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CONTENTS

In vitro cytotoxic and human recombinant caspase inhibitory effect of *Annona reticulata* leaves

Research is being carried out throughout the globe to find a lead compound which can block the development of cancer in humans. Nature has always been a great contributor towards this goal. Plant-derived natural products such as flavonoids, terpenoids and steroids have received considerable attention due to their diverse pharmacological properties, which include cytotoxic and chemopreventive effects.[1]

Caspases (cysteine-dependant aspartate specific proteases) are involved in apoptosis (programmed cell death), inflammatory responses and cellular proliferation and differentiation. The initiator and executioner caspases have gained considerable importance in drug research as caspase induction is known to have role in cancer cell death, while inhibition of caspases can be used in the therapy in acute cellular degenerative diseases such as ischemic damage and Alzheimer's disease.[2]

Annona reticulata, belonging to the family Annonaceae, is a medium-size plant found in countries with a tropical climate, such as India. The acetogenins present in the bark, fruit and leaves are known to have anticancer properties.[3-6] The present study aimed to determine the *in vitro* cytotoxicity as well as the recombinant caspase inhibitory effect of a methanol extract of *A. reticulata* leaves.

The leaves of *A. reticulata* were collected during its fruiting season from Bangaon, West Bengal, India and identified by the Botanical Survey of India, Howrah, India. A voucher specimen (PG-212) was retained in our laboratory for further reference. The leaves were dried under shade and powdered in a mechanical grinder. The powdered material was extracted successively with petroleum ether (60-80°C), chloroform and methanol, using Soxhlet apparatus. The methanol extract was concentrated *in vacuo* and kept in a vacuum dessicator for complete removal of the solvent. The yield was 7.5% w/w with respect to dried powder. Preliminary qualitative analysis of the methanol extract showed the presence of alkaloid, flavonoid, tannin, gum, saponins and triterpenes. The methanol extract of *A. reticulata* (MEAR) was used for the present study.

MEAR was dissolved in dimethyl sulfoxide (DMSO) as 25 mg/ml stock and further dilutions were made in DMSO to get lower concentrations as required for the different experiments. However, in all types of experiments, the final DMSO concentration was limited to 1%.

MEAR was investigated for cytotoxic property against Caco-2 (human colorectal adenocarcinoma), Hep G2 (human hepatocellular carcinoma) and HEK (human kidney carcinoma) cell lines, which were obtained from the National Center for Cell Sciences, Pune, India. Hep G2 and HEK cultures were maintained in DMEM at pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum (PAN Biotech GmbH, Germany), ciprofloxacin 5 μg/ml and gentamycin 40 μg/ml; Caco-2 was maintained with the above medium containing 10 μg/ml human transferrin (Sigma, USA). The cells were grown in 25 cm^2 tissue culture flasks (Tarsons Products Pvt. Ltd, Kolkata, India), maintaining a humidified atmosphere with 5% CO_2 at 37°C until confluent. The cells were dissociated with 0.2% trypsin and 0.02% EDTA in phosphate-buffered saline solution and resuspended in the respective media as stated above. Cells were plated at 20,000 cells per well in 96-well microtiter plates (Tarsons Products P Ltd, Kolkata, India) and incubated for 24 h at 37°C, with 5% CO_2 in a humidified incubator. Cells were then exposed to different concentrations (20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 μg/ml) of MEAR in triplicate. Doxorubicin 10 μM (Sigma, USA) was used as standard, while control wells received only maintenance media. Cells were incubated for 72 h and cell viability was determined using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).[7]

For the caspase inhibition assay all the chemicals were purchased from Sigma, USA. Initiator caspase (caspase-9) and executioner caspases (caspase-3 and 6) were studied. The specific substrates were Ac-LEHD-AFC, Ac-DEVD-AFC and Ac-VEID-AFC, respectively, while the standard inhibitors were Z-LEHD-FMK, Ac-ESMD-CHO and Z-VEID-FMK, respectively.

The caspases preferentially cleave their specific substrates, resulting in release of free 4-amido-4-trifluoromethyl coumarin (AFC), which can be measured by fluorometry.[8] The reaction buffers were prepared and the analyses performed as per the enzyme manufacturer's recommended procedure. In brief, 10 ng of caspase-3, 25 ng of caspase-6 or 500 ng of caspase-9 (in the respective assay buffers) along with MEAR (5 and 10 μg/ml) or the standard inhibitors (10 μM) were incubated at 37°C for 10 min, with shaking for allowing the extract or inhibitor to interact with the caspases. To obtain a signal-to-noise ratio at least 3 or more, different amounts of enzymes were used, as they differ in potency[2] Then the substrate solutions were added to the final concentrations of 20, 50 and 200 μM, respectively, for caspases 3, 6 and 9; this was followed by incubation at 37°C and shaking for 1 h. Fluorescence of free AFC was measured at excitation 370 nm and emission 480 nm. Percent caspase inhibition was calculated assuming 100% caspase activity with the wells having caspase and substrate but no inhibitor/MEAR[8] The experiments were performed thrice and the results averaged.

MEAR did not show cytotoxicity against HEK up to a concentration of 20 μg/ml, while it showed good dose-dependant cytotoxicity against both Caco-2 and Hep G2 cell lines [Table 1].

For human recombinant caspase inhibition assay, MEAR failed to show promising inhibition against caspase-3, while MEAR at 5 and 10 μg/ml exhibited 56.02 and 66.64% inhibition against caspase-6 and 76.35 and 87.03% inhibition against

Table 1

Cytotoxic activity of Methanol extract of *A. reticulata* on different human cancer cell lines

MEAR (µg/ml)	*% Inhibition**		
	Caco-2	*Hep G2*	*HEK*
20	46.99 ± 1.20	35.34 ± 3.35	1.56 ± 3.88
10	41.16 ± 0.74	24.74 ± 0.42	0.60 ± 2.47
5	35.91 ± 1.66	18.70 ± 1.62	−2.58 ± 7.64
2.5	30.99 ± 2.77	14.25 ± 0.29	−1.57 ± 3.35
1.25	14.33 ± 0.56	5.90 ± 1.41	1.58 ± 8.55
0.625	12.03 ± 0.50	−1.47 ± 3.50	−0.81 ± 4.78
0.3125	8.77 ± 0.69	−1.48 ± 0.53	0.11 ± 9.14
Doxorubicin 10 µM	99.92 ± 0.08	99.98 ± 0.01	99.60 ±0.22

*Average of three independent determinations, 3 replicates; values are mean ± SEM. MEAR - Methanol extract of *A. reticulata* leaves

Table 2

Inhibition of human recombinant caspases 3/6/9 by methanol extract of *A. reticulata*

	*% inhibition of human recombinant caspases**		
	Caspase-3	*Caspase-6*	*Caspase-9*
MEAR 5 µg/ml	5.50 ± 1.82	56.02 ± 2.21	76.35 ± 1.46
MEAR 10 µg/ml	4.93 ± 0.95	66.64 ± 1.12	87.03 ± 0.64
Ac-ESMD-CHO 10 µM	90.40 ± 0.99	-	-
Z-VEID-FMK 10 µM	-	99.44 ± 0.53	-
Z-LEHD-FMK 10 µM	-	-	92.64 ± 1.19

*Average of three independent determinations, 3 replicates; values are mean ± SEM. MEAR - Methanol extract of *A. reticulata* leaves

caspase-9, respectively [Table 2].

The results from the present study show that the extract is cytotoxic to colon and liver cancer cells and may possess anticancer activity.

Induction of caspase-3 is a common phenomenon in cancer cell death; MEAR did not show any inhibition, indicating that it has no action in preventing cancer cell death. However, marked inhibition of caspase-6 by MEAR is observed. Reports indicate that caspase-6 is effective in lamin B_1 proteolysis and keratin cleavage. But evidence for its importance in cancer cell apoptosis is lacking.[2] Caspase-6 is known to cause apoptosis in Alzheimer's disease, where its inhibition may produce a beneficial effect in such neurodegenerative diseases.

Similarly, caspase-9 inhibition may also indicate that the extract has a role in the therapy of cellular degenerative diseases. As the extract contains different type of phytochemicals it may be that the phytochemical(s) that show cytotoxicity is/are different from that which cause caspase-6/9 inhibition.

The results justify further investigation to identify the active cytotoxic principle and its inductive/inhibitory effect on different types of caspases.

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