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Methodology for Rapid Isolation of Moringin: Potential Anticancer Compound from the Seeds of *Moringa stenopetala*

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Abstract

The seeds of *Moringa stenopetala* which are known to be rich sources of glucosinolates, primarily glucomoringin, have been shown to have limited anticancer effects when tested *in vitro*. In the present study, however, the water extract of the seeds obtained following defatting by hexane displayed potent cytotoxic activity against HepG2 (IC_{50}, 6.28 ± 0.55 µg/ml) and the SH-SY5Y human neuroblastoma (IC_{50}, 9.81 ± 1.30 µg/ml) cells. The methanol extract displayed a weak cytotoxic activity against the HepG2 human hepatocellular cancer cells when tested upto 500 µg/ml, while extraction with hexane yielded the non-cytotoxic fixed oil which fatty acid composition was primarily oleic acid (75%). By monitoring the constituents of the water extract by HPLC and a one-step combiflash chromatographic system using C-18 silica gel system, the principal active constituent (glucomoringin isothiocyanate or moringin; 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate) was isolated. Moringin was 6.3 and 2.4 times more potent than etoposide in the HepG2 and SH-SY5Y cell lines respectively. The isolation of this potent potential anticancer compound from the seeds of *M. stenopetala* without the need for multi-step chromatographic and enzyme-digestion-based purification steps are discussed.

Keywords: *Moringa stenopetala*; Moringaceae; Seeds; Glucomoringin isothiocyanate; Moringin; Cancer; HPLC; Combiflash chromatography

Introduction

Moringa stenopetala, also called the African Moringa is native to the Southern region of Ethiopia and Northern Kenya. The plant has multipurpose usage that mirrors the well-known Moringa, *M. oleifera*, commonly known as the Indian Moringa. Being described as "The Miracle Tree", the plant is used as a source of food to humans and as animal fodder, medicine, edible oils, biofuel and applications for water sanitations [1,2]. The various parts of *M. stenopetala* are extensively employed in the Ethiopian and Kenyan traditional medicine for treating a range of illnesses such as, diabetes, hypertension, stomach pain, malaria, leishmaniasis, leprosy, epilepsy, diarrhoea, asthma, colds, as an anthelmintic, emetic and wound healing [3]. Some of the recent pharmacological studies that appear to validate the claimed medicinal uses of the plant, primarily the leaves, include antimicrobial [4,5], antihypertensive [6-8], and blood glucose lowering and other favourable blood biochemical parameters [1,2,6,9-13].

The seeds of *M. stenopetala* are packed full of fixed oils (over 30% by weight) which are characterised by the predominant oleic acid (around 75%) composition [14,15]. The seeds are also sources of the cationic proteins which are responsible for the flocculant activity and/or water purification potential of the plant [16,17]. The secondary metabolites of the seeds are represented by glucosinolates primarily glucomoringin (GMG; 4-(α-L-rhamnosyloxy)-benzyl glucoseinolate, 1; Figure 1) which is reported to constitute about 13% of the seeds by dry weight [18]. Plants producing glucosinolates are also equipped with a thio-linked glucose hydrolysis enzyme called myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) that liberate bioactive molecules such as isothiocyanates and nitriles and in the case Moringa, the principal enzyme hydrolysis product in the seeds is 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate (GMG-ITC) or moringin (Figure 1). To date, moringin obtained through enzyme catalyzed extraction process from plants such as *M. oleifera* has been shown to have numerous pharmacological effects including as chemoprotective agent against cancer progression and direct antitumoural effects along with antioxidant and antiinflammatory properties both *in vitro* and *in vivo* [19-25]. Despite all these established pharmacology for moringin (2), the anticancer potential of *M. stenopetala* seeds has not been established and preliminary study on the methanol extract has shown effect only at high doses (500 µg/ml) *in vitro* when tested against the HepG2 hepatocytes cell line [26]. Since, such a weak effect shown for the seeds was suspected to be due to the methodology of extraction employed, the organic extracts with high oil content masking/diluting the active principles, the present study was undertaken to demonstrate the anticancer potential of the seeds and isolation of the active principle(s).

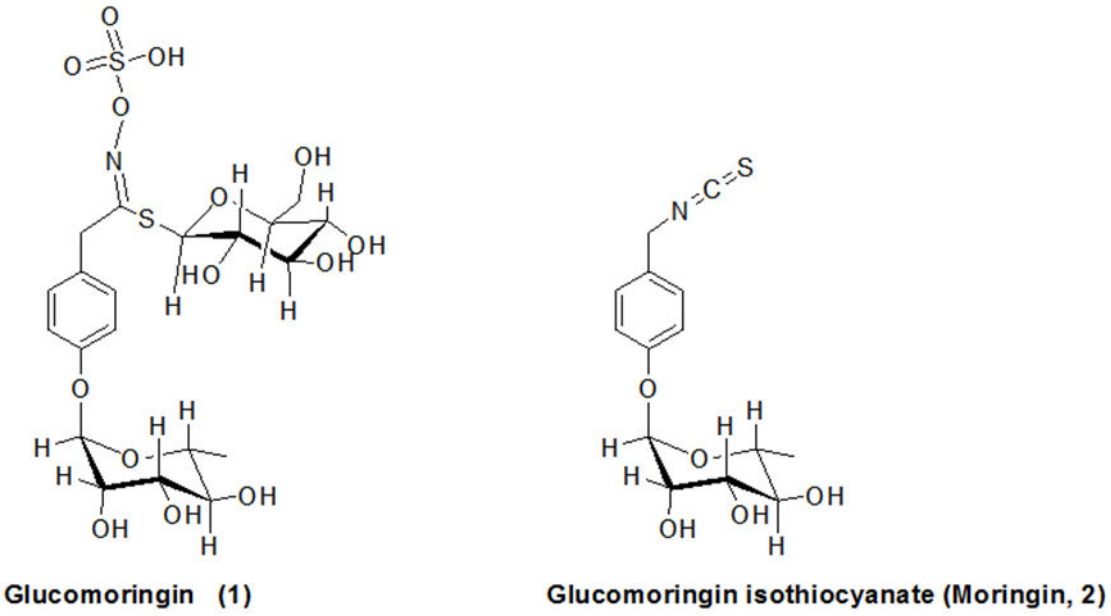

Figure 1: Structures of glucomoringin its isothiocyanate derivative, moringin.

Materials and Methods

Chemicals

All solvents used for the extraction and chromatographic analysis were obtained from Fisher Scientific UK Ltd (Loughborough, UK) while all other reagents were from Sigma (Sigma-Aldrich Chemical Company, Dorset, UK).

Extraction of the plant material

The seeds of *M. stenopetala* were purchased from Ethiopian supplier as described previously [1,2]. The seeds were crushed and made into small pieces and extracted at room temperature by either methanol or successively by hexane followed by water. In each case, the plant material (20 g) was soaked in the chosen solvent (1 L) and left overnight. After removing the extract, the remaining plant material was further extracted twice with the same solvent to maximize the yield. The bulked extract materials labelled as methanol, hexane, and water are taken for bioassays and further chromatographic analysis.

In vitro anticancer activity evaluations

The liver hepatocellular HepG2 and SH-SY5Y neuroblastoma cell cultures were obtained from the European Collection of Cell Cultures (Public Health England, Porton Down, Salisbury, UK). Cell cultures were routinely maintained with RPMI 1640 medium supplemented with 10% heat inactivated foetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere of 95% air-5% CO_2 at 37 °C. Cells were seeded in 96-well plates at a density of 5,000 cells per well and allowed to establish by incubating plates for 24 h. Various concentrations of the test compounds were then added and culture plates were further incubated for 24 h. Cell viability was assessed by measuring the fluorescence of Alamar Blue as described previously [27].

Isolation and structural assignments of compounds

Isolation: The RediSep C18 gold column (100 g, Presearch, Hampshire, UK) attached to a Teledyne Isco flash chromatography system was used to isolate moringin from the water extract of *M. stenopetala* seeds. The solvent system was composed of methanol and water: a linear gradient starting from 10% methanol to 100% over a period of 25 minutes was employed. The flow rate was maintained at 60 ml/min and the chromatogram was monitored by using dual wavelength of 214 and 254 nm. Through this methodology, analysis of 3.9 g of the extract yielded 30 mg of pure moringin.

HPLC analysis: The HPLC analysis was based on an Agilent 1200 series gradient system composed of degasser (G1322A), quaternary pump (G1322A), auto sampler (G1329A), thermostat column compartment (G1316A) maintained at 25°C and a diode array detector (G1315D). Test samples were injected (20 µL) into a reverse phase column (Agilent - Eclipse XDB-C18, 5 µm, 4.6 x 150 mm) and eluted with the mobile phase which was a mixture of water (A) and methanol (B). The composition of the mobile phase at a flow rate of 1 ml/min was rising from 10% to 90% B over a period of 50 minutes.

General analytical instrumentation and methodology: ^{1}H NMR, ^{13}C NMR and 2D-NMR (HMQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One bond ^{1}H-^{13}C connectivities were determined with HMQC while two- and three-bond ^{1}H-^{13}C connectivities were determined by HMBC experiments. Chemical shifts were reported in δ (ppm) using the solvent (MEOH-D_4) standard. The high resolution mass spectroscopy instrument, **thermofisher LTQ orbitrap XL** (Thermofisher Scientific, UK), with an

electrospray ionisation probe was used for accurate mass measurement over the full mass range of m/z 50-2000. Nano-electrospray analyses were performed in positive ionization mode by using NanoMate to deliver samples diluted into MeOH+10% NH4OAc. The temperature was set at 200°C, sheath gas flow of 2 units and capillary (ionizing) voltage at 1.4 kV. The accurate mass measurements obtained from this system were far better than 3 ppm.

GC-MS analysis: The fatty acid composition of seeds of *M. stenopetala* has been described previously [14,15]. Briefly, the fatty acid methyl ester derivatives of the seed oil obtained from the hexane extract was analysed by GC-MS using the Perkin Eilmer TurboMass spectrometer coupled with PE AutoSytem XL GC chromatography. The operating parameters were as follow: Column: Spec-DB-5MS (Length 30 metre and ID of 0.25 mm), Carrier gas: helium, flow rate: 1 ml/min, inlet temp: 250°C, Detector temp: 280°C, with temperature programming of 120-240°C over 40 min and library search from the NIST spectral database.

Moringin (2): Colourless gummy powder; UV (MeOH) λ_{max} nm: 223, 264 (sh); IR (Universal Attenuated Total Reflectance) ν_{max}; 3339 (br; OH), 2932, 2169, 2082, 1612, 1589, 1509, 1239, 1119, 1098, 1059, 1014, 980 cm^{-1}; ^{1}H and ^{13}C NMR (MeO-D_4): (Table 1) ESI-MS m/z [M +NH4]+ 329.1169 (calc $C_{14}H_{17}NO_5SNH_4$ 329.1166); 337.0732 observed for $[M+Na]^+$, $C_{14}H_{17}NO_5SNa$, (theoretical, 334.0720).

Statistical analysis

All cytotoxic measurements were carried out at least in quadruplicate set up and repeated at least four times. Results are shown as mean ± standard error of mean (SEM). Where necessary, unpaired student *t*-test was employed to assess significant differences between two mean values.

Results and Discussion

As part of our attempt to identify potential anticancer agents from natural sources, our laboratories have been investigating numerous crude and purified plant secondary metabolites [28-36]. The present study focused on the seeds of a plant which has been extensively advocated as a safe alternative means of flocculent agent for water purification and also sources of edible oil that could also be exploited as biofuel. The therapeutic potential of the leaves and seeds of *M. stenopetala* along with the socioeconomic significance of the plant have been the subject of intense scrutiny in recent years [3].

Despite the established presence of glucomoringin (1) in *M. stenopetala* seeds, the *in vitro* anticancer effect of the extracts of the seeds has been reported to be weak and manifested at high doses when tested against the HepG2 hepatocyte cell line [26]. On the other hand, the prevalent pharmacological activity of glucosinolates has been shown to be associated with the generation of more biologically active compounds, isothiocyanates, through action by the myrosinase enzyme. Hence, the large scale biological activity assessments of moringin (2) from *M. oleifera* seeds and other sources have been based on the isolation of the compound through enzyme-hydrolyzed extraction procedures. This involve several steps: first, the parent glucosinolate, glucomoringin (1), has to be purified from the seeds through sequential chromatographic steps such as isolation through anion exchange system followed by gel filtration chromatography. Moringin (2) is then produced through a hydrolysis reaction by using ` sourced from other plant species (e.g. from seeds of Sinapis alba [22,23]. It is herein demonstrated that these steps are not necessary to obtain the bioactive product moringin (2) from the seeds of *M. stenopetala.*

Extraction of the seeds of *M. stenopetala* by methanol gave the crude extract in the yield of 32% while extraction with hexane followed by water yielded 28.4 and 15% respectively. In order to reproduce the results already reported on the possible cytotoxicity of *M. Stenopetala* seeds against cancer cells, the same cell line (HepG2) was used for the initial assessment. As shown in Figure 2, the methanol extract was very week showing marginal activity at the highest concentration tested. Considering that around 30% of the seeds by weight constitute fixed oils with oleic acid as predominant but also other fatty acids composition [14,15], any biologically active compound present in the seeds is likely to be diluted by the oils and hence its biological activity could be masked. The other explanation for the weak biological activity of the methanol extract could be due to the fact that glucosinolates may not be as active as their highly potent derivatives isothiocyanates; the latter of which require enzymatic action to be liberated from their parent compounds (glucosinolates).

When the seeds of *M. stenopetala* were first extracted with hexane and then by water, instead of the common alcoholic extraction, a completely different profile of biological activity was observed. The hexane extract which is a source of fixed oil did not show cytotoxicity against cancer cells as expected up to the concentration of 500 µg/ml (Figure 2). The compositional analysis of the hexane extract by GC-MS also reveal the classical components consistent with what has already been reported for this plant [14,15]: Oleic acid (75.2%); palmitic acid (6.5); stearic acid (9.1); 7(Z)-hexadecanoic acid (2.1); arachidonic acid (4.6) and behenic acid (0.6%). The focus of isolating potential anticancer agent(s) from the seeds of *M. stenopetala* was thus centered in the defatted water extract of the seeds.

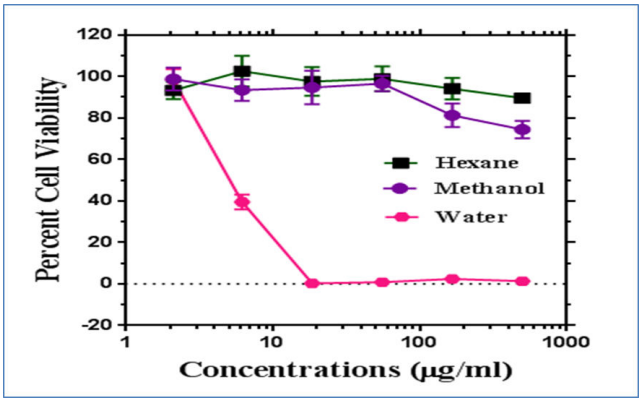

Figure 2: Cytotoxicity of the seeds extract of *M. stenopetala* against HepG2 cells. Notice that, when the seeds extract was first defatted by hexane followed by water extraction, the latter showed potent potential anticancer activity.

The components of the water extract of *M. stenopetala* seeds that was obtained through water extraction following defatting by hexane were monitored by HPLC. As shown in Figure 3, the HPLC chromatogram shows uncomplicated profile with one major components apparent through both UV detectors (215 and 280 nm) employed. The highly polar components such as glucosinolates, carbohydrates and proteins are normally eluted within the first 10 min while the isothiocyanates such as moringin (2) are nonpolar

components that are eluted later in the chromatographic analysis. Large scale extraction/isolation of compounds from water interface complex mixtures of crude natural products is always a challenge. First, the HPLC chromatogram of the water fraction (Figure 3) deceptively appears to be uncomplicated as only UV detectable compounds in the seeds that is packed full of sugars and inorganic materials in water medium are visible. Secondly, the HPLC system is really a quantitative method and does not represent a reliable means of natural products isolation on preparative scale. My laboratory effectively employ the Combiflash chromatographic system for the large scale isolation of biologically active natural products; including flavonoids from the leaves of the same plant, *M. stenopetala* [1,2]. Through just one-step procedure using a reverse phase silica gel flash chromatography system (Figure 4), the known biologically active compound, moringin (2) that corresponds to the retention time of 33.6 min in Figure 3, was isolated in good yield. Hence, the routine procedure of isolation of moringin (2) from *M. oleifera* or other plant sources through ion exchange and other chromatographic methods followed by enzyme catalyzed reaction appears to be unnecessary for obtaining this isothiocyanate compound from *M. stenopetala* seeds. The incredibly potent activity shown for the crude extract of *M. stenopetala* seeds when extracted under the method described herein also suggest that the plant could probably be developed as a cheap source of anticancer agent without even the need to isolate the active principle in its purest form.

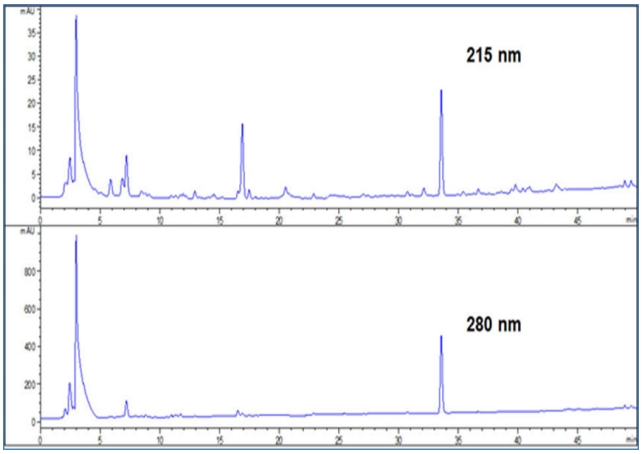

Figure 3: HPLC profile of the water extract obtained after defatting the seeds of *M. stenopetala* with hexane extraction. The water extract (5 mg/ml in methanol) was analysed by HPLC as describe in the materials and methods section.

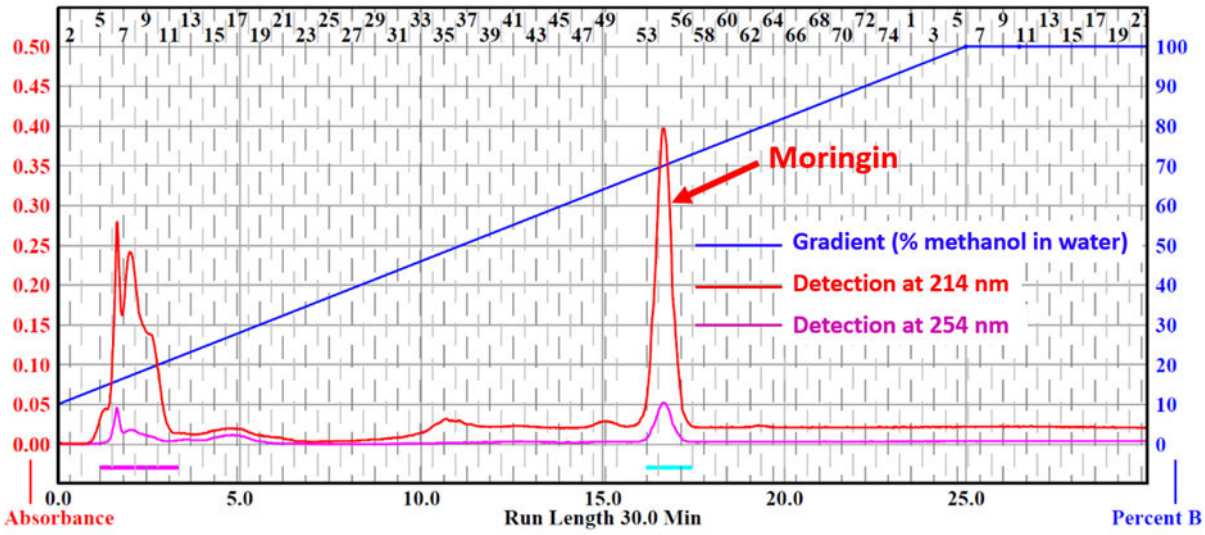

Figure 4: Combiflash chromatographic profile of the water fraction. Experiments are carried out as described in the materials and methods section. The preparative scale isolation of moringin is shown.

The identification of the isolated product as moringin (2) was based on comprehensive 2-D NMR studies including COSY, HMBC, HMQC as well as 1-D NMR (^{1}H, ^{13}C NMR and dept ^{13}C NMR analysis) experiments. The NMR data were generally in good agreement with those already published for 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate [37]. The data herein was however obtained by using methanol-D_4 instead of $CDCl_3$ [37] and hence presented in full (Table 1) to benefit the readers. Accurate mass analysis also allowed to confirm the structure as the $[M + Na]^+$ and $[M + NH_4]^+$ ions were in perfect agreement with those expected for the compound (see experimental section).

The activity of the crude extract and the isolated bioactive principle were tested for their anticancer effect *in vitro* with due comparison with a known clinically useful drug, etoposide. These data on HepG2 and SH-SY5Y neuroblastoma cells are shown in Table 2. It appears that the crude extract obtained by water was a potent anticancer agent with slightly less activity than etoposide. On the other hand, the purified active principle isolated from the water extract, moringin (2), was about 5 and 8 times more potent than the crude extract in the hepatocyte and neuroblastoma cell lines respectively. In these cell lines, moringin (2) was also far superior to etoposide as anticancer agent *in vitro* (Table 2). On molar basis, for example, moringin (2) was 6.3 and 2.4 times more potent than etoposide in the HepG2 and SH-SY5Y cell

lines respectively. The seeds of *M. stenopetala* could thus be used as a good source of a potent potential anticancer agent, moringin (2) that is currently obtained through various chromatographic methods of isolation coupled with enzymatic digestions.

Position	δ_H	δ_C	Key ^3J HMBC Correlations
Aromatic			
1	-	130.1	-
2, 6	7.27 d (8.7)	129.6	48.2, 117.6, 157.8
3, 5	7.07 d (8.7)	117.6	129.6, 157.8
4	-	157.8	-
-CH_2-	4.67s	48.2	130.0, 130.1
N=C=S	-	130.3	-
Sugar			
1'	5.42 d (1.85)	99.8	70.6, 157.8
2'	3.99 dd (3.2, 1.85)	72	72.2, 73.8,
3'	3.82 dd (9.6, 3.65)	72.2	72.0, 73.8
4'	3.45 t (9.6)	73.8	17.5, 70.6, 72.0, 72.2
5'	3.61 m	70.6	-
6'	1.2 (6.4)	17.5	70.6, 73.8

Table 1: ^1H, ^{13}C and ^3J HMBC NMR data for Moringin. All experiments were run in CD_3OD - δ values in ppm and coupling constant (*J*) in parenthesis are shown.

With IC_{50} value between 1-10 μM [38-40], the SH-SY5Y cells are generally known to be more sensitive to etoposide than HepG2 cells. In the latter case, etoposide has been shown to display activity with IC_{50} value around 30.0 μM [41,42]. Hence, the biological effect recorded for the positive control etoposide in the present study (Table 2) was in good agreement with previous reports. On the other hand, concentrations of moringin (2) higher than 12 μM have been shown to be effective in inducing apoptosis in human malignant astrocytoma cells through p53 and Bax activation and Bcl-2 inhibition [22]. The oxidative stress related Nrf2 transcription factor and its upstream regulator CK2 alpha expressions were also shown to be modulated at higher doses of moringin. At the same time, a significant reduction in 5S rRNA was observed with moringin (2) treatment [23]. Brunelli et al. [43] have also conducted *in vitro* and *in vivo* experiments on moringin (2). By inhibiting NF-kappaB activity and inducing apoptosis through a caspase-dependent pathway, moringin (2) has been shown to display anticancer activity in a myeloma model, both *in vitro* and *in vivo* [43]. As sown in Table 2, moringin also appear to be more potent than the clinically used drug etoposide further highlighting the anticancer potential of *M. stenopetala* seeds either in the crudest form or as source of moringin through the method of isolation described in the present study. Given the antitumour activity of moringin (2) has already been established in the last three years and the compound appears to be the major principle of *M. stenopetala* seeds, there was no need to undertake more experiments to establish the anticancer potential of the plant.

	IC_{50} values: μg/ml (μM)	
	HepG2	**SH-SY5Y**
Methanol extract	>500	>500
Hexane extract	>500	>500
Water extract of the defatted seeds	6.28 ± 0.55 (n=4)	9.81 ± 1.30 (n=7)
Moringin	1.21 ± 0.24* (3.90 ± 0.78)*(n=4)	1.23 ± 0.16* (3.39 ± 0.57)* (n=5)
Etoposide	14.50 ± 1.85 (24.65 ± 3.14) (n=4)	5.75 ± 0.16 (7.98 ± 2.12) (n=4)

Table 2: Cytotoxicity of extracts and seeds of *M. stenopetala*, the active principle (moringin, 2) and etoposide against cancer cells *in vitro*. * Indicate data was significantly different from the etoposide treatment in the same cell line ($p<0.01$).

Conclusion

The present study highlighted that the seeds of *M. stenopetala* has tremendous potential as anticancer agent if it is defatted first by non-polar extract before extraction with aqueous media. The active principle, moringin which was obtained from other sources and showed promising anticancer effect in various experimental models, could also be isolated from *M. stenopetala* seeds without the need to go through lengthy processes of purification coupled with enzyme-assisted glucosinolate degradation methods.

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