Pharmacogn. Mag.
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ORIGINAL ARTICLE

Naphthazarins as Cytotoxic Agents isolated from *Arnebia euchroma*

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Submitted: 14-03-2019 Revised: 18-05-2019 Published: 26-08-2019

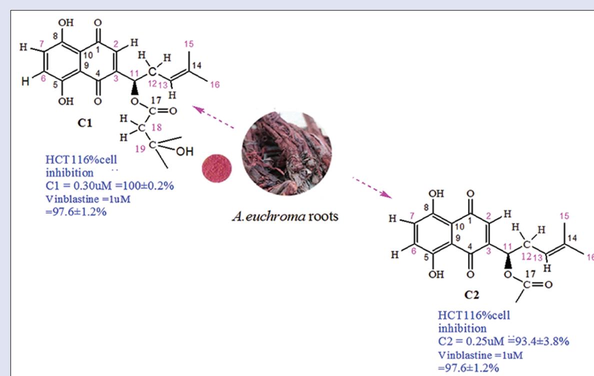
ABSTRACT

Background: Currently, many compounds of different medicinal plants are under investigation for curing various types of cancers. In extension with this ongoing research, *Arnebia euchroma* is taken for the study of cytotoxic compounds present in its roots. The sensitivity of human cervical cancer (SiHa), human epidermoid carcinoma (KB), and human colorectal cancer 116 (HCT116) cell lines toward C1 and C2 isolated from *A. euchroma* roots was evaluated. **Materials and Methods:** The root extract of *A. euchroma* was subjected to extraction, chromatographic separation to afford two naphthazarin esters C1 and C2 as well as two white-colored compounds. The structures of purified compounds were determined by electrospray ionization mass spectrometry and nuclear magnetic resonance analysis. **Results:** Both C1 and C2 showed potent cytotoxicity against SiHa, KB, HCT116 cells at different concentrations. **Conclusion:** C1 and C2 exhibited strong cytotoxicity with IC_{50} = 0.02 µM and 0.09 µM against SiHa cells and with IC_{50} = 0.11 µM HCT116 cells than the vinblastine standard, respectively.

Key words: *Arnebia euchroma*, cell lines, cytotoxicity, dietary supplements, naphthazarin esters

SUMMARY

- Two naphthazarin esters C1 (hydroxyisovalerylshikonin) and C2 (acetylshikonin) were isolated and purified from *Arnebia euchroma* roots. The cytotoxic action of C1and C2 was studied against human cervical cancer (SiHa), human epidermoid carcinoma (KB), and human colorectal cancer 116 (HCT 116) cell lines. The cytotoxicity of test compounds was evaluated using sulforhodamine B assay. In the results, C1 showed effective 93.4% ± 3.8% cell inhibition at the concentration of 0.25 µM and C2 showed 88.4% ± 2.5% cell inhibition at the concentration of 0.15 µM and 100.0% ± 0.2% cell inhibition at the concentration of 0.30 µM on HCT116 cell line. Further, it is found that the presence of an acetyl group may be decisive for the cytotoxicity, as C2 exhibited higher % cell inhibition on SiHa, KB, and HCT 116 cancer cell lines than C1. The C2 showed 100.0% ± 0.2% cell inhibition at a concentration of 0.30 µM on HCT116 cell line than C1 that showed 93.4% ± 3.8% at a concentration of 0.25 µM, respectively.



Abbreviations used: SiHa cells: Human cervical cancer cells; KB cells: Human epidermoid carcinoma cells; HCT116 cells: Human colorectal cancer cells; Ppm: Parts per million; µL: Microliter; µM: Micromolar; TLC: Thin-layer chromatography; ESI-MS: Electrospray ionization-mass spectrometry; TCM: Traditional Chinese medicine.

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DOI: 10.4103/pm.pm_115_19

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INTRODUCTION

Naphthazarin esters are well-known potent pharmaceutical compounds present in plants of genus *Arnebia*. Among various *Arnebia* species, *Arnebia euchroma* (Royle ex Benth.) is a perpetual member in the family *Boraginaceae*. It is widely distributed in the Alpine Belt and the Himalayas.[1] It is a herbaceous perennial plant. *A. euchroma* has thick roots of diameter 2 cm, a cluster of basal leaves and several flowering stems up to 50 cm tall.[2] It was listed in the 2005 Chinese Pharmacopoeia as the main resource of medicinal substances Zicao.[3] Zicao has been used for the treatment of throat sores, burns, cuts, and skin diseases such as macular eruption, measles, and carbuncles in China back from the fifth century.[4] Previous phytochemical studies on this plant reported the isolation of naphthoquinones monoterpenes, phenols, organic acids, and pyrrolizidine alkaloids.[1-9] Shikonin and its derivatives are naphthazarin esters of hydroxynaphthoquinones that are present as ester derivatives in the outer surface of the roots in *Arnebia* species.[10] Naphthazarin esters are used as natural colorants for food, cosmetics, and textiles. These naphthazarin are reported to have anti-inflammatory, antifungal, antioxidant, cytotoxic, and radical scavenging activities and enzyme inhibitory properties.[11-17] Further, acyl derivatives of isohexenylnaphthazarin have been investigated for topoisomerase I inhibition and proved to be potential anticancer agents.[18-21] Furthermore, the increasing problem of cancer in the developed world, leading to death

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Cite this article as: Sharma N, Gulati A, Kumar D, Padwad Y. Naphthazarins as cytotoxic agents isolated from *Arnebia euchroma*. Phcog Mag 2019;15:S366-70.

has motivated the extensive growth of cancer research in recent years. Mass screening programs of natural products and synthetic compounds by the National Cancer Institute of the USA have identified the Quinone moiety as a pharmacophore that normally affords the cytotoxic activity.[22] These results provided a framework to study other shikonin derivatives as promising compounds for cytotoxicity assays. Thus, further research in the field is certain to continue for the emergence of a clinically useful anticancer agent. Moreover, accumulating research evidence suggests that many dietary factors may be used alone or in combination with traditional chemotherapeutic agents to prevent the occurrence of cancer, their metastatic spread, or even to treat cancer. As naphthazarin are well-known dietary supplements with accepted antioxidant behavior, based on earlier reported literature on antioxidant and cytotoxic nature of naphthazarin, the study presented the potent percentage cell inhibition by naphthazarin esters hydroxyisovalerylshikonin and acetylshikonin against Human cervical cancer (SiHa), human epidermoid carcinoma (KB), human colorectal cancer 116 (HCT116) cell lines.

MATERIALS AND METHODS

The roots of *A. euchroma* were collected from Lahaul and Spiti Districts of Himachal Pradesh in Western Himalayas of India. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AVANCE III 600 MHz for ^{1}H and ^{13}C NMR, and chemical shifts were reported in parts per million δ downfield from internal standard Me_4Si (TMS) [Figures S1-4]. Multiplicities of signals are described as follows: S – singlet, br. S – broad singlet, d – doublet, t – triplet, and m – multiplet. Mass spectrums were on recorded Q-time-of-flight micromass liquid chromatography-mass spectrometry (MS) spectrometer [Figures S5 and 6].

Extraction and isolation of compounds

The roots of *A. euchroma* (100 g) was air-dried under shade temperature 27°C ± 2°C and relative humidity of 34% ± 3% for 5 days before extraction and analysis. The roots were powdered and exhaustively extracted with ethyl acetate (boiling point 60°C–80°C). The polar solvent extract was concentrated under vacuum to give a reddish-brown viscous residue (13.8 g). The extract was chromatographed on a column of Si gel and eluted with n-hexane in ethyl acetate (0%–100% gradient) and methanol to afford four fractions (C1–C4). Out of the four fractions, C3 and C4 are white colored fractions. The remaining two fractions C1and C2 were ultraviolet (UV) absorbing fractions with semi-viscous dark reddish colored and dry light red colored, respectively [Figure 1]. Initial separation and purification of different fractions were checked on normal phase Si gel thin layer chromatography (TLC) plate developed in solvent systems of hexane ethyl acetate in the ratio ranging from 0:100 to 100:0.

Cell lines and cell culture

SiHa and KB cell lines were obtained from the National Centre for Cell Science, Pune, India. HCT116 cell line, was obtained from the Indian Institute of Integrative Medicine, Jammu. All the cell lines were cultured in Dulbecco's Modified Eagle Medium (Invitrogen Biosciences, India), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Biosciences, India) and 1% antibiotic-antimycotic solution (Invitrogen Biosciences, India). The cell lines were maintained at 37°C in the CO_2 incubator.[23-25]

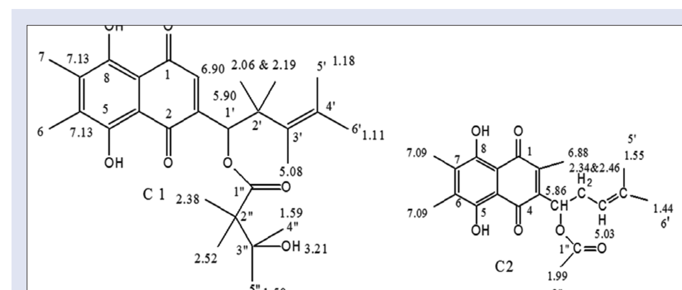

Figure 1: Structures of compounds

Sulforhodamine B assay

Cell lines were trypsinized and washed twice with phosphate buffered saline by centrifugation. SiHa and KB cells incubated at a density of 2×10^4 cells/well and HCT116 cells were incubated at a density of 1×10^4 cells/well in 96 well plates in 100 µL complete medium. Several dilutions (10, 25, 50, and 100 µg/mL) of the test compounds (in 100 µL of complete medium) were added. Vinblastine (1 µM) was used as a positive control, whereas cells alone supplemented with the complete medium used as a negative control. Plates were incubated at 37°C for 48 h in a CO_2 incubator. After 48 h, 50 µL of 50% trichloroacetic acid (Sigma Aldrich, India) was added, and the plates were kept at 4°C for 1 h. The plates were flicked and washed five times with water and then air-dried. Subsequently, 100 µL of sulforhodamine B (SRB) solution (Sigma Aldrich, India) was added and incubated for 30 min at room temperature. After incubation, plates were washed five times with 1% acetic acid, air dried. 100 µL of 10 mM Tris base (Sigma Aldrich, India) was added. The absorbance was measured using a microplate reader (BioTeK Synergy H1 Hybrid Reader) at a wavelength of 540 nm.[25] The IC_{50} values were calculated by "Quest Graph™ IC_{50} Calculator" AAT Bioquest, Inc.[26,27]

RESULTS AND DISCUSSION

The similar fractions of purified *A. euchroma* root extract were collected and combined by repeated TLC experiments, to afford purified compounds C1–C2. ^{1}H and ^{13}C NMR and electrospray ionization (ESI)-MS studies characterized compounds C1–C2 [Table 1 and Figures S1-6]. In the ^{1}H-NMR spectra of C1 and C2, the peaks belonging to side chains (C1′–C6′) were similar to each other. Compound 1 obtained as the red-colored powder was named as hydroxyisovalerylshikonin. UV spectrum

Table 1: ^{1}H and ^{13}C NMR data of compounds C1-C2

Position	(C1) δ_H	(C1) δ_C	(C2) δ_H	(C2) δ_C
OH	3.21 (s)			
C-5"	1.18 (s)	29.32		
C-4"	1.09 and 1.11 (s, t)	29.32		
C-3"		69.07		
C-2"	2.06 and 2.19 (d, m)		1.99 (s)	19.42
C-1"				170.29
C-6'		17.92	1.55	16.58
C-5'	1.60	26.38	1.44	24.48
C-4'	1.59	135.50		135.61
C-3'	5.08 (t)	117.92	5.03 (t)	117.77
C-2'	2.38 and 2.52 (m)	33.84, 41.12	2.34 and 2.46 (m)	32.42
C-1'	5.90 (dd)	69.07	5.86 (q)	69.40
C-9	-	111.40		
C-10	-	111.74		
C-1	-	175.99		175.79
C-2	-	147.69		147.53
C-3	6.90 (s)		6.88 (s)	130.84
C-4	-	177.18		177.33
C-5	12.42 (OH, singlet)	167.45	12.38 (OH, singlet)	167.89
C-6	7.13, s (aromatic)	132.61	7.09 s aromatic	132.49
C-7	7.13, s (aromatic)	132.77	7.09 s (aromatic)	132.74
C-8	12.24 (OH, singlet)	168.00	12.22 (OH, singlet)	167.89

of 1 showed λ_{max} at 214, 274, 485, 518, and 559 nm. Its molecular formula was deduced as $C_{21}H_{24}O_7$ by ESI-MS (m/z 388, 389 [C1+H], 411 [C1+Na]). There are 21 signals in the C13 spectrum in which peaks at δ_{C1}178.2, δ_{C2}136.4, δ_{C3}147.5, δ_{C4}178.3, δ_{C5}167.3, δ_{C8}166.9, δ_{C6-7}132.1 and 132.6, δ_{C9-10}111.6 implied that C1 was naphthazarin ester derivative of hydroxynaphthoquinones. The ^{1}H and ^{13}C NMR spectra of C1 showed typical phenolic OH groups at δ_H 12.24 and δ_H 12.42. Characteristic signals of aromatic H6-H7 and H3 protons of naphthazarin moiety are seen at δ_H7.13, δ_C132.1, δ_C132.6 and δ_H6.90, δ_C136.40, respectively. In addition to the singlets of methyl protons, H5' and H6′ at δ_H 1.59 and 1.50, δ_C 17.9, 25.7 and H4" and H5" at δ_H 1.18, 1.11, δ_C 28.7 the ^{1}H NMR spectrum displayed methylene protons H2' at δ_H 2.06–2.24 (multiplet, bd, J = 3 Hz,) δ_C 32.8 and H2" at δ_H 2.42–2.58 multiplet, δ_C 46.4, H3' at δ_H 5.08 (triplet, J = 7.0 Hz, δ_C 123.0) and H1at δ_H 5.90 (1H, multiplet) δ_C 70.7. The structure of hydroxyisovaleryl chain (C3-O3-H9) and its connectivity to naphthoquinone ring in the molecular structure was further confirmed by the observed signals at δ_H 3.21 (s, OH) and δ_H 2.42–2.58 (2H, multiplet) δ_C 46.4. Compound 2 obtained as the red-colored powder was named as acetyl shikonin. UV spectrum of 2 showed λ_{max} at 213, 273.50, 483, 495, and 562.00 nm. Its molecular formula was deduced as $C_{18}H_{18}O_6$ by ESI-MS (m/z 330, 331 [C2+H], 353 [C2+Na]). There are 15 signals in the C13 spectrum in which peaks at δ_{C1} 175.7, δ_{C2}130.8, δ_{C3}147.5, δ_{C4}177.3, δ_{C5}167.8, δ_{C6-7}132.4, and 132.7 shows that C2 was ester derivative of hydroxynaphthoquinones. The ^{1}H NMR spectra of 2 showed typical phenolic OH groups at δ_H 12.22 and δ_H 12.38. Characteristic singlets of aromatic H6-H7 and H3 protons of naphthazarin moiety are seen at δ_H 7.09, δ_C 132.4, 132.7 and δ_H 6.88, δ_C 130.84, respectively. In addition to the singlets of methyl protons H5 and H6 at δ_H 1.44-1.55, δ_C 16.58, 24.48 and the ^{1}H NMR spectrum displayed methylene protons H2' at δ_H 2.34–2.46, multiplet, δ_C 32.42 and H2"at δ_H 1.99, 19.42, H3' at δ_H 5.03 triplet, δ_C 117.7 and H1' at δ_H 5.86 (1H, quartet) δ_C 69.40 [Table 1].

Cytotoxicity assay

The cytotoxicity of C1and C2 against SiHa, KB, and HCT116 cell lines was evaluated using the SRB assay. These two compounds were tested for cytotoxicity at different concentrations (10, 25, 50, and 100 μg/mL) against the entire cell lines with vinblastine as a positive control. Results are shown in Table 2. C1 was effective with IC_{50} = 0.02 μM against SiHa cell lines. It displays inhibition on KB cells with IC_{50} = 0.30 μM and against HCT116 cells with IC_{50} = 0.23 μM. C2 displays significant inhibition on SiHa cells with IC_{50} = 0.096 μM and with IC_{50} = 0.36 μM against KB-cell lines. It showed notable inhibition against HCT116 cells with IC_{50} = 0.11 μM, respectively. Both the compounds come up as potent scavengers against all the three-cell lines as compare to positive control vinblastine [Figures 2-4 and Table S1].

As shown in Table 2, C1 showed prominent inhibitory concentration with IC_{50} = 0.02 μM against SiHa cell lines and C2 prominent inhibition

Table 2: IC_{50} values (uM) of compounds C1-C2 against human cervical cancer, human epidermoid carcinoma, and human colorectal cancer 116 human cancer cell lines

	IC_{50} (uM)		
Compound	**SiHa**	**KB**	**HCT**
C1	0.02	0.30	0.23
C2	0.096	0.366	0.11
Vinblastine	0.59	0.60	0.51

KB: Human epidermoid carcinoma; HCT: Human colorectal cancer; SiHa: Human cervical cancer

against SiHa cells with IC_{50} = 0.09 μM and HCT116 cells with IC_{50} = 0.11 μM. Similar cytotoxic results from callus and cell suspension cultures of *A. euchroma* were reported by Damianakos *et al.*[28] Authors from this study tested the cytotoxic activity of acetylshikonin and hydroxyisovalerylalkannin against HCT116 cells along with other cell lines in 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-

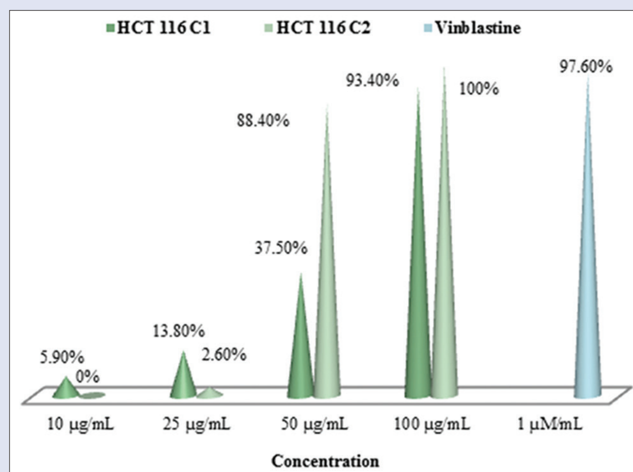

Figure 2: Effect of C1 and C2 on HCT116 cells

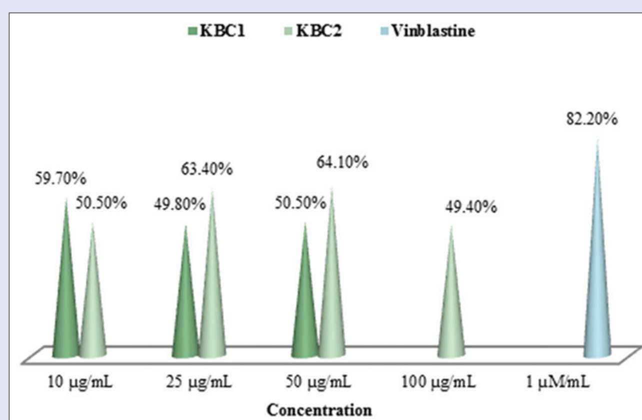

Figure 3: Effect of C1 and C2 on KBC cells

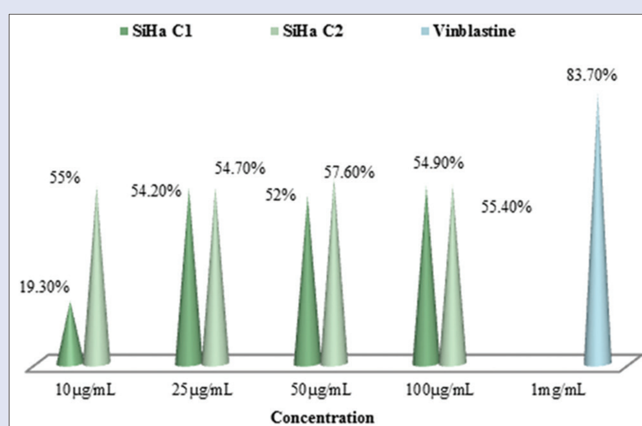

Figure 4: Effect of C1 and C2 on SiHa cells

5-carboxanilide inner salt (XTT) viability assay. While acetylshikonin shows inhibition with IC_{50} of 9.02 μM, hydroxyisovalerylkannin depicted cytotoxic effect at IC_{50} = 12.50 μM against HCT116 cells. Moreover, these compounds were reported to inhibit human cancer cell lines without or little effect on normal cells.[29-31] The nontoxicity of compounds further compliments their use as selective cytotoxic drugs against specific cancer cell lines. There are some mechanisms, which are proposed for the cytotoxic action of naphthoquinones such as oxidative stress, which arises from the capacity of these compounds to enter into redox cycle to generate-free radicals. The generation of free radicals may also cause cellular damage.[12] The inhibition of enzymes by quinones vital for replication may be the other cause. Ahn and coworkers identified structure-activity relationships of these derivatives to find their order of capturing cellular nucleophiles as 6-substituted derivatives >2-substituted derivatives >naphthazarin.[32] The most reasonable mechanism behind the cytotoxicities of C1 and C2 proposed based on the above results is bioreductive alkylation. C1 and C2 quinones are substituted with a side-chain bearing good leaving group (X) hydroxyisovaleryl in C1 and acetyl in C2 at the 2-position of the substituent, the quinone methide formation can result in the elimination of HX from the naphthazarin. The alkylating agent quinone methide is believed to act as Michael acceptor of a biologically important nucleophile (Nu: DNA, protein, carbohydrate, etc.,) which may eventually lead to cell death. Comparing C1 and C2, it was found that C2 exhibited higher % cell inhibition on SiHa, KB, and HCT 116 cancer cell lines. These results indicate that the presence of an acetyl group may be critical for the cytotoxicity. It is believed that the acetyl group in C2 contributes significantly to its cytotoxicity as it showed 100.0 ± 0.2% cell inhibition at a concentration of 0.30 μM on HCT116 cell line [Table S1]. Toxic and electrophilic C1 and C4 carbonyl carbons of quinones react preferentially with the genetic material of macromolecule due to their high susceptibility toward a nucleophilic attack clarify the underlying chemistry involved in this critical event.

CONCLUSION

The use of traditional medicinal plants has become essential as most of the anticancer compounds are identified and isolated from plants. Various genus of medicinal plants have been explored for isolation of chemotherapy drugs. In this study, two compounds hydroxyisovalerylshikonin and acetylshikonin isolated from *A. euchroma*, an important traditional Chinese medicinal herb were tested for inhibitory effect against SiHa, KB, HCT116 human cancer cell lines. Both the compounds showed promising % cell inhibition against HCT116 cell lines.

Acknowledgements

We thank Director IHBT Dr. Sanjay Kumar for all the necessary facilities provided by the institute. We thank the National Centre for Cell Science, Pune, and Indian Institute of Integrative Medicine, Jammu. This is IHBT communication number - 4137.

Financial support and sponsorship

NS gratefully thanks and acknowledges the Department of Science and Technology New Delhi for financial support (SR/WOS-A/CS-08/2014).

Conflicts of interest

There are no conflicts of interest.

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