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Research Article

PHYTOCONSTITUENTS FROM *RICHARDIA SCABRA* L. AND ITS BIOLOGICAL ACTIVITIES

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ABSTRACT

Objective: The present chemical investigation is carried out for the isolation of active phytoconstituents of *Richardia scabra* Linn. belongs to Rubiaceae family from petroleum ether and methanol extract and found out *in vitro* cytotoxic potential of novel compound heraclenin.

Methods: Air dried plant material was macerated with petroleum ether and defatted plant was introduced in Soxhlet apparatus with methanol. Both extracts were subjected to column chromatography for isolation of active chemicals.

Result: Two novel coumarins from the methanol extract and one fatty acid from the petroleum ether extract were isolated from *Richardia.* Oleic acid, heraclenin, and isopsoralen were isolated for the first time in the genus *Richardia.* Furthermore, the cytotoxicity of the heraclenin was evaluated with HeLa cell line and cell cycle arrest by fluorescent assisted cell sorting analysis.

Conclusion: The result revealed that the heraclenin has potent cytotoxicity with the IC_{50} value was 46.09 µg/ml, and it induced cell cycle arrest in G0/G1 phase about (51.2%), S phase (30.2), and resulted in a decline in the percentage of G2-M phase. All the compounds were isolated first in the genus *Richarda* and specie *Scabra.*

Keywords: Cell cycle, Cytotoxicity, Heraclenin, Isopsoralen, Oleic acid, *Richardia scabra.*

INTRODUCTION

Plant-based systems continue to play an essential role in the primary health care of about 80% of the world's population [1]. An increasing number of chemotherapeutic agents are discovered as a result of chemical studies directed toward the isolation of the active substances from plants used in traditional medicine [2]. In the ancient system of medicine, the plants were used as a primary source for health and healing of many diseases. Since the advent of modern allopathic medicine, the use of traditional medicine has declined to a considerable extent. However, in recent years, traditional medicine has made a comeback for a variety of reasons, including side effects and toxicity of modern synthetic drugs, inability of modern medicine to find effective cures for a number of diseases and high cost [3]. India is one of the 12 mega biodiversity nations; many plants are used for curing a lot of diseases by rural people and also have thousands of unexplored plant species. Research has been focused on the therapeutic values and active phytoconstituents of the unexplored species to find out low cost and side effect free drugs.

Richardia scabra Linn. belongs to the Rubiaceae family is widely distributed in the tropical and temperate region and is known as Florida pusley native to North America and is an invaded specie in India. The Rubiaceae family is considered the biggest one of the order Gentianales [4] and presents around 637 genera and 10,700 species [5]. The phytochemistry of the important *Richardia* species is only scarcely investigated [6-9]. *Richardia* is a genus of about 15 species in the family Rubiaceae, only *Richardia brasiliensis* Gomes, *Richardia grandiflora* (Cham & Schltdl.) Steud. have been studied.

The farmers have a greater struggle to remove *R. scabra* weed from their farms, but our findings suggested that it will be used for curing many diseases. It displays a broad range of uses in traditional medicine. The extract of this plant is used to cure skin diseases in South India [10] and in Northeast India, shoot paste is applied externally on the abdomen to get relief from abdomen pain in urinary tract infection [11]. So, far only one report for isolation of lipids is available from this plant [5]. These medicinal uses constitute an important indication of the presence of various constituents responsible for the biological effects.

In this study, we isolated for the first time the oleic acid from petroleum ether extract and two novel coumarins heraclenin and isopsoralen from the methanol extract of *R. scabra* and *in vitro* cytotoxicity of one of the isolated compound heraclenin. Coumarins have been identified from a number of natural sources, especially green plants. Cell proliferation and Apoptosis assays are particularly useful for basic cancer research and drug discovery. So, every possible phytoconstituents isolated was tested for cytotoxicity, and one of the isolated coumarins was tested its cytotoxicity, and cell cycle arrest was analyzed by fluorescence-activated cell sorting (FACS) analysis.

METHODS

Plant materials

The plant was collected locally in Pollachi, Tamil Nadu, South India. The specie for this study was identified as *R. scabra* Linn., family Rubiaceae by the Botanical Survey of India, Coimbatore, Tamil Nadu (BSI/SRC/5/23/10-11/tech.972).

Chemicals

Petroleum ether, ethyl acetate, methanol, silica gel G, silica gel (100-120 mesh), iodine, dimethyl sulfoxide, 4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS), etoposide, and HeLa cervical cancer cell lines were purchased from NCCS, Pune, was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluence at 37°C in 5% CO_2 in a humidified atmosphere in a CO_2 incubator. All the chemicals and reagents used were of LR grade. All the solvents used for chromatography were purified by standard procedures. Melting points were measured on electrical melting point apparatus (Guna) and were uncorrected.

Extraction process

The plant (1.5 kg) was shade dried, and coarse powdered material was defatted with petroleum ether by cold maceration and was subjected to vacuum distillation and was concentrated to yield a greenish residue (15 g). The defatted plant leaves were again extracted with methanol using a Soxhlet apparatus. The extract was subjected to vacuum distillation and was concentrated to yield a brownish residue (40 g).

Isolation of compound from petroleum ether extract

The column was packed with 150 g of silica gel (100-120 mesh), and around 12 g of *R. scabra* was dissolved in a minimum amount of petroleum ether and was uniformly poured into the column. Then, the column was eluted with a mixture of petroleum ether and ethyl acetate in the order of increasing polarity. Each fraction of 20 ml was collected and monitored by thin layer chromatography (TLC), and iodine vapor was used as the spraying agent. Fractions eluted with petroleum ether and ethyl acetate (9:1) yielded a total of 128 fractions; the fractions (78-111) showed single spot in TLC analysis and concentrated under rotary vacuum evaporator yielded pale white powder of R_f value 0.69/cm named as Compound 1 (35 mg).

Compound 1

It was obtained as pale white powder (35 mg): m.p. 16-17.5°C

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 0.80 (*t*, 3H, J=6.8 Hz, CH3-18), 2.29 (*t*, 2H, J=7.6 Hz, CH2-2), 5.29 (*m*, 1H, H-9) and 5.25 (*m*, 1H, H-10).

^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 179.1 (C-1), 33.82 (C-2), 24.3 (C-3), 29.4 (C-4), 29.2 (C-5), 29.3 (C-6), 29.6 (C-7), 27.2 (C-8), 130.0 (C-9), 128.9 (C-10), 27.1 (C-11), 29.5 (C-12), 29.7 (C-13), 29.7 (C-14 and C-15), 31.9 (C-16), 24.7 (C-17), 14.1 (C-18).

Isolation of compound from methanol extract

The column was packed with 450 g of silica gel (100-120 mesh), and the methanol residue (25 g) was dissolved in a minimum quantity of the solvent and loaded into the column. Then, the column was eluted with a mixture of chloroform and methanol in the order of increasing polarity. Each fraction containing 20 ml was collected and monitored by TLC. The fractions 5-15 chloroform:methanol (8:2) yield single spot in TLC analysis and concentrated and yielded shiny white crystals of R_f value 0.23/cm named as Compound 2 (68 mg).

The fractions 27-33 eluted with chloroform:methanol (6:4) yields two major spots of 2.5 g and was further subjected to column chromatography over silica gel (100-120 mesh, 150 g) bed using petroleum ether with increasing amount of chloroform and methanol as eluent. Total 5 fractions were collected, the second fraction yielded single spot in TLC with R_f value of 0.56/cm and was concentrated under vacuum evaporator gave white powder named as Compound 3 (80 mg).

These compounds were identified by Fourier transform infrared, ^{1}H-NMR, ^{13}C-NMR, and EI-MS spectral techniques.

Compound 2

It was obtained as shiny white crystals (68 mg): m.p.134-138°C

^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm): 6.31 (*d*, 1H, J=9.6 Hz, H-3), 7.73 (*d*, 1H, J=9.6 Hz, H-4). 7.30 (*d*, 1H, J=8.5Hz, H-5), 7.36 (*dd*, 1H, J=8.2 Hz & 0.8 Hz, H-6), 7.63 (*dd*, 1H, J=2.4 Hz & 0.8 Hz, H-1'), 7.07 (*dd*, 1H, J=2.4 Hz and 0.8 Hz, H-2'),^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 160.78 (C-2), 114.15 (C-3), 144.45 (C-4), 123.80 (C-5), 108.70 (C-6), 157.39 (C-7), 116.96 (C-8), 145.8 (C-1'), 104.11 (C-2'), 148.55 (C-9), 113.96 (C-10).

Compound 3

The compound was obtained as white amorphous powder (80 mg); m.p. 102-106°C

IR vmax (KBr): 2926 (Aromatic-stretching) 2345 (CH Stretching), 1717 (C=O).

^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm): 6.39 (*d*,1H, J=9.3 Hz, H-3), 7.78 (*d*, 1H, J=9.6 Hz, H-4), 7.42 (*s*, H-5), 7.72 (*d*, 1H, J=2.4 Hz, H-2'), 6.84 (*d*, J=2.8 Hz, H-3'), 4.59 (*t*, H-1", J=5.4 Hz), 3.35 (*t*, H-2", J=5.5 Hz), 1.35 (*s*, H-4"), 1.26 (*s*, H-5").

^{13}C-NMR (100 MHz, $CDCl_3$) δ: 160.5 (C-2), 114.4 (C-3), 144.3 (C-4), 113.6 (C-5), 126.2 (C-6), 148.3 (C-7), 131.6 (C-8), 143.6 (C-9), 116.7 (C-10), 146.7 (C-2'), 106.8 (C-3'), 72.8 (C-1"), 61.4 (C-2"), 58.2 (C-3"), 24.6 (C-4"), 18.9 (C-5").

MASS (EI): m/z: 286.7[M]$^+$85, 105, 174, 202, 215, 252.

***In vitro* cytotoxicity by MTT assay**

MTT is a yellow water-soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 hr of incubation, 15 µl of MTT (5 mg/ml) in PBS was added to each well with test compound and incubated at 37°C for 4 hrs. The medium with MTT was then flicked off, and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using a microplate reader. The % cell inhibition was determined using the following formula.

% cell inhibition=100−Abs (sample)/Abs (control)×100.

Nonlinear regression graph was plotted between % cell inhibition and Log10 concentration, and IC_{50} was determined using GraphPad Prism software.

DNA profile and flow cytometric analysis

To investigate the effect of the compound 3 on the cell cycle distribution, cells (1×10^5 cells/ml) were treated with IC_{50} concentration of extract and cultured for 24 h. The treated cells harvested, washed with PBS and fixed in 75% ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40 µg/ml of propidium iodide (PI) and 0.1 mg/ml RNase and followed by 200 µl of Muse cell cycle reagent. The tubes were incubated for 30 minutes at dark and analyzed on Muse flow cytometer (Millipore, USA).

RESULTS

The three known compounds oleic acid (1) [12,13] from petroleum ether extract, isopsoralen (2) [14,15], and heraclenin (3) [16-18] were isolated from the methanol extract of *R. scabra,* and their identities were established by comparison of their spectroscopic data with reported values in the literature. The three compounds were isolated the first time in genus *Richardia* and *R. scabra* is given Fig. 1.

To evaluate the *in vitro* cytotoxicity of the Compound 3 isolated from *R. scabra,* because of scarce reports in the literature about the heraclenin on cancer cell lines, we employed MTT assay, which measures cell viability for screening the cytotoxicity of the natural products, and the results showed that the Compound 3 caused dose-dependent activity against treated HeLa cells (Human cervical cancer cell line) when treated with various concentrations (6.25, 12.5, 25, 50, and 100 µg/ml) with the IC_{50} value was 46.09 µg/ml. This is the first report of its kind to test the cytotoxicity of the heraclenin. The percentage of cell inhibition of the each concentration was given in the Fig. 2, and it induced cell cycle arrest in G0/G1 phase about (51.2%), S phase (30.2), and resulted in a decline in the percentage of G2-M phase.

DISCUSSION

The results indicated that the Compound 3 has potent cytotoxic in nature [19]. The effect of the Compound 3 on HeLa cells was investigated by flow cytometry after staining reveals the cell cycle phases. Results showed in Fig. 3. Cell cycle plays an important part in the processes of cell proliferation and growth as well as of cell division [20]. One of the hallmarks of cancer is the malfunction within the regulation of cell cycle, such that injured or mutated cells which are normally killed are allowed to progress the cell cycle [21]. Cell cycle arrest is an important way to inhibit the proliferation of cancer cells except for apoptosis and is considered as a potential approach for cancer treatment [22]. After 24 hr treatment of the Compound 3 with concentration (25 µg/ml) which induced cell cycle arrest in the G0-G1, S phase, and resulted in a decline in the percentage of cells in the G2/M phase. The results indicated that

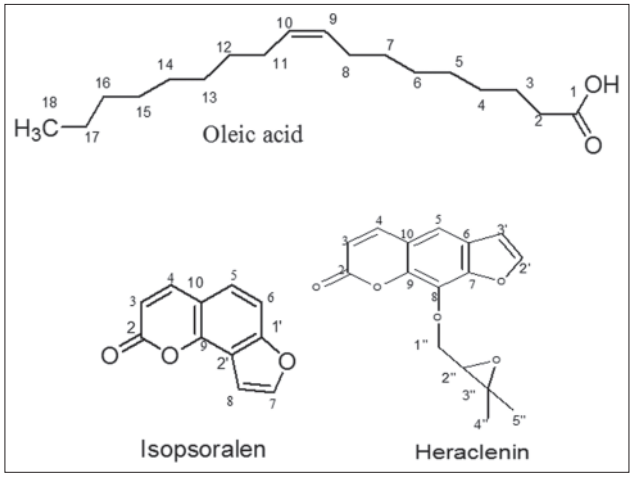

Fig. 1: Structures of the compounds 1-3

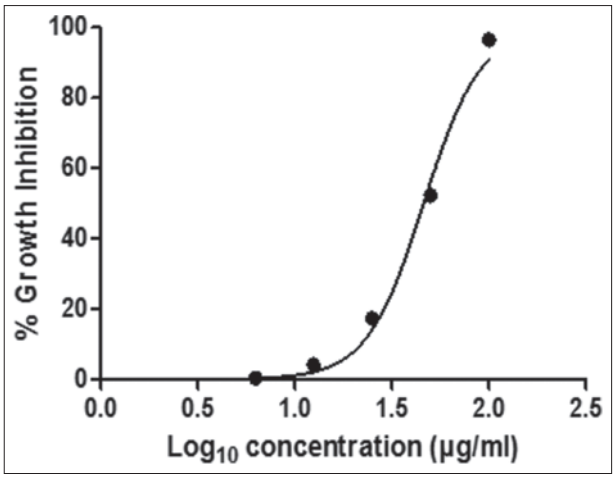

Fig. 2: % of cell inhibition of the compound 3 on HeLa cell line

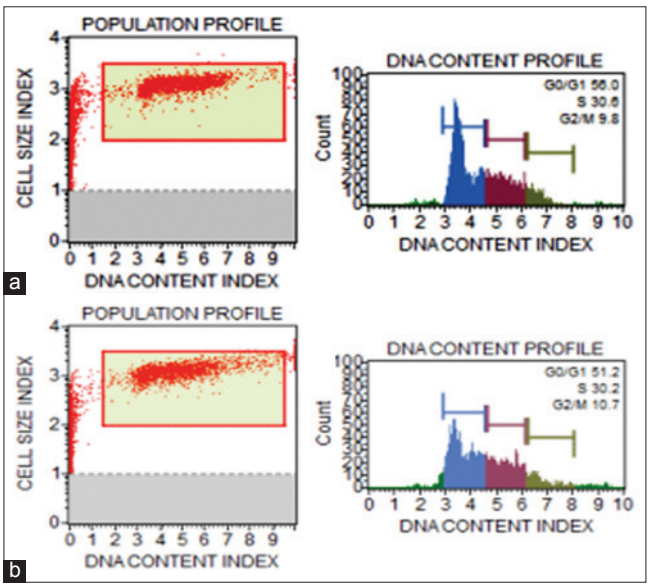

Fig. 3: Effect of compound 3 on the cell cycle distribution profile of the HeLa cells by flow cytometry. (a) Control. (b) Heraclenin at 25 µg/ml

the Compound 3 was very effective in preventing the cell replication by arresting the cells maximum in the G0-G1 region because cell cycle is an ordered process of events that occurs in four stages. During the two gap phases, G1 and G2, the cell is actively metabolizing but not dividing. In S (synthesis) phase, the chromosomes duplicate as a result of DNA replication. During the M (mitosis) phase, the chromosomes separate in the nucleus and the division of the cytoplasm (cytokinesis) occurs. There are checkpoints in the cycle at the end of G1 and G2 that can prevent the cell from entering the S or M phases of the cycle. Cells that are not in the process of dividing are in the G0 stage, which includes most adult cells. FACS analysis showed that the Compound 3 interferes with cell division, leading to inhibition of cell proliferation ultimately leading to cell death. These findings indicate that at the ranges of concentration studied, the antiproliferative effect of the Compound 3 on HeLa cells could be attributed primarily to the induction of apoptosis with less or no contribution from cell cycle arrest.

CONCLUSION

Two novel coumarins from methanol extract and oleic acid from petroleum ether extract were isolated first time in *Richardia*. The *in vitro* cytotoxicity of heraclenin was performed using HeLa cell line by MTT assay further cell cycle analysis provided insight into the potential mechanism of action of heraclenin in cancer cells; it arrests the cancer cells via an apoptosis pathway. Molecular docking studies are in progress.

REFERENCES

1. Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z. Medicinal plants in therapy. Bull World Health Organ 1985;63(6):965-81.
2. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. J Nat Prod 1997;60(1):52-60.
3. Azaizeh H, Saad B, Cooper E, Said O. Traditional arabic and islamic medicine, a Re-emerging health aid. Evid Based Complement Alternat Med 2010;7(4):419-24.
4. Coelho VP, Agra MF, Barbosa MR. Estudo farmacobotânico das folhas de *Tocoyena formosa* (Cham.& Schltdl.) *K. Schum* (Rubiaceae). Rev Bras Farmacogn 2006;16:170-7.
5. Mongrand S, Badoc A, Patouille B, Lacomblez C, Chavent M, Bessoule JJ. Chemotaxonomy of the Rubiaceae family based on leaf fatty acid composition. Phytochemistry 2005;66(5):549-59.
6. Pinto DS, Tomaz AC, Nunes XP, Mesquita RF, Almeida JR, Cavalcanti LS, *et al*. Antioxidant activity of *Richardia brasiliensis* gomes, XX Symposium on Medicinal Plants of Brazil X. São Paulo: International Congress of Ethnopharmacology, May 16 to September 19; 2008.
7. Tomaz ZC, Bezerra R, Nogueira SS, Pinto DS, Agra MF, Souza MF, Cunha EV. Chemical constiuents from *Richardia grandiflora* (Cham.& Schltdl.) Steud. (Rubiaceae). Brazil J Pharmacogn 2008;18(1):47-52.
8. Morais CE, Nogueira FB, Leite LA, Lucena FF, Figueredo GF, Guedes MM. Evaluation of antimicrobial and modulatory activity of the extract of *Richardia brasilensis* gomes. Indian J Tradit Knowl 2013;12(4):619-22.
9. Adekunle AA. Antifungal property of the crude extracts of *Brachystegia eurycoma* and *Richardia brasiliensis*. Nig J Nat Prod Med 2000;4:70-2.
10. Ayyanar M, Ignacimuth S. Medicinal plants used by the tribals of Tirunelveli hills, Tamilnadu to treat poisonous bites and diseases. Indian J Tradit Knowl 2005;4(30):229-36
11. Nath M, Dutta BK, Hajra PK. Medicinal plants used in major diseases by dimasa tribe of barak valley. Assam Univ J Sci Technol Biol Environ Sci 2011;7(1):18-26.
12. Matern U, Lue P, Kreusch P. In: Barton D, Nakanishi K, Meth-Cohn O, Sankawa U, editors. Biosynthesis of Coumarins. Oxford, UK: Elsevier Science Ltd.; 1999. p. 623-7.
13. Casset F, Hamelryck T, Loris R, Brisson JR, Tellier C, Dao-Thi MH, *et al*. NMR, molecular modeling, and crystallographic studies of lentil lectin-sucrose interaction. J Biol Chem 1995;270:25619-28.
14. Jacobsen NE. NMR-Spectroscopy Explained; Simplified Theory; Applications and Examples for Organic Chemistry and Structural Biology. New Jersey: John Wiley & Sons; 2007. p. 16-20.
15. Liu R, Li A, Sun A, Kong L. Preparative isolation and purification

of psoralen and isopsoralen from *Psoralea corylifolia* by high-speed counter-current chromatography. J Chromatogr A 2004;1057(1-2):225-8.
16. Lu H, Zhang L, Liu D, Tang P, Song F. Isolation and purification of psoralen and isopsoralen and their efficacy and safety in the treatment of osteosarcoma in nude rats. Afr Health Sci 2014;14(3):641-7.
17. Kocka AB. The analysis of furano coumarins in fruits of *Hercleum sibiricum* L. Acta Pol Phrm Drug Res 1999;56(5):399-401.
18. Sharma YN, Zaman A, Kidwai AR. Chemical examination of *Heracleum candicans*-I. Isolation and structure of a new furocoumarin-heraclenin. Tetrahedron 1964;20(1):87-90.18.
19. Wang XB, Li GH, Lei LI, Zheng LJ, Huang R, Zhang KQ. Nematicidal coumarins from *Heracleum candicans*. Wall Nat Prod Res 2008;22:8, 666-71.
20. Rastogi S, Pandey MM, Rawat AK. Determination of heraclenin and heraclenol in *Heracleum candican* D.C. by TLC. Chromatographia 2007;66(7-8):631-4.
21. Bagwell CB. Theoretical aspects of data analysis. In: Bauer KD, Duque RE, Shankey TV, editors. Clinical Flow Cytometry Principles and Application. Baltimore: Williams & Wilkins; 1993. p. 41-61.
22. Ross JS. DNA content analysis. In: DNA Ploidy and Cell Cycle Analysis in Pathology. New York: Igaku-Shoin; 1996.