*SHORT REPORT*

A C G publications

*Rec. Nat. Prod.* 9:1 (2015) 164-168

records of natural products

# Anticancer and Alkaline Phosphatase Inhibitory Effects of Compounds Isolated from the Leaves of *Olea ferruginea* Royle

**Muhammad Ali Hashmi[1], Hamid Saeed Shah[2], Afsar Khan[1,*], Umar Farooq[1], Jamshed Iqbal[2,**], Viqar Uddin Ahmad[3] and Shagufta Perveen[4]**

[1] *Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad-22060, Pakistan;*
[2] *Center for Advance Drug Research, COMSATS Institute of Information Technology, Abbottabad-22060, Pakistan;*
[3] *H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[4] *Department of Pharmacognogy, College of Pharmacy, King Saud University, Riyadh, P. O. Box 2457, Riyadh 11451, Saudi Arabia*

*(Received December 5, 2013; Revised June 3, 2014; Accepted July 8, 2014)*

**Abstract:** One flavonoid, one ursane type triterpene, and two seco-iridoids were isolated from the leaves of *Olea ferruginea* Royle. The compounds were screened against TNALP and CIALP enzymes for their *in vitro* alkaline phosphatase inhibitory studies and HeLa cancer cell lines to measure their anticancer potential. Compound **1** showed the highest activity of 89.5 ± 1.5 nM against CIALP enzyme. All the compounds showed little activity against TNALP enzyme which shows the specificity of these compounds for CIALP enzyme only. Compounds **1**, **3**, and **4** exhibited anticancer activity comparable to the reference drug vincristine (VNCT). All the compounds showed minimum toxicity against vero cells at 10 µM concentration.

**Keywords:** *Olea*; *Olea ferruginea*; secoiridoid; anticancer; flavonoid. 

## 1. Plant Source

Alkaline phosphatases (ALPs) have become important targets in drug discovery due to their importance in clinical diagnosis [1]. Elevated levels of ALPs are normally reported during the active growth of bones. The main pathological bone conditions of its elevated levels may be fractures, primary bone tumors or invasion of bone by other cancers and diseases that increase bone turnover [2]. Physicians have long recognized that if ALP levels are found elevated in cancer patients, then it is a sign that the disease has spread to the patients' liver and bones. Due to these reasons inhibition of ALPs is a hot area of research nowadays.

*Olea ferruginea* Royle (Oleaceae) known as Kahoo or Khoona in Pakistan is one of the most important plants in folk medicine [3]. Leaves of *O. ferruginea* Royle were collected from Abbottabad, Pakistan. Plant was authenticated by a taxonomist, Dr. Gul Jan, Department of Botany, Hazara

* Corresponding author: E-Mail: afsarhej@gmail.com; Phone: +92-992-383591–5.
** Co-corresponding author: E-Mail: jamshediqb@googlemail.com; Phone: +92-992-383591–5

The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 09/23/2014 EISSN: 1307-6167

University, Mansehra, Pakistan. A voucher specimen (No. 3544) is available in the herbarium of the same department.

## 2. Previous Studies

Screening of plants as anticancer agents dates back to 1950s, when *Vinca* alkaloids, i.e. vinblastine and vincristine were discovered [4]. Due to the problems associated with elevated levels of ALPs and cancer in humans, finding out compounds which not only suppress the ALPs levels but also fight against cancer might be a groundbreaking idea in the field of cancer therapy [5]. Oleuropein, isolated from *O. europaea*, inhibited proliferation of human colon cancer HT-29 cells *in vitro* at micromolar ($\mu$M) range [6] while maslinic acid triggered decrease in HT-29 cell viability by inducing significant changes in the cytoskeleton of the cell-line [7].

## 3. Present Study

Interesting biological activities of the crude extracts and pure compounds from *O. europaea* are reported in the literature, however, its closely related species *O. ferruginea* Royle is very little explored phytochemically which prompted us to investigate it for secondary metabolites and carry out the biological activities of those metabolites. Herein, we report the isolation of quercetin (**1**), $\beta$-amyrin (**2**), oleuropein (**3**), and ligstroside (**4**) (Fig. 1) and their effect on HeLa cancer cell-lines as well as alkaline phosphatases (ALPs).

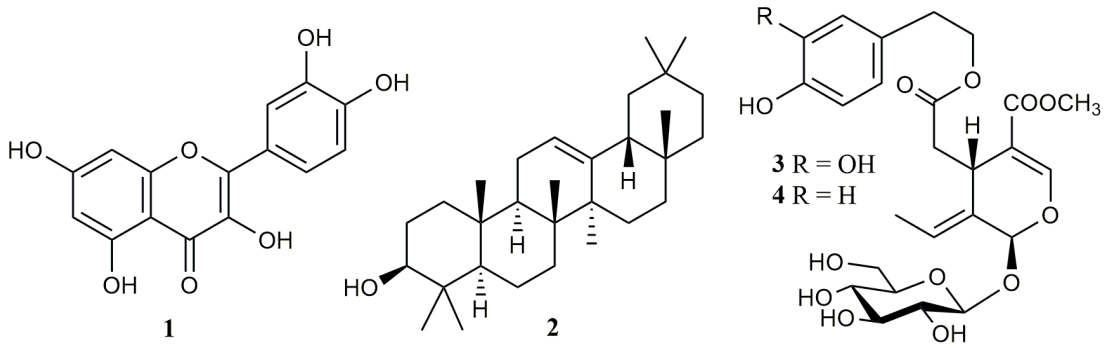


**Figure 1.** Compounds isolated from *Olea ferruginea* leaves

Plant material was air and shade dried and ground to a fine powder. The powdered material (16 Kg) was soaked in MeOH and the filtrate was condensed under reduced pressure to obtain a brownish gum (1285 g) which was suspended in distilled water and sequentially partitioned between *n*-hexane, $CHCl_3$, EtOAc, and *n*-butanol. The resulting extracts were dried in a rotary evaporator and stored under refrigeration conditions. The EtOAc extract (162 g) was subjected to silica gel CC with a gradient of $CHCl_3$/MeOH (100:0–0:100) to give 14 major fractions (Fr. A1–A14). Fr. A5 (30 g) was further chromatographed on a silica gel column eluted with $CHCl_3$/MeOH (95:5–0:100) to produce 9 subfractions (A5-i–A5-ix). Subfractions A5-iv–A5-vi were subjected to repeated column chromatography (silica gel CC, $CHCl_3$/MeOH, 100:0–0:100; Sephadex LH-20, $CHCl_3$/MeOH, 1:1) and compound **1** (15 mg) was obtained from these subfractions. Fraction A5-iii (250 mg) was subjected to silica gel CC, eluted with a gradient of $CHCl_3$/MeOH (97:3–20:80) to afford compound **2** (7.1 mg). Subfraction A5-ix was eluted on a silica gel column with a gradient of $CHCl_3$/MeOH (95:5–20:80) to afford subfractions Q1–Q9. Subfractions Q6 and Q7 were subjected to recycling preparative HPLC to yield compound **4** (6 mg) with a retention time ($t_R$) of 30.5 minutes and compound **3** (129 mg) with a retention time ($t_R$) of 32 minutes (Fig. 1).

*3.1. Instruments:* The $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded at 600 MHz and 150 MHz, respectively on a Bruker Avance AV-600 NMR spectrometer. Mass spectra were recorded on QStar XL Hybrid LC/MS/MS (Applied Biosystems) mass spectrometer. Absorbance was recorded on ELx800 Absorbance Microplate Reader (Bio-Tek Instruments, Inc. USA). Absorbance for

anticancer studies was measured at 490 and 30 nm as in SRB assay while for alkaline phosphatase assay it was recorded at 490 nm. The High Pressure Liquid Chromatography was performed with LC-908W Recycling Preparative HPLC (Japan Analytical Industries (JAI) Co. Ltd.) which was equipped with Hibar LiChrosorb RP-18 column (7 µm, Θ 25 mm × 250 mm; Merck) using a JAI RI-5 refractive index detector and a JAI UV-310 detector (254 nm), simultaneously. The flow rate was kept constant at 3 mL/min.

*3.2. Chemicals and Reagents*: Commercial grade soluents were used for extraction after double distillation. Silica gel (70-230 mesh) was purchased from E-Merck. Tissue non-specific alkaline phosphatase enzyme (TNALP), L-phenyl alanine, levamisole, Sephadex LH-20, Tris–HCl, $MgCl_2$, $ZnCl_2$, RPMI-1640, fetal bovine serum (FBS), glutamine, penicillin, streptomycin, and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich. Tissue specific alkaline phosphatase enzyme extracted from calf intestine (CIALP) was obtained from Calbiochem, Germany. Human cervical adenocarcinoma cell line (HeLa) was purchased from ATCC (ATCC CRL-5802) and vero cell line used as normal cell line was acquired from RIKEN Bio Resource Center, Japan.

*3.3. Preparation of Materials for Biological Assays*: The enzymes were diluted in a buffer (pH 9.5) made up of 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 50% glycerol. The solution of substrate (*p*-nitro phenyl phosphate) of these enzymes was also prepared in assay buffer (pH 9.5) having the same composition except glycerol. Levamisole was used as standard inhibitor for TNALP [8] and L-phenylalanine for CIALP [9]. The compounds having more than 50% inhibition were subjected for further testing by making 3-fold dilutions to determine a dose-response curve. Each compound was pre-incubated for 10 minutes at 37 °C with 10 µL of enzyme and 70 µL of assay buffer and then 10 µL of enzyme substrate was added and incubated for further 30 minutes. The amount of product (*p*-nitrophenolate) formed was measured with the help of an Absorbance Microplate Reader ELx800 at a wavelength of 430 nm. All readings were taken in triplicate. The respective K*i* values were calculated by using the statistical software Prism 5.0 (GraphPad Software, San Diego, CA, USA).

*3.4. Cytotoxicity analysis by sulforhodamine B (SRB) assays*: A previously described method by Skehan *et al.* [10] was implemented to perform cytotoxicity assay using HeLa and vero cells. In short, the cells were seeded in 96 well plates and kept for 24 h in an incubator. The compounds having different concentrations (10, 1, 0.1 and 0.01 µM) were inoculated in the test wells of 96 well plate and incubated for up to 48 h. Similarly, the control and blank wells were also prepared containing reference drug vincristine (VNCT) and culture media, respectively. Then the cells were fixed with 50 µL of 80% ice cold TCA solution for 1 h at 4 °C. The plates were washed 5 times with PBS and air dried. The fixed cells were further treated with 0.4% w/v SRB dye prepared in 1% acetic acid solution and left at room temperature for 30 min. Then the plates were rinsed 4 times with 1% acetic acid solution and allowed to dry. In order to solubilize the dye, the dried plates were treated with 10 mM unbuffered Tris base (pH 10.5) at room temperature for 5 minutes. The absorbance was measured at 490 nm subtracting the background measurement at 630 nm [11].

*3.5. Cell-Lines and Cell Cultures*: HeLa cells and vero cells were cultured in a medium containing RPMI-1640, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) accompanied with 10% heat-inactivated fetal bovine serum in a humidified atmosphere at a constant temperature of 37 °C in 5% $CO_2$ incubator. After achieving an 80% confluence of both adherent cell lines, the cells were cultivated in 96-well plates at a seeding density of $1\times10^4$ cells/100 µL to conduct cytotoxicity experiments.

*3.6. In vitro Alkaline Phosphatase Assay Results*: The isolated compounds were tested against TNALP and CIALP for their potential effects as inhibitor of these enzymes. The increased level of these enzymes was seen in liver and bone diseases (osteoporosis) and cancer which metastasize and affect the bones as seen in prostate cancer [2]. Compound **1** showed the highest activity against CIALP which was found to be 89.5 ± 1.5 nM while compounds **3** and **4** also demonstrated a good inhibition

potential of 134.4 ± 7.7, 162.2 ± 24 and 233.3 ±7 nM, respectively (Table 1). Compound **2** was found to be the least inhibitor of CIALP and TNALP with K*i* values of 1433 ± 172 and 2650 ± 627 nM, respectively. The potent inhibitors of CIALP along with other compounds were tested against TNALP to evaluate the specificity against alkaline phosphatases (ALPs). The potent inhibitors **1**, **3**, and **4** showed higher K*i* values 2452 ± 116, 2582 ± 82.6, 1563 ± 127, and 1750 ± 126 for TNALP which confirm their specific inhibition against CIALP (Fig. S1).

**Table 1.** Activities of the tested compounds against CIALP and TNALP enzymes

| S. No. | Compound | $K_i$ (nM) ± SEM | |
|---|---|---|---|
| | | CIALP | TINALP |
| 1 | **1** | 89.5 ± 1.5 | 2452 ± 116 |
| 2 | **2** | 1433 ± 172 | 2650 ± 627 |
| 3 | **3** | 134.4 ± 7.7 | 2582 ± 82.6 |
| 4 | **4** | 162.2 ± 24 | 1563 ± 127 |
| 5 | L-Phenyl alanine | 73.2% | - |
| 6 | Levamisole | - | 70.8% |

*3.7. In vitro Anticancer Results*: The isolated compounds were tested and compared with reference anticancer drug vincristine (VNCT) to evaluate their cytotoxic potential and non-toxic behavior against human cervical cancer cells (HeLa) and normal cells (vero), respectively. All the compounds showed >50% cytotoxic potential while compounds **1**, **3**, and **4**, respectively, showed 74.29 ± 9.23, 76.16 ± 10.62, and 70.68 ± 11.81 percent inhibition against HeLa cells which are even greater than the inhibition of VNCT 69.48 ± 9.3 at 10 µM concentration. The above described compounds showed minimum toxicity against vero cells when tested at 10 µM concentrations (Table 2). Compound **2** showed lower cytotoxic behavior (62.91 ± 8.94) that may be due to the presence of less hydroxyl groups compared to compounds **1**, **3**, and **4**. Among these compounds, **1** showed 53% inhibition even at 0.01 µM concentration (Fig. S2). The compounds **1**, **3** and **4** isolated from the crude extracts and oil of *O. europaea* have also been studied for their anticancer potential in HT-29 and MRC-5 cell lines *in vitro* [12-14].

**Table 2.** Cytotoxic potential of isolated compounds against HeLa and Vero (normal) cells

| Conc. (µM) | VNCT | Compound 1 | Compound 2 | Compound 3 | Compound 4 |
|---|---|---|---|---|---|
| | **Percent inhibition against HeLa cells** | | | | |
| 10 | 69.48 ± 09.30 | 74.29 ± 09.23 | 62.91 ± 08.94 | 76.16 ± 10.62 | 70.68 ± 11.81 |
| 1 | --- | 67.53 ± 08.47 | 56.21± 11.54 | 68.56 ± 08.53 | 63.94 ± 09.15 |
| 0.1 | --- | 59.86 ± 11.37 | 40.07± 10.21 | 61.70 ± 06.62 | 50.28 ± 10.21 |
| 0.01 | --- | 53.74 ± 07.81 | 30.63 ± 09.46 | 48.54 ± 10.92 | 42.85 ± 06.73 |
| | **Percent inhibition against Vero cells** | | | | |
| 10 | 10.48 ± 03.86 | 10.03± 6.25 | 18.03 ± 05.69 | 13.59 ± 04.28 | 12.03 ± 03.74 |

## 4. Conclusion

The compounds quercetin (**1**), *β*-amyrin (**2**), oleuropein (**3**), and ligstroside (**4**) isolated from *Olea ferruginea* Royale leaves showed good inhibition against TNALP and CIALP enzymes as well as a very good anticancer activity on HeLa cell-line. Compound **2** showed comparable anticancer results to the reference drug vincristine while compounds **1**, **3**, and **4** showed a more potent anticancer activity than the reference drug.

Natural compounds have always provided good leads for synthetic drugs. We, hereby, predict the hit generation, the secoiridoid nucleus for certain chemical modifications which may result in even more potent anticancer agents.

## Acknowledgments

MAH and HSS acknowledge the funding from Higher Education Commission of Pakistan for their PhD studies.

## Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

## References

[1] R. H. Christenson (1997). Biochemical Markers of Bone Metabolism: An Overview, *Clin. Biochem.* **30**, 573-593.
[2] K. B. Whitaker, D. Eckland, H. J. Hodgson, S. Saverymuttu, G. Williams and D. W. Moss (1982). A variant alkaline phosphatase in renal cell carcinoma, *Clin. Chem.* **28**, 374-77.
[3] M. A. Ginai (1968). Alteratise on Horticulture, *Bureau of Agriculture Information, Publication Division, Department of Agriculture, Government of West Pakistan, Lahore*. 450.
[4] G. M. Cragg and D. J. Newman (2005). Plants as a source of anti-cancer agents, *J. Ethnopharmacol.* **100**, 72-79.
[5] Z.-B. Li, J.-Y. Wang, B. Jiang, X.-L. Zhang, L.-J. An and Y.-M. Bao (2007). Benzobijuglone, a novel cytotoxic compound from Juglans mandshurica, induced apoptosis in HeLa cervical cancer cells, *Phytomedicine*. **14**, 846-852.
[6] A. Cárdeno, M. Sánchez-Hidalgo, M. A. Rosillo and C. A. de la Lastra (2013). Oleuropein, a Secoiridoid Derived from Olive Tree, Inhibits the Proliferation of Human Colorectal Cancer Cell Through Downregulation of HIF-1α, *Nutr. Cancer*. **65**, 147-156.
[7] E. E. Rufino-Palomares, F. J. Reyes-Zurita, L. García-Salguero, K. Mokhtari, P. P. Medina, J. A. Lupiáñez and J. Peragón (2013). Maslinic acid, a triterpenic anti-tumoural agent, interferes with cytoskeleton protein expression in HT29 human colon-cancer cells, *J. Proteomics*. **83**, 15-25.
[8] H. Van-Belle (1976). Alkaline phosphatase. I. Kinetics and inhibition by levamisole of purified isoenzymes from humans, *Clin. Chem.* **22**, 972-6.
[9] W. H. Fishman (1974). Perspectives on alkaline phosphatase isoenzymes, *Am. J. Med*. **56**, 617-650.
[10] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney and M. R. Boyd (1990). New colorimetric cytotoxicity assay for anticancer-drug screening, *J. Natl. Cancer Inst.* **82**, 1107-12.
[11] G. S. Longo-Sorbello, Say, G. m, D. Banerjee and J. R. Bertino (2005). Cytotoxicity and cell growth assays, *Cell Biology, Four-Volume Set: A Laboratory Handbook*. **1**, 315.
[12] C. I. R. Gill, A. Boyd, E. McDermott, M. McCann, M. Servili, R. Selvaggini, A. Taticchi, S. Esposto, G. Montedoro, H. McGlynn and I. Rowland (2005). Potential anti-cancer effects of virgin olive oil phenolson colorectal carcinogenesis models in vitro, *Int. J. Cancer*. **117**, 1-7.
[13] R. Acquaviva, C. Di Giacomo, V. Sorrenti, F. Galvano, R. Santangelo, V. Cardile, S. Gangia, N. D'Orazio, N. G. Abraham and L. Vanella (2012). Antiproliferative effect of oleuropein in prostate cell lines, *Int. J. Oncol.* **41**, 31-38.
[14] Q. Long, Y. Xiel, Y. Huang, Q. Wu, H. Zhang, S. Xiong, Y. Liu, L. Chen, Y. Wei, X. Zhao and C. Gong (2013). Induction of apoptosis and inhibition of angiogenesis by PEGylated liposomal quercetin in both cisplatin-sensitive and cisplatin-resistant ovarian cancers, *J. Biomed. Nanotechnol.* **9**, 965-75.