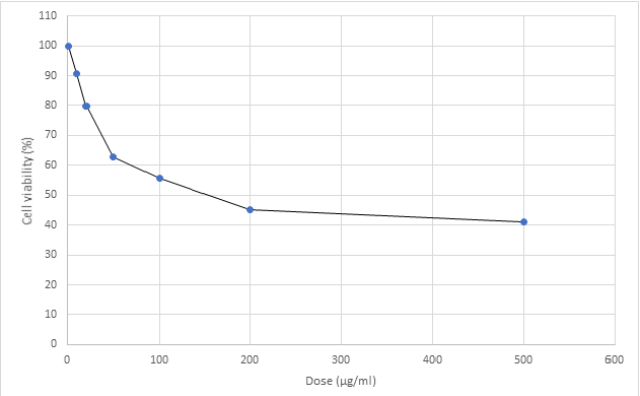

Figure 5: Effects of the *M.oleifera* Ethanol 99.7% extract against normal breast cell line (MCF-10A) with 72 hours of incubation.

Therefore, bioassays of crude plant extracts are usually carried out in order to determine the biological activity of the plant itself (10). Phytochemicals may potentially be applied in cancer prevention and therapy as well as in other diseases with similar etiology (11). Single compound may not be effective in combating cancer due to the superfluous number of triggering mechanism involved. Isolating pure compounds from crude plant extracts decreases the pharmacological activity of the compound, not mentioning the astronomical costs to purify the compounds. On the other hand, due to the lack of consistency in pharmacological response of the crude plant extracts, standardization of the extract is required. This step will ensure repeatability and consistency of the pharmacological outcome of the same product with a clear pharmacokinetic and pharmacological profiles (12). The toxicity or adverse effects of conventional drugs that are usually prescribed for cancer patients in hospitals has shifted its focus to phytochemicals of medicinal plants (3).

Apart from conventional drugs, the currently available chemotherapy causes several serious adverse effects like infertility, nephrotoxicity, anemia, nausea, skin irritation and hair loss (13, 14). This pitfall used up beautifully by natural products that are derived from herbal plants which have potential pharmacological effects with

less toxicity and greater biocompatibility. *M. oleifera* is one of the extensively studied medicinal plants due to its anticancer potential and many other therapeutic properties (9). An important aspect in this research work was to identify a plant species that has well up scaling capability in terms of the ease of plantation, short duration of cultivation and high extractive value for commercial viability. With that in mind, two specific criteria in the selection of plant species were considered. These include strong anti-proliferative property and good anti-migration activity.

Solvent extraction is the most commonly used method to extract bioactive constituents from plants. The bioactive compounds of *M. oleifera* have been routinely extracted using many solvents, namely ethanol and methanol (8, 9). Some studies focused on organic solvent extraction due to its greater efficacy than aqueous extraction (9, 15). Generally, organic solvents are able to dissolve high quantity of beneficial organic substances such as phenolic compounds present in plant material. In fact, different solvents possess different extraction potency and distinct spectrum of solubility as well for bioactive constituents (15). Solvent extract of *M. oleifera* was much competent for hepatocarcinoma cells than a buffer extract (16). In this study, 3 solvent systems (99.7% ethanol, 50% ethanol and deionised water) with increasing polarity were used to prepare crude extracts of *M. oleifera*. The extraction was assisted by ultrasound wave, thus referred as Ultrasound Assisted Sequential Extraction (UASE). Physical characteristic examination of yield extracts showed 99.7% ethanol extract appeared to be semi solid whereas 50% ethanol and deionised water extracts appeared as a solid form. The highest extractive value achieved by 50% ethanol extract (68.76%) and the least was deionised water extract (56.76%). There are a lot of previous studies of cytotoxicity effect of this plant on human cancer cells such as pancreas (Panc-1), breast (MCF-7) (3), colon cancer cells (SW480 and HCT18) and keratin forming tumor cells (9). The present study is the very first study to assess *in vitro* cytotoxicity of *M. oleifera* extracts (99.7% ethanol, 50% ethanol and deionised water) against a breast adenocarcinoma cancer cell line (MCF-7). Of all these extracts, 99.7% ethanolic extract of MOL showed the most potent anti-proliferative effect on MCF-7 cells with IC_{50} value of 25µg/mL after 72 hours exposure. As shown in this study, the inhibitory effects of *M. oleifera* on MCF-7 *in vitro* was through cell proliferation. The anti-proliferative of *M. oleifera* occurred at a concentration of < 30 µg/mL. Although the anti-proliferative effect exhibited by 50% ethanol and deionised water extracts were lower, their activities remained significant, thus suggesting the presence of other potentially active compounds within the intermediate to low solvent polarity. The lowest IC_{50} value was recorded by 99.7% ethanol extract after 72 hours exposure. This might be due to the presence of both polar and non polar metabolites in the extract. According to Jung, Lee (9), organic solvent was able to extract both polar and non polar metabolites of the crude extract. The obtained IC_{50} value of 25µg/mL confirmed the potent anti cancer effect of 99.7% ethanolic extract of *M. oleifera*. As mentioned in previous study by Hermawan, Nur (17), an anticancer agent derived from plant should have IC_{50} lower than 100µg/mL, if not it is only considered to be a chemopreventive agent. The possible metabolites that contributed to the anticancer properties were niazimicin, niaziminin, glucosinolates and isothiocyanates. In addition to that, *M. oleifera* leaves also contained thiocarbamates, carbamates and nitrile glycosides (18). Niazimicin and 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate were verified as natural anticancer agents according to a study on assessment of prostate cancer cell in which these compounds recorded comparable effect as the chemotherapeutic drug called Estramustine (19). However, 99.7% ethanolic extract of *M. oleifera* gave the lowest IC_{50} value after 72 hours of exposure. This portrayed the potent anticancer effect was due to the fact that the extract was rich in phenolic constituents with radical scavenging property that induced cytotoxicity in cancerous cell. The anti-oxidant property was indicated by the presence of the high amount of antioxidant, glutathione (GSH) at the lower dose and vice versa (20).

Generally, the quality of extract that encompasses anti-oxidant properties is greatly related to the strength and polarity of solvents. The organic solvents like ethanol and methanol give higher quality of extract and high anti-oxidant activity. *M. oleifera* contained antioxidants which were extensively extracted with organic solvents. These antioxidants suppressed the reactive oxygen species (ROS) and reduced oxidative stress. Lipid peroxidation due to the ROS wass evaluated by quantifying MDA level. Study showed significant elevation of MDA level in *M. oleifera* treated cells as compared with the untreated cells (0.269 ± 0.013 µM vs 0.197 ± 0.016 µM, $p < 0.001$). Apparently, the cytotoxicity only occur in cancerous cells whereas healthy cell are not affected (18). The IC_{50} value of deionised water extract was higher than in other solvents during the first timeline. The water extract contains both non phenolic and phenolic constituents which might be able to suppress the sole activity of phenolic compounds. Furthermore, there is a possibility for water insoluble phenolic compounds to be present in this plant. Hence, there are chances for the test sample (extract) not to really merge with the cells and medium to evoke a therapeutic response. This made difficult to administer the test sample (extract) orally in rodents. This statement is in agreement with (9). Hence, the *M. oleifera* extract from deionised water was found not to be a promising anticancer agent through this study. To determine the specificity of the cytotoxic effect of *M. oleifera*, a normal MCF 10A cells, was also subjected to M. oleifera treatments. The study showed that this plant was not toxic to normal cells, thus considered safe for human and animal uses. Previous study verified 72 hours

treatment as the right choice of treatment as 24 hours treatment study revealed weak cytotoxic effect on HeLa cells with less cell death induction (17). A study reported the IC_{50} value of 166.7µg/mL for aqueous extract after 24 hours of treatment (18). Our finding indicated the IC_{50} value of deionized water extract was 180µg/mL after 72 hours of exposure. Besides that, a study reported *M. oleifera* leaf aqueous extract at the respective doses of 200, 300 and 400 µg/mL showed growth inhibition of 21%, 65% and 93% after 48 hours treatment (9). The present study, however showed cell viability to be 88% and 92% for the respective doses of 400 and 200 µg/mL.

CONCLUSION

M. oleifera has been unequivocally shown to be a potent anticancer agent. This study showed that *M. oleifera* is cytotoxic to human breast adenocarcinoma cell lines. The test results further showed no apparent cytotoxicity response towards normal cell lines. This give a good indication that the anti-proliferative response observed in the MTT assay is a characteristic of a true cell proliferation inhibitor and not because of the cytotoxic feature of the compound. Hence, it can be conclude that *M. oleifera* could be a potential preventative and/or therapeutic agent for breast cancer, either used alone or as an adjunct to the standard chemotherapeutic drugs.

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