International Journal of Plant Research 2012, 2(3): 57-60
DOI: 10.5923/j.plant.20120203.02

Cytotoxicity of Aporphine Alkaloids from the Roots of *Annona Reticulata* on Human Cancer Cell Lines

H M Suresh[1,*], B Shivakumar[2], S I Shivakumar[3]

[1] Department of Pharmacognosy,HKE Society's, College of Pharmacy, Gulbarga 585 105, India and Department of Biotechnology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India
[2] Department of Pharmaceutical Chemistry, BLDEA's College of Pharmacy, Bijapur 586 103, Karnataka, India
[3] Department of Pharmacology, HKE Society's, College of Pharmacy, Gulbarga 585 105, India

Abstract Phytochemical and pharmacological activities of Annona reticulata components suggest a wide range of clinical application in lieu of cancer therapy. Present study includes investigation of bioactive constituents from roots of Annona reticulata for cytotoxic properties against different cancer cell lines. Three aporphine alkaloids liriodenine (AAR-01), norushinsunine (AAR-02) and reticuline (AAR-03) isolated from the roots of Annona reticulata. The structures of the compounds were established by ^{1}HNMR, ^{13}CNMR and Mass spectral analysis. All the isolated compounds subjected for cytotoxicity evaluation against A-549, K- 562, HeLa, MDA-MB cancer cell lines and normal cell lines (Vero cells) by MTT assay. All the isolated compounds exhibited prominent dose-dependent cytotoxicity against all the cancer cell lines at dilutions 5, 10 and 20 µg/ml, whereas AAR-02 showed strong cytotoxicity against cancer cell lines with IC50 values ranging from 7.4 to 8.8 µg/ml. Simultaneously, the effect of all the isolated compounds against Vero cell lines was lower in comparison with the cancer cell lines. The prominent cytotoxicity of three aporphine alkaloids viz liriodenine (AAR-01), norushinsunine (AAR-02) and reticuline (AAR -03) may be because of isoquinoline moiety in their structures. The appearance of hydroxyl group at C-7 in AAR-02 could be favorable for increased cytotoxicity against cancer cell lines and lower cytotoxicity against Vero cell line seems that the aporphine alkaloids (AAR-01, AAR-02 and AAR-03) may be used as chemopreventive agents in cancer therapy.

Keywords Annona Reticulata, A-549, K- 562, Hela, Mda-Mb, Vero Cell Lines, Mtt Assay

1. Introduction

Aporphine is one of a class of quinoline alkaloids widely distributed among the plants of annonaceae family. Many potent relatives of this compound have been purified from the plants. They possess various pharmacological activities such as antiplatelet, antitumor, cytotoxic and antibacterial activities[1,2]. Cells of the body routinely grow and divide as and when the body needs them. This orderly process is disturbed when new cells form that the body was not needed and old cells don't die when they should, leading to infinite multiplication and spread within the body of abnormal forms of the body's own cells. These extra cells lump together to form a growth or tumour. Though cancer treatment by modern system of medicines using synthetic drugs is better, search for newer natural drugs continuesbecause of some complications like cell injury, bonemarrow depression, impair growth, sterility and hair loss associated with synthetic drugs[3]. Annona reticulata linn, commonly called as bullock's heart or raamphal plant, is widely distributed all over India and are tall, with many branches, bearing nutritious fruits. The leaves are used as insecticides, anthelmintic, styptic and also used externally as suppurant. The bark as a powerful astringent is used as antidysenteric and vermifuge. Root bark, leaves and stem possess isoquinoline alkaloids[4]. In our early report, we investigated the in vitro antiproliferative activity of ethanol extract of roots against A-549, k- 562, HeLa and MDA-MB human cancer cell lines[5]. In continuation of our research work on evaluation of bioactive constituents from the roots of this plant for cytotoxic properties, three aporphine alkaloids liriodenine (AAR-01), norushinsunine (AAR-02) and reticuline (AAR -03) were isolated and evaluated for cytotoxicity against A-549, K-562, HeLa, MDA-MB and Vero cell lines by performing MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay.

* Corresponding author:
srsh_hm@rediffmail.com (H M Suresh)
Published online at http://journal.sapub.org/plant

2. Materials and Methods

Plant Material

The roots of *Annona reticulata* were collected from local areas of north Karnataka and identified by Dr. Srinath Rao of

Botany department, Gulbarga University, Gulbarga. A botanically authenticated voucher specimen (HGUG No. 5007) has been deposited at the botany department herbarium of the Gulbarga University, Gulbarga, India.

Material and reagent

MTT assay kit was purchased from Roche Applied Sciences, Germany. A-549 (Human lung carcinoma) , K-562 (Human chronic Myelogenous Leukemia Bone Marrow), HeLa (Human Cervix) and MDA- MB (Human Adenocarcinoma Mammary Gland) and Vero (African green monkey kidney Normal cell) cell lines, free from any bacterial and fungal contamination were procured from NCCS , Pune. All the chemicals and reagents viz Propanol (Qualigens), Fetal Bovine Serum (Bioclot) and MTT dye were used for the study.

Extraction and isolation

Air dried and coarsely pulverized roots (2 kg) of *Annona reticulata* extracted with ethanol (95 %). The obtained extract was concentrated to dryness in a rota evaporator at room temperature to obtain ethanol extract (600 gm). About 50 gm of concentrated ethanol extract with 2× 250 ml petroleum ether (40 -60 ^{0}) to separate the fats. The complete defatted marc obtained after extraction was dried and made alkaline with 2× 250 ml ammonium hydroxide (NH_4OH) and filtered. The alkaline filtrate further fractionated with 4× 250 ml of chloroform. The organic fractions were pooled and together made alkaline with ammonium hydroxide (NH_4OH) washed with water, dried and the solvents removed to yield gummy residue (20 gm). It was partitioned with dichloromethane to remove the neutral components. This residue showed alkaloidal composition on TLC and further used for isolation of alkaloids by column chromatography (neutral alumina) using solvent system toluene: ethyl acetate: diethyl amine (70: 20: 10). Elution carried out gave a light brown colored substance. After washed with methanol, it was recrystallised from MeOH: acetone (1:1) to give (AAR-01) pale yellow mass (101 mg).Elution further carried with chloroform: diethylamine (9:1) resulted with another single alkaloidal compound. After removing solvent from the mixed fraction, a residue resulted as a mixture of alkaloid compound which was pale mass (477 mg) followed by rechromatography on alumina bed using solvent system petroleum ether-chloroform (7: 3) afforded two pure alkaloid compounds designated as AAR- 02 pale brown amorphous powder (116 mg) and AAR -03 yellow amorphous mass (99 mg).

Liriodenine (AAR -01) Pale yellow powder (C_2H_5OH), MS m/z: 274 $[M + H]^+$. ^{1}HNMR (400 MHz, $CDCl_3$) : δ 9.14 (1H,s, NH) ,9.24 – 7.0 (6H, m, aromatic protons), 6.8 (2H, d, O-CH_2-O). ^{13}CNMR (100 MHz, $CDCl_3$) δ 179.22 (C-6), 179.127, (C-5), 165.618 (C-2), 164.506 (C-1), 135.9 (C-13), 135.5 (C-12), 135.5 (C-15), 132.4 (C-9), 129.2 (C-14), 129.17 (C-7), 129.1(C-10), 125.6 (C-8), 124.5 (C-4), 119.6 (C-16), 118.3 (C-17), 104.3 (C-3), 101.2 (C-11)

Norushinsunine (AAR-02) Light brown solid mass (C_2H_5OH), MS m/z: 280$[M + H]^+$. ^{1}HNMR (400 MHz, $CDCl_3$) : δ 6.6 (2H, d, OCH_2) , 4.0 - 7.9 (11H, m, aromatic protons), 3.9 (1H, s, C-OH), 1.7 (1H, s, NH). ^{13}CNMR (100 MHz, $CDCl_3$) δ 139.5 (C-2), 138.5 (C-1), 137.8 (C-14), 129.0 (C-15), 128.9 (C-9), 125.8(C-8), 123.3 (C-12), 120.2 (C-10), 118.30 (C-17),115.0 (C-7), 111.0 (C-16), 100.0 (C-3),78.07 (C-11), 70.9 (C-6), 62.8 (C-13), 43.3 (C-5), 31.2 (C-4).

Reticuline (AAR-03) Yellow powder (C_2H_5OH), MS m/z: 329$[M + H]^{+1}$HNMR (400 MHz, $CDCl_3$): δ 6.6- 8.3 (6H, m, aromatic protons), 5.7 (2H, d, OH), 3.2 (6H, m, OCH_2), 1.2-2.8 (9H, m, protons of rings CH_2 and side chain CH_2).^{13}CNMR (100 MHz, $CDCl_3$) δ 161.3 (C-10), 160.30 (C-9), 158.4 (C-3), 150.7 (C-2), 149.2 (C-15), 129 (C-16), 119.8 (C-13), 117.4 (C-12), 103.7 (C-8), 89.6 (C-1), 79.6 (C-11), 79.3 (C-14), 59.2 (C-17 & C-18), 58.2 (C-6), 52.13 (C-19), 51.0 (C-7), 42.6 (C-4), 41.3 (C-5).

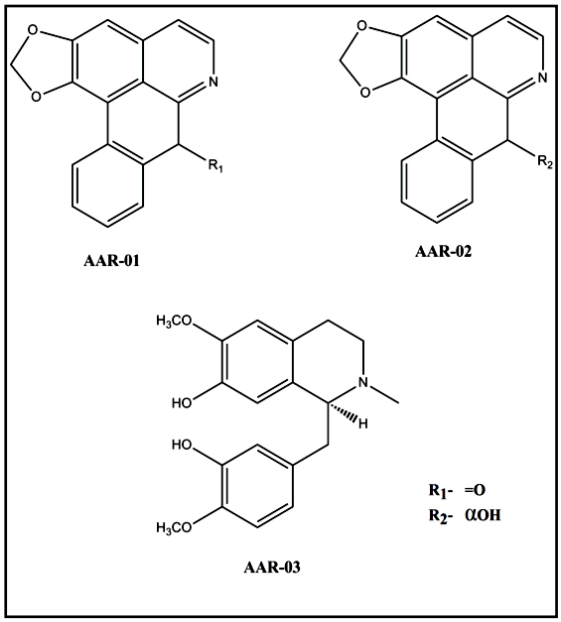

Figure 1. Structures of Isolated Aporphine Alkaloids

Evaluation of cytotoxicity by MTT assay

Both ethanol and aqueous extracts were evaluated for in vitro cytotoxicity study on MDA- MB - 435 (Human melanoma cells) and Vero (African green monkey kidney Normal cell) cell lines by employing MTT assay[6]. The monolayer cell culture was trypsinized and the cell count was adjusted to 3-lakhcells/ ml using medium containing 10% newborn calf serum. To each well of 96 well microtitre plates, 0.1ml of diluted cell suspension of different cell lines was added separately. After 24 hours, when the monolayer formed the supernatant was flicked off and 100 µl of AAR-01, AAR-02 and AAR-3 each at the concentration 5, 10 and 20 µg in buffered DMSO were added to the cells in microtitre plates separately and kept for incubation at 37ºC in 5 % CO_2 incubator for 72 hour and cells were periodically checked for granularity, shrinkage, swelling. After 72 hour, the sample dilution in wells was flicked off and 50µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37℃ in 5% CO_2 incubator. The

supernatant was removed, 50 µl of Propanol was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 490 nm[7].

Graph of absorbance against concentration of test substance was plotted and inhibitory effect (IC_{50}) was calculated as the drug concentration that is required to reduce absorbance to half that of the control, based on dose - response curve for different isolated substances. The reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of viability of cells. Absorbance values that are lower than the control cell lines reveals decline in the rate of cell proliferation. Conversely, a higher absorbance indicates an increase in the cell proliferation. Untreated microtitre plates of cell lines with only vehicle (0.3 % v/v DMSO in water) is considered as proliferative control.

The percent inhibition of cell proliferation by the isolated compounds was calculated based on formula [100–(Mean OD of individual test substance / Mean OD of control group)] × 100.

3. Results and Discussion

Table 1. Cytotoxicity of isolated compounds from roots of *Annona reticulata* against cancer cell lines

Treatment	Inhibitory effect (IC_{50}) on cancer cell lines (µg/ml)				
	A-549	*K-562*	*HeLa*	*MDA-MB*	*Vero*
AAR 01	18.2 (27.27)	16.2 (31.81)	12.0 (42.85)	12.2 (51.38)	26.0 (21.73)
AAR-02	8.8 (52.77)	7.4 (57.97)	7.6 (57.14)	8.4 (51.51)	20.0 (33.33)
AAR-03	19.8 (31.74)	15.8 (37.50)	17.4 (31.81)	13.0 (40.90)	22.0 (28.78)

Values in the parenthesis indicates percent growth inhibition

In the present study three aporhine alkaloids, liriodenine (AAR-01),norushinsunine(AAR-02) and reticuline (AAR-03) were isolated from the crude ethanol extract of *Annona reticulata*. The structures of all the compounds were elucidated by spectroscopic methods, including ^{1}HNMR, ^{13}CNMR and Mass spectra and depicted in [Figure 1]. However, they were identified by analysis of their spectral data and by comparison with those previously reported in the literature[8,9]. All the three isolated compounds (AAR-01, AAR-02 and AAR-03) exhibited dose – dependent cytotoxicity against different cancer cell lines at dilutions 5, 10 and 20 µg/ml. Mean cytotoxicity (IC_{50}) and percent inhibition of cell growth results of the isolated compounds against human cancer cell lines (A-549, K-562, HeLa , MDA- MB) and normal cells (Vero cell lines) are shown in the **table 1**. Among the isolated compounds, liriodenine (AAR-01) and reticuline (AAR-03) showed similar pattern of cytotoxicity against all cancer cell lines with IC_{50} values ranging from 12.0 to 18.2 and 13.0 to 19.8 µg/mlrespectively. Interestingly, norushinsunine (AAR-02) exhibited strongest cytotoxicity against A-549, K-562, HeLa and MDA- MB cancer cell lines with IC_{50} values ranging from 7.4 to 8.8 µg/ml. Cytotoxicity of test compounds on Vero cell line was constantly less at experimented dilutions as compared with cancer cell lines with IC50 values ranging from 20.0 to 26.0 µg/ml.

Compound AAR-01 (Liriodenine) ; light yellow powder with molecular formula $C_{17}H_9NO_3$, compound AAR-02 (Norushinsunine) ; light brown solid mass with molecular formula $C_{17}H_{15}NO_3$ (Reticuline) and compound AAR-03 ; yellow powder with molecular formula $C_{19}H_{23}NO_4$ as determined from their molecular ion peaks at m/z 274 $[M + H]^+$, 280$[M + H]^+$ and 329$[M + H]^+$ respectively. The structures of the isolated compounds based on spectral data conclusively revealed that they were aporphine alkaloids and identified as liriodenine (AAR-01), norushinsunine (AAR-02) and reticuline (AAR-03). Aporphine alkaloids are mostly discovered from annonaceae than other families[10]. Among the isolated compounds, liriodenine (AAR-01) is oxoaporphine of 7-substituted aporphines, norushinsunine (AAR-02) is 7- hydroxy aporphine and reticuline (AAR-03) isbenzyltetrahydroisoquinoline[11,12]. Prominent cytotoxic ity results of all the isolated compounds may be because of isoquinoline moiety in their structures. More pronounced cytotoxicity of AAR -01 than AAR-03 against all the cancer cell lines suggests that appearance of oxygenated substitution at C-7 in AAR-01 may be favorable for cytotoxicity. Remarkably, AAR-02 showed strongest cytotoxicity than AAR -01 and AAR-03 against all the cancer cell lines (A-549, K-562, HeLa , MDA- MB). This reveals that the hydroxyl group at C-7 with cis- configuration may be responsible for enhanced cytotoxicity. Another interesting point we have considered is that all the isolated compounds exhibited weaker cytotoxicity against normal cell lines (Vero cells). The isolated compounds - mediated cytotoxicity was more confined to the cancer cell lines rather than to the normal cell lines. Apoptosis is physiologically programmed process of active cellular self-destruction responsive to gene expression[13].The specific cytotoxicity of isolated compounds against cancer cell lines may be due to apoptosis in response to defective gene expression in cancer cell lines rather than the normal cell line.

5. Conclusions

In summary, the results of the present study provides convincing evidence that the aporphine alkaloids present in the roots of *Annona reticulata* may be one of the responsible compounds for cytotoxicity potential against cancer cell lines. The increased cytotoxicity of AAR- 02 reveals the importance of appearance of hydroxyl group at C-7 in 7-substituted aporhine alkaloids than others and can be used as a prototype for the development of new synthetic / semi-synthetic analogues for cancer treatment. However, the less prominent cytotoxic effect of isolated compounds on Vero cell line seems that these compounds (AAR-01,

AAR-02 and AAR-03) may be used as chemopreventive agents in cancer therapy. However, the precise mechanism by which they exert this effect needs further investigations.

ACKNOWLEDGEMENTS

We thank Dr. S. Appalaraju, Principal HKES's Matoshree Taradevi Rampure Institute of Pharmaceutical Sciences, Gulbarga for encouragement and support in research.

REFERENCES

[1] Stevigny C, Bailly C, Quetin- Leclerq. Cytotoxicity and antitumor potentialities of aporphinoid alkaloids. Cur Med Chem, Anti-cancer agents.2005; 5 (2):173-82

[2] Pyo MK, So H, Yun-Choi, Hong YJ. Antiplatelet activities of aporhine alkaloids isolated from leaves of Magnolia obovata. Planta Med. 2003; 69: 267-9

[3] Nadkarni K M. Indian Materia Medica. Mumbai: Popular Prakashan; 2002; 115-16

[4] Suresh HM, Shivakumar B, Hemalatha K, Heroor SS, Hugar DS. In vitro antiproliferative activity of *Annona reticulata* roots on human cancer cell lines. Phcog Res. 2011; 3: 9-12

[5] Xu L, Sun N, Kong J, Zhonqquo Zhong Yao Za Zhi. 1992; 17: 295-96

[6] Sanjay Patel S, Nirav Gheewala, Ashok Suthar , Anand Shah " In vitro Cytotoxicity activity of *Solanum nigrum* extracts against Hela and Vero cell lines", Int J Pharm and Pharmaceutical Sci. 2009, 1: 38-46

[7] Feleszko W, Mlynarczuk I, Olszewska D, Jalili A, Grzela T , Lasek W. Int J Cancer. 2002; 100: 111-118

[8] Uraiwan Songsiang, Chariya Hahnvajanawong and Chavi Yenjai. Cytotoxicity of chemical constituents from the stems of Dalbergia parviflora. Fitoterapia. 2011; 82:1169-74

[9] Zuo-Fu Guo, Xiao-Bing Wang, Jian-Guang Luo, Jun Luo, Jun-Song Wang and Ling-Yi Kong. A novel apophine alkaloid from Magnolia officinalis. Fitoterapia. 2011; 82:637-41

[10] Leboeuf M, Cave A, Bhaumik PK, Mukherjee B and Mukherjee R. The Phytochemistry of the Annonaceae. Phytochem. 1982; 21(12): 2783-2813

[11] Bhaumik PK, Mukherjee B, Juneau JP, Bhacca NS and Mukherjee R. Alkaloids from leaves of Annona squamosa. Phytochem. 1979; 18:1584-1586

[12] Francinete R, Regiane L, Cristiane L, Andersson , Maria B. Isoquinoline alkaloids from leaves of Annona sericea (Annonaceae). Biochem Syst and Eco. 2008; 36:804-806

[13] Deodhare S G. General Pathology and Pathology of systems. 2nd ed. Mumbai: Popular prakashan; 2002; 1572-74