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# Identification of an Anticancer Compound Contained in Seeds of *Maesa lanceolata*, a Medicinal Plant in Ethiopia

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**Abstract** *Maesa lanceolata* is an ethno-medicinal plant distributed in Central and East Africa, especially Ethiopia and Kenya. It has been used as a traditional medicine against bacterial infections in the small intestine and viral infections in the liver and throat, as well as treatment for rheumatic arthritis. Even though the activity of *M. lanceolata* leaves has been studied in angiogenesis, which is significantly related to cancer and tumorigenesis, tangible evidence for cancer inhibiting activity of *M. lanceolata* seeds has not yet been obtained. To the best of our knowledge, this is the first study in which an anti-cancer compound has been identified from the seeds of *M. lanceolata* and its anti-tumor activity evaluated in HCT116 human colon cancer cells.

**Keywords** anticancer · clonogenic assay · colon cancer · *Maesa lanceolata* · quercitrin

## Introduction

Phytochemicals can be defined as secondary metabolites found throughout the plant kingdom (Cushnie and Lamb, 2005), and their application as traditional medicines is well rooted in time. *Maesa lanceolata* belongs to the Myrsinaceae family and is a shrub found in countries in Central Africa, including Ethiopia and Kenya, where it has been used as an ethno-medicine for a long time (Sindambiwe et al., 1998). According to a survey of the Maasai tribe in Kenya, the Maasai name for *M. lanceolata* is Iloodua and it is used as a traditional medicine in a mixture of individual parts such as the flowers, leaves, stem, bark, seeds, and roots. A mixture of seeds and ground fruit is applied as an anti-helminthic, seeds and crushed fruit for backache, seeds and dried fruit for loss of appetite, boiled water extracts of seeds and fruit for malaria, roots boiled in water for reduced overall body strength and as a nutrient, and seeds and ground/crushed fruit in water for sexually transmitted diseases such as syphilis and gonorrhea (Kiringe, 2006). With regard to remedial applications, the seeds might be an important element; however, the effects of *M. lanceolata* seeds have not yet been studied.

Most of the studies on *M. lanceolata* have been focused on saponins, called maesasaponins, which were shown to affect angiogenesis in the chick chorioallantoic membrane assay even though some saponins (Sindambiwe et al., 1996; Apers et al., 1998; Apers et al., 1999), flavonol glycosides (Manguro et al., 2002) from leaves, alkylated benzoquinones from fruit, and bark extracts including nutrients (Okemo et al., 2003) were identified as ingredients, (Foubert et al., 2012). Angiogenesis is associated with tumor growth and metastasis (Folkman, 1971) and is considered as one of many discrete steps in tumor progression (Bergers and Benjamin, 2002). Because inhibition of angiogenesis is an indirect antitumor therapy, direct therapy based on cytotoxic effects needs to be developed for cancer treatments (Rakesh, 2005). Therefore, the present study focused on the discovery of direct evidence for the presence of a major anticancer constituent in the seeds of *M. lanceolata* by its anticancer activity based on the inhibition of survival and proliferation of cancer cells as well as chromatographic and structural analysis methods.

## Materials and Methods

The seeds of *M. lanceolata* were cultivated and harvested in

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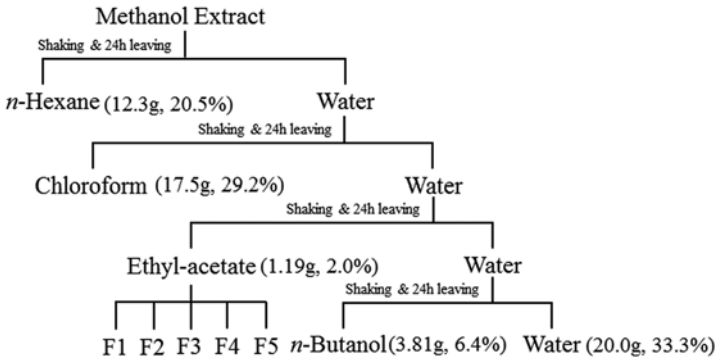


**Fig. 1** Schematic representation of the extraction and separation of a bioactive compound from *Maesa lanceolata* seeds based on its polarity.

Oromia regional state, Ethiopia. The dried seeds of *M. lanceolata* were extracted with methanol three times for 3 days, and the methanolic extract was fractionated with *n*-hexane, chloroform, ethyl acetate (EA), *n*-butanol, and water. According to results of activity-guided assay, the EA fraction was selected. It was freeze-dried and further separated by preparative high-performance liquid chromatography (HPLC). All chemical reagents for HPLC analysis were purchased from local chemical companies; flavonoids used as internal standards were purchased from INDOFINE Chemical Company (USA). The preparative HPLC analysis was carried out on an Agilent 1260 Infinity (Agilent Technologies, USA) fitted with a RP-C18 column (Luna C-18 (II), 5 μm, 10.0 ×250 mm; Phenomenex, USA), and analytical HPLC was carried out on an Agilent 1100 series fitted with a RP-C18 column (Gemini, 5 μm, 10.0×250 mm; Phenomenex) at room temperature. UV/VIS detector (Agilent Technologies, 260 nm), 25% acetonitrile with 0.1% trifluoroacetic acid, and flow rate of 3.0 mL/min, was used. All nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance 400 MHz (9.4T) spectrometer (Bruker, Germany). Samples for NMR experiments were dissolved in deuterated dimethylsulfoxide (DMSO-$d_6$) and then transferred to a 2.5-mm NMR tube. The NMR experiments including $^{1}$H-NMR, $^{13}$C-NMR, distortionless enhancement by polarization transfer, correlated spectroscopy, heteronuclear multiple quantum coherence, and heteronuclear multiple bonded connectivities (HMBC), were performed as described previously (Hwang et al., 2011).

HCT116 human colon cancer cells were obtained from the American Type Culture Collection (USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) at 37°C in a 5% $CO_2$ atmosphere. Cell viability was determined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, USA) as described by Hyun et al. (2012). The clonogenic long-term survival assay was performed as follows: HCT116 cells were seeded ($5\times10^3$ cells/well) onto 24-well tissue culture plates (BD Falcon, USA) in DMEM supplemented with 10% FBS. The cells were then treated with different concentrations (0, 20, 40, 60, and 100 μM) for 6 days. The cells were then fixed with 6% glutaraldehyde and stained with 0.1% crystal violet. The clonogenic survival density was measured using densitometry (Multi-Gauge, Fujifilm, Japan) (Shin et al., 2013).

## Results and Discussion

The methanolic extract (60.0 g) obtained from seeds of *M. lanceolata* was fractionated with *n*-hexane (20.5%), chloroform (29.2%), EA (2.0%), *n*-butanol (6.4%), and water (33.3%). Among the fractions, the EA fraction showed the highest biological activity. Preparative HPLC was applied for the isolation of a pure compound. Because several peaks observed in the chromatogram of the EA fraction showed similar anticancer activity, the main peak at the retention time of 11.1 min (MLEA-F5) was selected (Fig. 2). To confirm if MLEA-F5 is a single compound, the chromatogram and spectrum collected at variable wavelengths using the photodiode array detector were analyzed. HCT116 cells were treated with different concentrations of MLEA-F5 to determine its effect on cancer cell viability, which was analyzed using the aforementioned Cell Counting Kit-8. Purified MLEA-F5 decreased cell viability in a dose-dependent manner (Fig. 3A) . To further investigate the antitumor activity of MLEA-F5, a long-term clonogenic assay was performed. In the presence of MLEA-F5, dose-dependent inhibition of the colony-forming activity of HCT116 cells was observed (Fig. 3B). The clonogenic assay revealed the $GI_{50}$ (50% growth inhibition) to be 72.3 μM. These results suggest that MLEA-F5 isolated from seeds of *M. lanceolata* has intrinsic antitumor activity.

NMR spectroscopy was carried out for structural elucidation of MLEA-F5 (Supplementary data). $^{13}$C NMR analysis revealed 21 carbon peaks, some of which were typically correlated with monosaccharides specifically rhamnose (101.9, 71.2, 70.6, 70.4, 70.1, and 17.6 ppm). Based on the HMBC analysis for the partial structure of MLEA-F5 with protons in the aromatic region (6.20, 6.39, 6.86, 7.25, and 7.30 ppm), two aromatic rings including four hydroxyl groups were verified, which were connected to three conjugated carbons including a carbonyl group. From the results of the NMR assignments MLEA-F5 was identified as quercetin

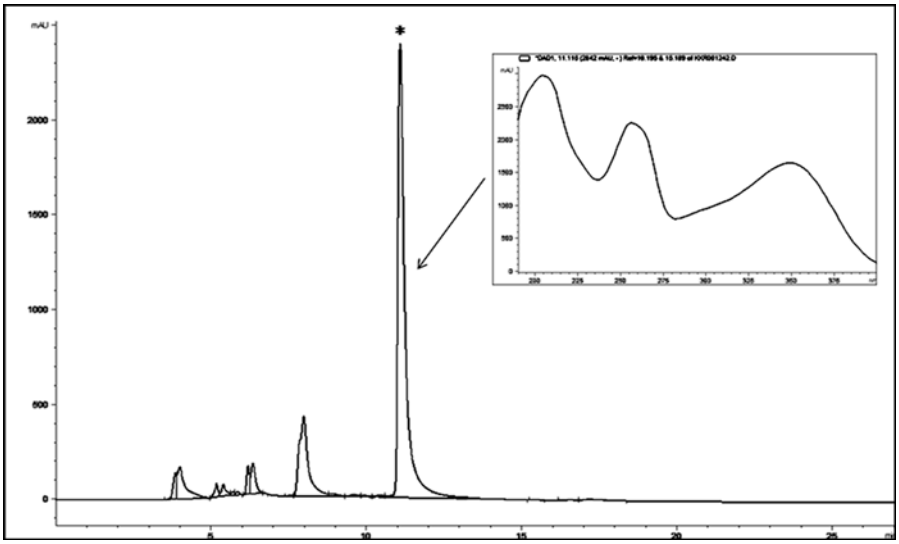

**Fig. 2** HPLC chromatogram of extracts of *Maesa lanceolata*, measured at 260 nm. * represents the purified fraction of *M. lanceolata* extracts for the analysis of its anticancer effects.

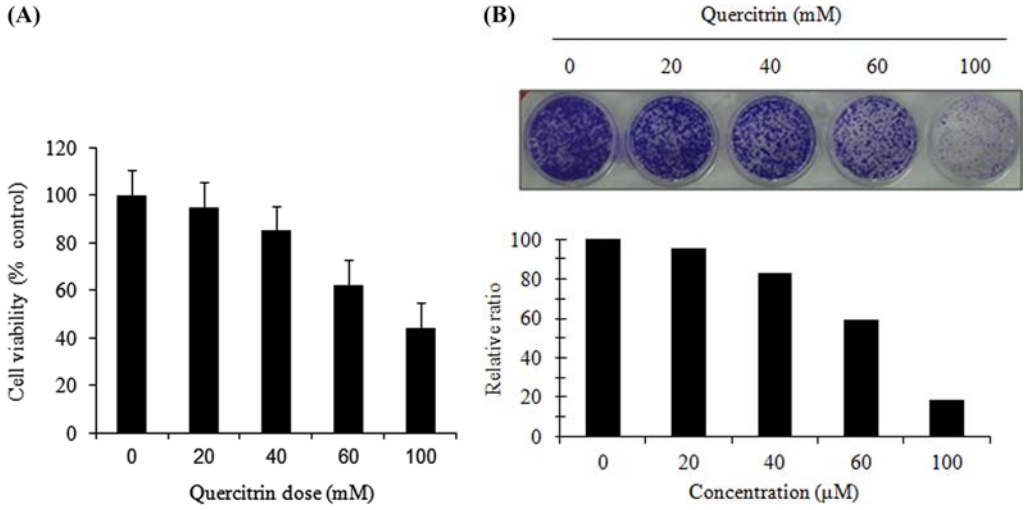


**Fig. 3** Effect of quercitrin on cell viability (A) and the inhibition of colony-forming activity (B). (A) For the viability assay, HCT116 cells ($1\times10^3$ cells/sample) were treated with 0, 20, 40, 60, and 100 μM quercitrin for 24 h. Data represent the mean +/- SD of two independent experiments performed in triplicate. For the inhibition of colony-forming activity, HCT116 cells ($5\times10^3$) were seeded and cultured in the presence of 0, 20, 40, 60, and 100 μM quercitrin. (B) Densitometry analysis was calculated based on the control.

and rhamnose (Table 1). To confirm the connecting position between quercetin and rhamnose, the correlations of both anomeric proton (5.25 ppm) and carbon (134.2 ppm) were used. Finally, MLEA-F5 was confirmed as quercetin-3-*O* rhamnose, namely quercitrin. To confirm the structure of MLEA-F5, an authentic quercitrin was compared with its NMR spectroscopy data.

These findings are in line with previous research on *M. lanceolata* leaves, in which flavonoids including glycosides, were separated, one of which was identified as quercitrin (Manguro et al., 2002). The most studied biological ingredients of *M. lanceolata* are saponins or extracts for antifungal and antilarval effects (Okemo et al., 2003; Adamu et al., 2013). However, the anticancer effects of the seeds of *M. lanceolata* and the components responsible for these effects have not yet been studied. Even though indirect evidence such as angiogenesis for anticancer activity has been studied (Folkman 1971; Apers et al., 1998; Foubert et al., 2012), no study has reported direct evidence such as cytotoxicity and reproductive failure. Therefore, the anticancer effect of quercitrin from *M. lanceolata* seeds was studied in the hope of obtaining direct evidence. Quercitrin is known to protect cells against apoptosis and cell death resulting from intracellular $H_2O_2$ production, ultraviolet radiation, antibacterial infections, and antiallergic reactions (Yin et al., 2013). Absorption of glycones attached to flavonoids are known to be crucial determinants of absorption in the intestine and

**Table 1** Assignments of NMR spectroscopy for quercitrin in DMSO-*d*6

| Position | δ of $^{13}$C | Multiplicity | δ of $^{1}$H |
|---|---|---|---|
| 2 | 157.3 | s | - |
| 3 | 134.2 | s | - |
| 4 | 177.8 | s | - |
| 5 | 161.3 | s | - |
| 6 | 98.8 | d | 6.20 (d, 1.2) |
| 7 | 164.4 | s | - |
| 8 | 93.7 | d | 6.39 (d, 1.2) |
| 9 | 156.5 | s | - |
| 10 | 104.1 | s | - |
| 1' | 120.8 | s | - |
| 2' | 115.7 | d | 7.30 (d, 2.1) |
| 3' | 145.5 | s | - |
| 4' | 148.5 | s | - |
| 5' | 115.5 | d | 6.86 (d, 8.3) |
| 6' | 121.2 | d | 7.25 (dd, 2.1, 8.3) |
| 1" | 101.9 | d | 5.25 (d, 1.3) |
| 2" | 70.1 | d | 3.98 (dd, 2.8, 1.3) |
| 3" | 70.4 | d | 3.51 (dd, 3.2, 9.2) |
| 4" | 71.2 | d | 3.13 (d, 9.2) |
| 5" | 70.6 | d | 3.21 (dd, 9.2, 6.0) |
| 6" | 17.6 | q | 0.81 (d, 6.0) |

subsequent biological activity even though there have been controversies concerning the absorption of flavonoids (Hollman et al., 1995; Beecher, 2003; and Hollman et al., 2004). The function of glycone-induced absorption is important in the use of flavonoid-based medicines and adjuvants. Furthermore, for these reasons, quercitrin extracted from the seeds of *M. lanceolata* can be considered a significant anti-cancer therapeutic.

In the present study, quercitrin, an anti-cancer metabolite, was separated for the first time, purified, and identified from the seeds of *M. lanceolata*. Our direct evidence-based results for anticancer effects suggest that the seeds of *M. lanceolata* can be potentially applied in anti-cancer therapy.

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