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# Analysis of Oleanolic Acid and Ursolic Acid, Potential Antifertility Agents in Moringa (*Moringa oleifera*) Seed

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**Abstract:** Oleanolic acid (OA) and ursolic acid (UA) are isomeric triterpenes that are difficult to separate chromatographically. Studies have indicated that both OA and UA have antifertility properties in several livestock species, suppressing attainment of puberty, gonad function and gamete production. Being able to determine the presence and quantity of the OA and UA in moringa and pawpaw seeds will allow for calculation of optimal inclusion levels of these acids in animal diets for antifertility-activity. The purpose of the study was thus to quantify the levels of OA and UA in Moringa and pawpaw seed powder by means of ultra-high performance liquid chromatography with electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS). Only OA was detected in moringa seeds at a concentration level of 0.508 μg/g ± 0.032 μg/g dry weight. UA and OA could not be detected in pawpaw seeds used in this study. The presence of OA in moringa seeds suggest that the seeds can be included in tilapia diets as a potential antifertility treatment to control precocious breeding in tilapia culture in small scale farming systems in Sub-Saharan Africa. Further studies are warranted to determine the levels of OA and UA composition in moringa seeds from other parts of the Sub-Saharan Africa (other than Zambia).

**Key words:** Triterpenoids, OA, moringa seed, antifertility, UA, liquid chromatography.

## 1. Introduction

Oleanolic acid (OA) and ursolic acid (UA) which are hydroxyl pentacyclic triterpenoic acids (HPTAs), are considered as ubiquitous triterpenoids in the plant kingdom [1]. OA and UA are isomers with similar chemical structures and often exist simultaneously in the same plant as aglycones of saponins, and as free acids [2]. These isomeric triterpenic acids occur mostly in medicinal herbs and plants, and naturally in a large variety of vegetarian foods forming an integral part of the human diet [1, 3]. OA and UA have great variation in their distribution between different families, genera and species [4]. The HPTAs have shown in hepatoprotective [5], antiallergic [6], anti-ulcer [7], cardioprotective [8], antimicrobial [9], anti-inflammatory and analgesic [10] activities. OA and UA also present an antiparasitic activity against *Leishmania* species [11] and *Trypanosoma* species [12], and an antiprotozoal potential against *Plasmodium falciparum*, which causes malaria [13]. OA and UA have also been used in the treatment of kidney diseases [14] and hypertension [15]. In addition, OA and UA have been associated with protective effects against periodontal pathogens, antiviral activity against HIV [7] and an anti-tubercular potential against *Mycobacterium tuberculosis* [16] which have been reportedly associated with OA and UA. Also, these triterpenoid compounds have been linked to immunomodulatory, hypoglycemic [17] and antifertility activities [18].

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Rajasekaran et al. [19] tested the antifertility activity of OA isolated from the flowers of *Eugenia jambolana* in male albino rats. They reported that OA arrested spermatogenesis but did not cause any abnormality to spermatogenic cells, Leydig cells and Sertoli cells in the rats. In a review of OA, Liu [1] reported an antifertility effect in rats that OA inhibited testosterone 5α-reductase activity. In a study by Mdhuli and Horst [20], OA was administered orally to a male Wister rat for 30 days followed by a 14-day withdrawal period. Their study indicated that OA induced reversible sterility in the male Wistar rats without any adverse effects on libido. Das et al. [21] evaluated the spermicidal activity of oleanolic acid 3-β-D-glucuronide (OAG) an active constituent isolated from root extracts of *Sesbania sesban*, which showed some significant spermicidal activity. The antifertility effect of these triterpene acids is attributed to their antiestrogenic activity which may be responsible for arresting spermatogenesis [21, 22], and inhibitory effect on implantation of ovum in females [23] in the tested animal models.

Other studies have identified phytochemicals, such as the triterpenic acids of OA and UA [19], and their derivatives, e.g., glycoside [24], oleanolic acid-3 β-glucoside and OAG [21], and saponin (steroidal or triterpenoid glycoside) [25, 26] isolated from plants to have abortifacient properties, indicating their potential to be used as an antifertility treatment in animals.

UA has been found to be the most abundant triterpenic acid in the leaves of the plant *Ocimum sanctum* [19, 22]. UA has been shown to have the potential of inhibiting sperm motility [18], and also possess antifertility activity in rats and mice [22].

Medicinal plants such as moringa and pawpaw have the potential to be used for the manipulation of gender differentiation in tilapia, due to the fact that they contain bioactive components which have an antifertility action in animals. Some of these plants, for example pawpaw have been used successfully to induce sterility in laboratory animals [23, 27] and humans in various countries [28]. Pawpaw (*Carica papaya*) seed administered orally to male albino rats, effectively controlled their reproduction [29] and reduced sperm motility [30]. Aqueous extracts of the root and bark of *Moringa oleifera* have been found to be effective in preventing implantation in rats [31]. And the ethanolic extracts of *M. oleifera* leaves have demonstrated 100% abortifacient activity in female albino rats (Charles Foster strain) [32]. Anwar et al. [33] reported that the root of *M. oleifera* also has antifertility properties with the root and flowers containing the abortifacient compounds.

Therefore, there is scientific merit in the assessment of similar effects of medicinal plants on reproduction of tilapia. These studies suggest that dietary inclusion of plant nutrients with antifertility or abortifacient activity could be used by resource-poor producers to combat problems of precocious breeding of tilapia in production ponds.

High pressure liquid chromatography combined with tandem mass spectrometer detection (HPLC-MS/MS) or ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) has been found to be the most powerful method for the separation, quantification and structural determination of position isomers with very similar structures, such as OA and UA [34-40]. According to Swartz [41], UHPLC-ESI-MS/MS retained the basic principles and the practicality of HPLC, whilst increasing the overall interlaced attributes of speed, sensitivity and resolution. The UHPLC-ESI-MS/MS technique is thus the appropriate method for separation of OA and UA in plant samples as used in a variety of botanical raw materials [39, 40] particularly, in biological fluids such as dyes [42] and also in pharmacokinetic studies [43, 44].

The aim of the present study was to use UHPLC-ESI-MS/MS to rapidly and accurately identify and determine the levels of OA and UA in moringa seed powder.

## 2. Materials and Methods

### *2.1 Plant Materials*

Dry moringa seeds were supplied by the Agribusiness in Sustainable Natural African Plant Products (ASNAPP) branch in Zambia, which also authenticated it. The seeds (Fig. 1a) were blended into a paste using a laboratory blender (ATO MIX, Serial #703449, MSE Model, London). The paste was then oven-dried for 12 h at 60 °C in an electrocool oven (Envirowatch 5, CFE, South Africa), at a relative humidity of 15%. A fine powder form (Fig. 1b) was obtained by sieving the dried powder with a sieve (Universal Laboratory Test Sieve, SABS ISO3310, Model Minor, Serial 100, Number 157-07), and loading the powder into air-tight double zipper plastic bags (Ziploc, SC Johnson & Son, SA (Pty) Ltd.), which were then labelled and stored in a cool dry area until further use.

### *2.2 Reagents and Chemicals*

The purified forms of OA, UA and other media (ethanol, methanol, acetonitrile and chloroform) required for analysis were obtained from Sigma-Aldrich (GmbH, D-91625, Schnelldorf, Germany). Purified water, which was filtered from Milipore water purification system was used throughout the experiment.

### *2.3 Preparation of Standard Solutions*

A 150 mg/L or parts per million (ppm) stock solution of OA and UA was prepared in a mixture of 950 μL methanol and 50 μL dichloromethane, and stored at 4 °C. Between six sets and 10 sets of the stock solution were prepared beforehand and used as required. Calibration standards (0.0012 mg/L, 0.0024 mg/L, 0.012 mg/L, 0.06 mg/L, 0.12 mg/L and 0.60 mg/L) were prepared from the stock solution by serial dilution of methanol [34].

### *2.4 Extraction*

A mixture of 50% acetonitrile, 45% methanol and 5% chloroform (v/v/v) modified from references [34, 45] was used as the extraction solvent. The dried powder (1.0 g) of moringa seed was accurately weighed using a RADWAG 2007 scale (Model WLC1/A1, RADWAG®). Each 1.0 g of powder sample was placed in a capped 50 mL centrifuge plastic tube and 20 mL of extraction solvent was added. The mixture was mixed thoroughly for 50-60 s by a Vortex mixer (VORTEX GENIE 2, Model G-560 E, Bohemia, NY, USA). The mixed samples were then transferred to 500 mL flat-bottom flask (Erlenmeyer) and immersed in the water bath component of the sonication device (Sonication Bath, Lasec Laboratory & Scientific Equipment). Sonication was done for 30 min, whereafter, the samples were centrifuged at 7,500 rpm, 4 °C for 20 min (Eppendorf Centrifuge 5430R, Eppendorf AG 22331, Hamburg, Germany).

After extraction and centrifugation, the supernatant was aspirated. The extraction process was repeated to obtain a solution of 35-40 mL before being dried [34]. For the drying out of the samples, about 5-7 mL of the collected supernatant were repeatedly transferred into 14 mL centrifuge plastic bottle and exposed to a stream of nitrogen gas ($N_2$) until completely dried. The dried sample was stored at 4 °C, until the later analysis. The samples were resuspended in 4 mL methanol before UHPLC-MS analysis.

### *2.5 UHPLC-ESI-MS/MS Analysis*

The levels of OA and UA were analysed using UHPLC-ESI-MS/MS. The separation was carried out using a waters acquity UHPLC with a Waters Acquity Bridged Ethyl Hybrid (BEH) C18 1.7 μm, 2.1 mm × 100 mm column. OA and UA were separated using an isocratic elution with a mobile phase consisting of 10 mm ammonium acetate (in water)-methanol (25:75, v/v) at a flow rate of 0.38 mL/min and at a column temperature of 50 °C.

The Xevo TQ (Waters Corporation, Milford, MA, USA) was operated in ESI negative mode and the m/z


(a) (b)

**Fig. 1 Moringa (a) fresh dehusked seeds, (b) dried powder, used for the analysis.**

455 was monitored (the $[M-H]^-$ ion for both OA and UA). Although OA and UA showed no significant collision-induced dissociation (CID), the mass spectrometer was still operated in multiple reaction monitoring (MRM) mode, monitoring the "transition" from m/z 455 to 455. The cone voltage and collision energy were 30 V and 15 eV, respectively. The peak area was used to calculate the amount of OA and UA from the standard curve.

*2.6 Recovery*

To test the extraction recovery, to the 1.0 g dried plant seed powder, 50 μL of the standard solution (made up of OA and UA, concentration of 12.0 mg/L) was added before and extraction was done through the same procedure as described above. The amount of solvent used for a single extraction was 40 mL (which has been concentrated 10 times), so a spiked sample extractant has a concentration of the stocked solution 0.15 ppm (or mg/L). Each final extractant dried for analysis was designated as either spiked or non-spiked, e.g., extractant from moringa powder spiked with pure chemical was designated as spiked moringa, MS and vice versa-moringa non-spiked, MNS.

Therefore, there were two sets of solutions, spiked (S) and non-spiked (NS). Out of these, UHPLC-ESI-MS/MS analysis was conducted. Detection of the pure chemicals of OA and UA from the spiked samples as well as in the non-spiked samples was determined by using the analytical software called MassLynx, version 4.0 [46]. The recovery of OA and UA was determined as follows:

$$\text{Recovery } (\%) = [(A - B)/C] \times 100\% \quad (1)$$

where, $A$ was the result after addition, $B$ was the amount of samples without adding standards and $C$ was the added amount of the standards.

Recovery was studied in three replicates of the samples (both spiked and non-spiked) and the average or mean values ± SD were recorded (i.e., standard deviation, as recovery or relative standard deviation (RSD)). The data were evaluated using the MassLynx version 4.0 software [46] and the results were presented as a chromatogram and in tables. The chromatograms of OA and UA in spiked samples and non-spiked samples are shown in Figs. 2 and 3.

*2.7 Calibration and Quantification of Triterpenoic Acids*

2.7.1 Calibration Curve

A calibration standard of 0.0012, 0.0024, 0.012, 0.06, 0.12 and 0.60 mg/L were prepared for OA and UA from the stock solution by the serial dilution of methanol and corresponding peak area from the chromatogram generated. Using Microsoft Excel (Office 2010), these values were used to construct calibration curve for OA. The calibration curve was prepared by determining the best fit of the oleanolic peak area against concentration [47].

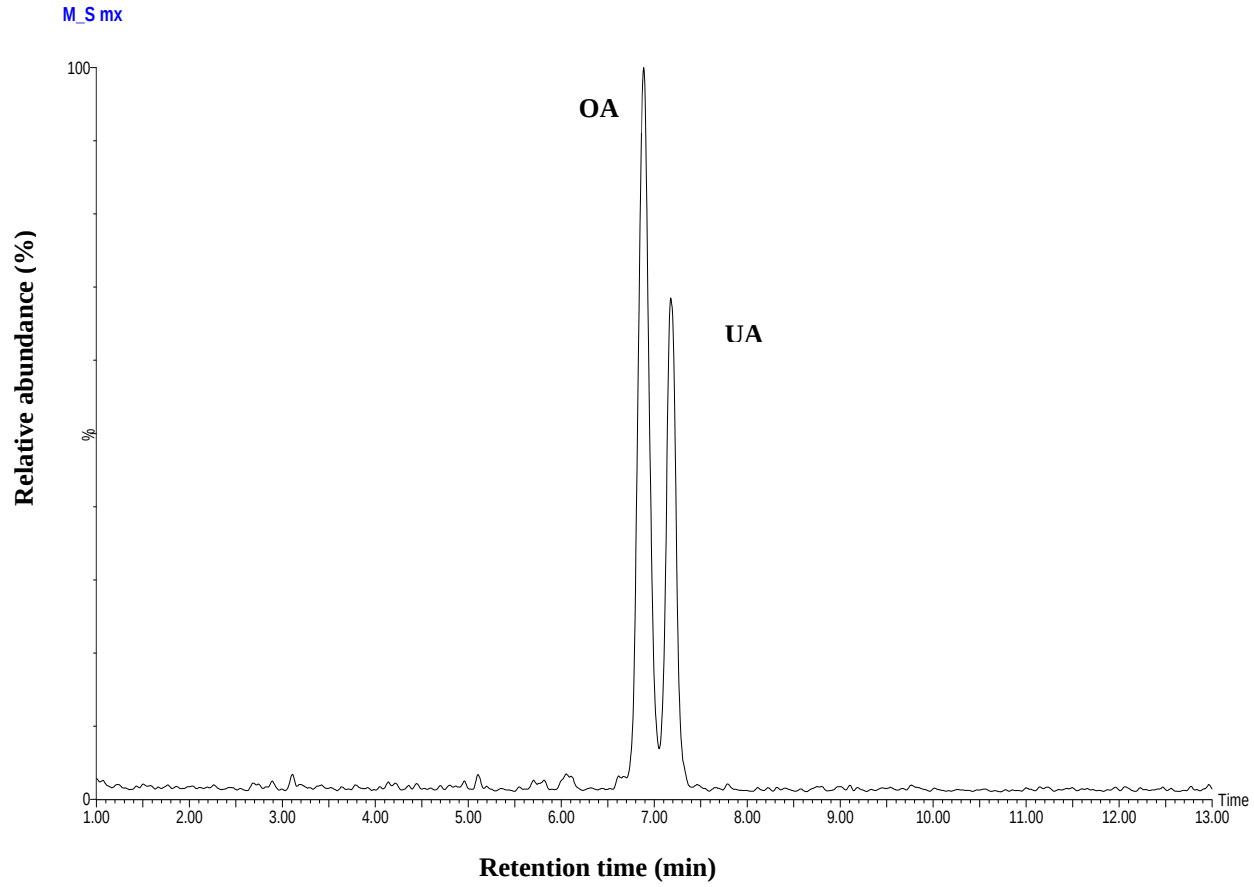


**Fig. 2 ESI-MS chromatogram of OA and UA of the spiked moringa sample.**

2.7.2 Quantification

The content was computed from the relation below:

Amount of Chemical in Sample (μg/g dry weight) = [*C* (ppm)/(1 g)] × [(40 mL solvent)/(10x concentrated solvent)] (2)

where, *C* is the concentration of injected sample (non-spiked).

## 3. Results

### *3.1 Separation of OA and UA*

OA and UA which were mixed in the standard solution could be successfully separated using the water acquity BEH C18 column and a mobile phase consisting of 10 mm ammonium acetate (in water), methanol at a ratio of 25:75 (v/v) from the standard solution. The chromatogram of OA and UA in a spiked moringa seed sample was shown in Fig. 2, and the separation of the two compounds was approaching the baseline [34]. Retention time for OA and UA was 6.9 min and 7.2 min, respectively. Both compounds were detected as a quasi-molecular ion peak at m/z 455 $[M-H]^-$.

### *3.2 Recovery of OA and UA in the Spiked Specimens*

Good recoveries of OA and UA in the spiked samples were obtained in the moringa seed powder. The recovery of OA and UA in the spiked moringa seed sample was presented in Tables 1 and 2. Mean recovery of OA and UA in moringa seed were: 96.467% ($n$ = 3), RSD (%) = 9.8 and 90.3% ($n$ = 3), RSD (%) = 5.7, respectively.

### *3.3 Detection and Quantification of OA in Moringa Seed*

The chromatogram of OA from the moringa seed was shown in Fig. 3. Through the UHPLC-MS analysis, only OA was detected in the non-spiked

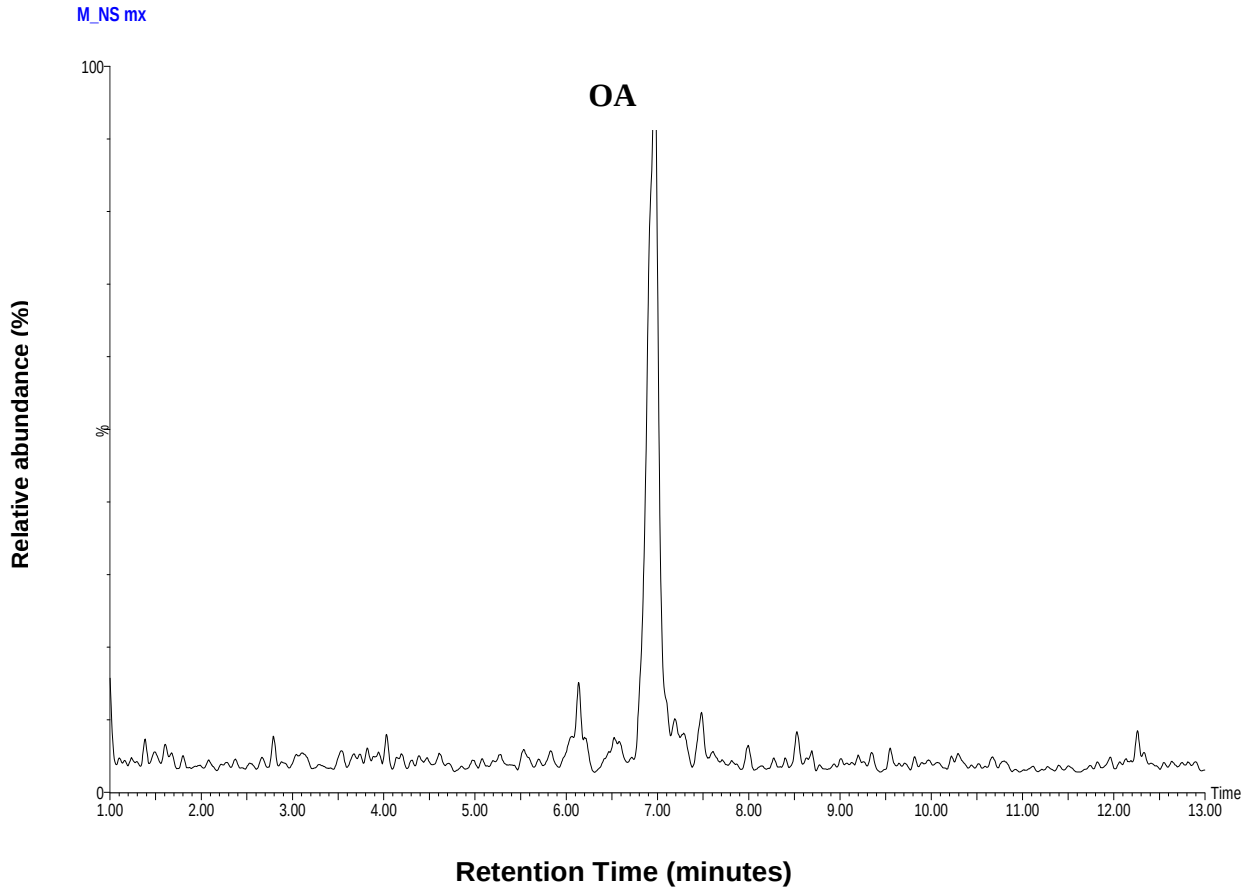


**Fig. 3 Chromatogram confirming the presence of OA in moringa seed non-spiked.**

**Table 1 OA recovery based on spiked (0.15 mg/L of standard solution) and non-spiked samples of moringa seed powder.**

| Moringa non-spiked (ppm) | Spiked moringa* (ppm) | Recovery (%) | RSD (%) |
|---|---|---|---|
| 0.133 | 0.272 | 92.600 | |
| 0.129 | 0.264 | 89.600 | |
| 0.118 | 0.279 | 107.200 | |
| | Mean (± SD) ($n$ = 3) | 96.467 ± 9.416 | 9.760 |

* Samples were spiked with a final concentration of 0.15 ppm OA.

**Table 2 UA recovery based on spiked (0.15 mg/L of standard solution) and non-spiked samples of moringa seed powder.**

| Moringa non-spiked (ppm) | Spiked moringa* (S + 0.15 ppm) | Recovery (%) | RSD (%) |
|---|---|---|---|
| n. d. | 0.143 | 95.500 | |
| n. d. | 0.128 | 90.200 | |
| n. d. | 0.135 | 85.200 | |
| | Mean (± SD) ($n$ = 3) | 90.300 ± 5.151 | 5.704 |

n. d. = not detected; * Samples were spiked with a final concentration of 0.15 ppm UA.

moringa seed powder (MNS) (Table 3). UA was not detected or found in moringa seed. The average content of OA in moringa seed powder was 0.508 μg/g ± 0.032 μg/g dry weight. The OA calibration curve that was used for quantification was presented in Fig. 4. The calculated regression equations between the OA peak area ($y$) against concentration ($x$) and their coefficients of determination ($R^2$) were as follows: OA,

$$y = -1074.5\,x^2 + 5760.9\,x + 3.5 \quad (R^2 = 0.9993) \quad (3)$$

**Table 3 UHPLC-MS detection of OA in moringa seed powder based on non-spiked samples.**

| Non-spiked (ppm) | Content (μg/g dry weight) |
|---|---|
| 0.118 | 0.472 |
| 0.133 | 0.533 |
| 0.129 | 0.517 |
| Average ($n$ = 3) | 0.508 ± 0.032 |

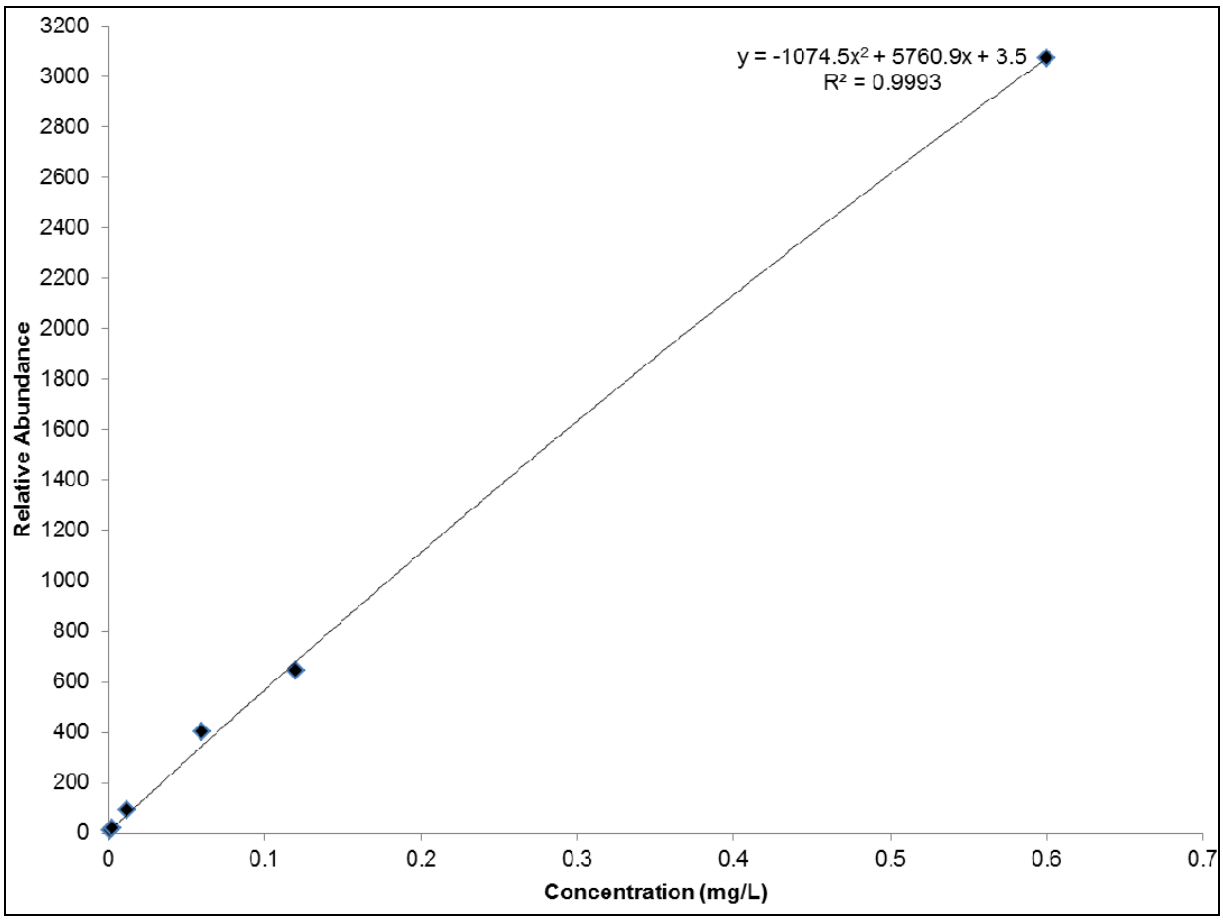


**Fig. 4 Regression between the chromatographic peak area ($y$) and OA concentration ($x$).**

## 4. Discussion

### *4.1 Recovery and Separation*

In this study, using the reported extraction procedure the mean recovery of OA from moringa seed was 96.5% with a RSD (%) of 9.8, while that of UA was 90.3% with a RSD (%) of 5.7. The limit of detection (LOD) was similar at 0.0006 ppm (0.6 ppb).

Studies have shown that several factors determine the effective recovery of a compound of a complex mixture and such factors might include the affinity of the compound for the solvent, the solvent to sample volume ratio, type and concentration of extracting solvent [35, 45, 48, 49]. The recovery of the spiked OA and UA from the samples showed a clear affinity between the solvents used and the respective compounds with ≥ 90% recovery. OA and UA are isomeric triterpenic acids with similar chemical structures that commonly existing in the same plant. Therefore, it is very difficult to separate them when determining a complex mixture with high performance liquid chromatography (HPLC). However, an ultra-performance liquid chromatography (UHPLC), which runs on chromatographic principles designed to

have a column packed with very small particles (with increased speed, superior sensitivity and high resolution) enables rapid separation and quantification [41, 50]. Using an UHPLC-ESI-MS/MS, OA and UA could be recovered and separated when added to a plant extract.

Several forms of HPLC methods such as reversed-phase high performance liquid chromatography (RP-HPLC) and HPLC in tandem with mass spectrometry (HPLC-MS/MS) have been used to separate OA and UA in complex mixtures found in medicinal plants and herbs. However, the retention time tends to be long. For instance, using RP-HPLC, Wang et al. [34] reported a retention time between 10 min and 20 min for OA and UA, respectively. In the study of Xu et al. [49], retention time for OA was 20.58 min, whiles that reported for UA was 21.57 min. Liang et al. [35] used HPLC with a mobile phase made up of methanol-0.2% ammonium acetate in water (83:17), to completely separate OA and UA from the herb O*ldenlandia diffusa* at a retention time between 25 min and 30 min. In all of these studies, OA appeared earlier on the chromatogram than the UA. In this study, the retention time for OA was 6.9 min, whereas that for UA was 7.2 min using standard solutions. This therefore indicated that OA and UA can be separated.

### *4.2 Detection of OA and UA in the Specimens*

According to Tiwari et al. [48], the type of solvent and extraction procedure influenced the successful determination of biologically active compounds from plant materials. Some of the merits of a proper plant extraction solvent included the ease of evaporation at low heat and promotion of rapid physiological absorption of the extract. Xia et al. [45] found that maximum extraction of OA and UA could be achieved from plants using a mixed solvent of 95% ethanol and 5% water at a liquid to solid ratio of 20:1.

In this study, a mixture of 50% acetonitrile, 45% methanol and 5% chloroform constituting the extraction solvent which was used in liquid-solid ratio of 20:1 (v/w). The use of combinations of solvents conforms to the studies [35, 45, 48, 49]. The triterpenic acids, OA and UA often existed simultaneously in the same plants. From three sets of samples, extracts of the moringa seed powder was subjected to the same test described above. The same procedure can be used for pawpaw seed powder where a recovery of 78% and 66% were achieved for OA and UA, respectively (results not shown). The chromatogram (Figs. 2 and 3) obtained were used to identify OA and UA using the retention time in the two figures. In Fig. 2, two peaks were observed with OA at 6.9 min, whiles UA eluted at 7.2 min. Only one peak was observed in the moringa extract (Fig. 3). By comparison of retention time with that of the standard it was authenticated that OA was present in moringa seed powder. Thus, only OA could be detected in the moringa seed powder. The average content of OA in moringa seed powder obtained was 0.5079 μg/g ± 0.032 μg/g dry weight ($n$ = 3), with a correlation coefficient of linear regression analysis (i.e., coefficient of determination, $R^2$ of 0.9993).

According to Swartz [41], UHPLC technology is an improved form of HPLC to provide increased resolution, speed and sensitivity to accurately detect micro-contents of complex matrix efficiently. In this study, only OA was detected in the non-spiked moringa seed with a clear distinct chromatographic peak (Fig. 3), but UA was not detected. The over 90% of recovery for OA and UA from the spiked samples clearly demonstrated the efficiency of the extraction procedure and solvent. Several investigators [35, 45, 48, 49] have used UHPLC to separate and quantify OA and UA in plant samples successfully.

Non-detection of UA in moringa could mean that it was not present or present at levels too small quantity to detect. It has been reported that the triterpene acids of OA and UA occurred both as a free acid and as an aglycone precursor for triterpenoid saponins in which

it was linked to one or more sugar chains in plants [40]. They also accumulated at very low concentrations in plants, which could explain the reason why UA was not detected in moringa. Also OA and UA could exist as derivatives [21, 24].

It has been established that moringa plant components contained phytoestrogens including β-sitosterol, caffeoylquinic acid, quercetin and kaempferol [33, 47, 51, 52]; saponin in the seed powder [53]; steroids, triterpenoids, flavonoids, saponins and anthraquinones in the leaves [54]; steroids, triterponoids and saponins in the bark [55], and saponins, flavonoids and steroids in the leaves [56]. This study has also established that OA was present in the moringa seed.

## 5. Conclusions

It could be concluded from available literature that the findings from this study is the first report on the identification of OA in moringa seeds. The presence of OA in moringa seeds adds more credence to its potential use as an antifertility agent, which could further be explored in the control of precocious breeding of tilapia. Also, to address toxicological concerns among the fish-consuming public, stability of OA (as a potential endocrine disrupter) needs to be investigated under experimental conditions (e.g., at different temperatures and in different media) to determine whether this phytoestrogen can survive food processing procedures (such as cooking, baking and frying of tilapia). Further studies are warranted to determine the levels of OA and UA composition in moringa seeds from other parts of the Sub-Saharan Africa (other than Zambia).

The extraction method adopted followed standard procedures; however, UA was not detected in moringa. As to why UA could not be recovered in the moringa seed cannot be explained at this point. A plausible explanation is that triterpenes are usually present in plants at micro levels and also may exist in their glycoside forms.

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