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Phytochemical Potential of *Annona reticulata* Roots for Antiproliferative Activity on Human Cancer Cell Lines

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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), reticuline (AAR-03) and one acetogenin neoannonin (TAR -01) isolated from the roots of *Annona reticulata*. The structures of the compounds were achieved by ^{1}HNMR, ^{13}CNMR and Mass spectroscopic methods. 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 TAR -01 showed strong cytotoxicity against cancer cell lines with IC_{50} values ranging from5.8 – 6.9µg/ml. Simultaneously, the effect of all the isolated compounds against *Vero* cell lines was lower in comparison with the cancer cell lines. The better cytotoxicity of all the isolated compounds the appearance of hydroxyl group at C-7 in AAR-02 could be favourable for increased cytotoxicity against cancer cell lines among aporphine alkaloids and the presence of two hydroxyl groups adjacent to the tetrahydrofuran ring in TAR-01 may be responsible for enhanced activity. The lower cytotoxicity against *Vero* cell line seems that the isolated constituents AAR-01, AAR-02, AAR-03 and TAR -01 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 and acetogenins are polyketides of many carbons formed by chain extension to form tetrahydrofuran and lactone rings. Both are widely distributed among the plants of annonaceae family. Many potent relatives of these compounds have been purified from the plants. They possess various pharmacological activities such as antiplatelet, anti-tumor cytotoxic and antibacterial activities [1, 2]. Though cancer treatment by modern system of medicines using synthetic drugs is better, search for newer natural drugs continues because of some complications like cell injury, bone marrow 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 are also used externally as suppurant. The bark as a powerful astringent is used as antidysentric 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)and one acetogenins (TAR-01) 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.

2. Materials and Methods

2.1. 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

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the Gulbarga University, Gulbarga, India.

2.2. 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.

2.3. Isolation of Aporphine Alkaloids

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 (600gm). About 50 gm of concentrated ethanol extract with 2× 250 ml petroleum ether (40 -60°) 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 re-chromatography 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).

2.4. Isolation of Acetogenin

About 50 gm of ethanol extract partitioned with ethyl acetate subjected for column chromatography [F-254, mesh 60] using solvent system with increasing order of polarities of n: hexane, ethyl acetate and methanol. This resulted with acetogenin mixture followed by re-chromatography with n: hexane: acetone (3:1) solvent system yielded a single acetogenin**TAR-01** compound (210 mg) based on TLC confirmation (benzene: acetone; 8:2).

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, $CDCl_3$) δ161.3 (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).

Neoannonin **(TAR-01)** Pale waxy solid residue (C_2H_5OH), MS m/z: 578 $[M + H]^{+}$,^{1}HNMR (400 MHz, $CDCl_3$): δ 7.2 (1H,s,C= CH), 4.76 (1H, m,CH-CH_3 at H34), 4.73 (2H, m , $2CH_2$ protons, H-17, H-18), 3.7 – 3.68 (2H, m, $2CH_2$ protons, H-14, H-21), 3.63 (2H,m,CH-OH protons, H-13,H-22), 2.62 - 0.8 (46H, m,23xCH_2 protons), 1.9 – 1.6 (4H, m, methylene protons, H-15, 16,19,20) , 1.3 (3H, m, O-C-CH_3). ^{13}CNMR (100 MHz, $CDCl_3$) δ178.1 (C-1), 155.8 (C-33), 130.15 (C- 2), 78.1(C- 17), 78.2(C-18), 77.3 (C-14), 77.3(C-21), 76.5(C-13), 76.5(C-22), 76.3(C-34), 39.6(C- 26), 39.5(C-30), 37.9(C-20), 37.9(C-15), 33.0(C- 12), 31.8(C-23), 31.4(C- 25), 29.7(C-5), 29.64(C- 6), 29.60(C-7), 29.4(C- 8), 29.2(C-9), 29.1(C- 10), 29.0(C-16),29.0(C-19), 28.67(C-31), 27.3(C-27), 27.3(C- 28), 27.1(C- 29), 25.5(C- 4), 25.3(C-31), 24.2(C- 3), 22.6(C- 11), 18.37(C- 24), 14.0(C- 32).

2.5. 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 micro titre 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 100µl of AAR-01, AAR-02 and AAR-3 each at the concentration 5, 10 and

20µg in buffered DMSO were added to the cells in micro titre plates separately and kept for incubation at 37°C 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 micro plate 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 micro titre 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.

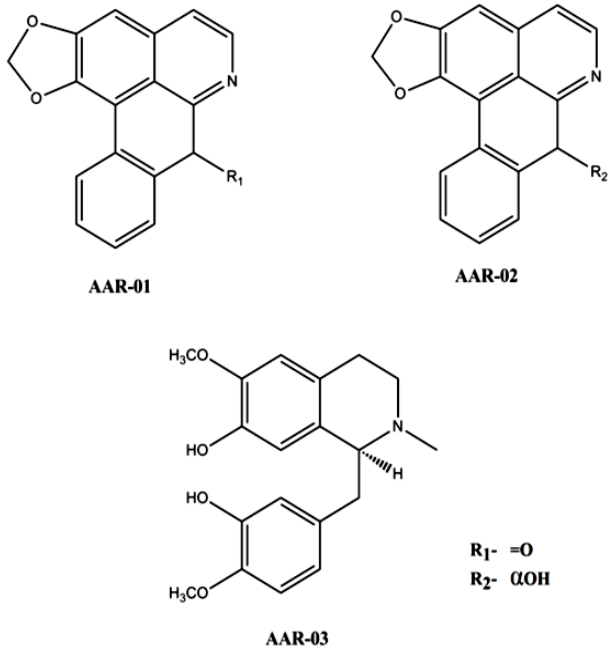

Figure 1. Structures of Isolated Aporphine Alkaloids

3. Results

In the present study three aporhine alkaloids, liriodenine (AAR-01), norushinsunine (AAR-02), reticuline (AAR-03) and one acetogenin, neoannonin (TAR-01) were isolated from the crude ethanol extract of *Annona reticulata*. The structures of aporhine alkaloids and acetogenin were elucidated by spectroscopic methods, including ^{1}HNMR, ^{13}CNMR and Mass spectra and depicted in Figure 1and 2 respectively. However, they were identified by analysis of their spectral data and by comparison with those previously reported in the literature[8,9].All the isolated compounds (AAR-01, AAR-02, AAR-03 and TAR -01)exhibited dose – dependent cytotoxicity against different cancer cell lines at dilutions 5, 10 and20 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, neoannonin (TAR-01) exhibited strongest cytotoxicity with IC_{50} values ranging from 5.8 to 6.9 µg/ml against *A-549, K-562, HeLa* and *MDA- MB* cancer cell lines. Interestingly, among the aporphine alkaloids, norushinsunine (AAR-02) showed better cytotoxicity 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 IC_{50} values ranging from 13.8 to 26.0 µg/ml.

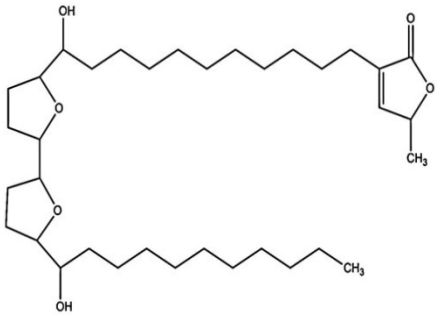

Figure 2. Structure of TAR-01

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)	20.00 (33.33)
AAR-03	19.8 (31.74)	15.8 (37.50)	17.4 (31.81)	13.0 (40.90)	22.0 (28.78)
TAR-01	5.8 (65.07)	6.9 (63.63)	6.4 (62.27)	5.9 (63.88)	13.8 (39.68)

Values in the parenthesis indicates percent growth inhibition

4. Discussion

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 $C_{17}H_{15}NO_3$, compound AAR-03(Reticuline); yellow powder with molecular formula $C_{19}H_{23}NO_4$

and compound TAR-01 (Neoannonin); Pale waxy solid residue with molecular formula $C_{35}H_{62}O_6$, as determined from their molecular ion peaks at *m/z* 274 $[M + H]^+$, 280$[M + H]^+$, 329 $[M + H]^+$ and 578 $[M + H]^+$ respectively. The structures of the isolated compounds based on spectral data conclusively revealed that they were liriodenine (AAR-01), norushinsunine (AAR-02), reticuline (AAR-03) and neoannonin (TAR-01). Aporphine and acetogenin compounds are mostly discovered from annonaceae family[10].Among the isolated compounds, liriodenine (AAR-01) is oxoaporphine of 7- substituted aporphines, norushinsunine (AAR-02) is 7- hydroxyaporphine and reticuline (AAR-03) is benzyltetrahydroisoquinoline[11,12]. Further, neoannonin (TAR-01) is dihydroxybistetrahydrofuran fatty acid lactone. Prominent cytotoxicity results of the isolated aporphine alkaloids may be because of isoquinoline moiety in their structures. More pronounced cytotoxicity of AAR -02 among aporhine alkaloids against all the cancer cell lines suggests that appearance of hydroxyl group at C-7 with *cis*-configuration may be responsible. Remarkably, the strongest cytotoxicity of TAR-01compared to other compounds may be because of presence of two hydroxyl groups adjacent to tetra hydro furan ring. The 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. This indicates that the specific cytotoxicity may be due to apoptosis inducing ability of isolated compounds in response to defective gene expression in cancer cell lines rather than the normal cell line because apoptosis is physiologically programmed process of active cellular self destruction responsive to gene expression [13].

5. Conclusions

In summary, the results of the present study provide convincing evidence that the aporphine alkaloids and acetogenins present in the roots of *Annona reticulata* may be responsible compounds for cytotoxicity potential against cancer cell lines. The prominent cytotoxic compounds TAR-01 (neoannonin) and AAR-02 (norushinsunine) 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 may be used as chemopreventive agents in cancer therapy but the precise mechanism by which they exerts this effect needs further investigations.

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