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Guanidinium Rich Dendron- Appended Hydnocarpin Executes Superior Anti-Neoplastic Effects Through Caspase Mediated Apoptosis

Bincy Mariyam Mathai[1], Manu M. Joseph[1], Santhi Maniganda[1,2], Jyothi B. Nair[1,2], Arya J.S[1]., Varsha Karunakaran[1,2], K .V. Radhakrishnan*[1,2], Kaustabh Kumar Maiti*[1,2]

Medicinal plants have truly demonstrated their potential as a repository of active biomolecules with promising therapeutic potential and represent an important pool for the identification of novel drug leads. *Hydnocarpus wightiana* Blume is a popularly known medicinal plant and its acetone extract of the seed demonstrated superior free radical scavenging property with a high total phenolic and flavonoid content. Hydnocarpin (Hy), which has been isolated and purified from the acetone extract, promotes moderate cytotoxicity on cancer cells. In an attempt to increase the efficiency of Hy as an anticancer agent, chemical coupling with a highly efficient, non-toxic cell penetrating guanidinium rich poly-(propylene imine) dendron (G8) was attempted. The resultant modified construct (Hy-G8) executes superior cytotoxicity preferentially on cancer cells through the induction of apoptosis mediated by caspases. The hybrid construct was also found to be a promising anti-metastatic agent. Therefore , Hy-G8 exhibited primarly as a hit compound which requires extensive interdisciplinary approches and legitimate engineering to accomplish a futurastic lead candidate in cancer chemotherapy.

Introduction

A copious of natural resources[1] for restorative uses exist around the world, of which numerous have not yet been successfully tapped for the development of conceivable drug candidates. Despite extensive research on compounds of natural origin to produce new drug substances occurs, research specifically aimed at naturally derived medicines to optimize dosages for the intended route of administration and to design the most effective dosage forms, has become a greater challenge. Many of the traditional medicines have real, beneficial effects and extracts of these crude combinations lead to the discovery of their active ingredients and eventually to the development of modern chemically pure drugs. In general, phytochemicals identified from traditional medicinal plants are presenting exhilarating opportunities for the development of new drug candidates. Several studies revealed that chemically modified natural compounds, especially from medicinal plants[2] generated better response as candidate lead molecule in the field of cancer therapy. Cancer[3] continues to be one of the major causes of death worldwide with only modest progress being made in reducing the morbidity and mortality of the disease. Phytochemicals from extracts of roots, bulbs, barks, leaves, stems and others parts of plants have shown promising potential as anti-cancer drugs or for serving as lead compounds in the synthesis of new drugs. They are often utilized as traditional medicines in the form of homemade tinctures, teas or crude extracts, but accompanied by drawbacks, including batch to batch variation in preparation methods and hence the chemical composition.

Hydnocarpus wightiana Blume[3-6] (Flacourtiaceae), is widely cultivated in Southeast Asia mainly in China, Taiwan, Indonesia, Malaysia, and Thailand. Four species occur in India, which grows up to 50 feet and is commonly known as Chaulmoogra with globose fruits containing 15-20 seeds. Chaulmoogra oil, extracted from seeds is traditionally being effectively used in the treatment of leprosy, chronic skin affections, ophthalmia, and for dressing of wounds and ulcers. The plant has also been listed with anti-obesity activity and the seed[3-7] oil also used in traditional applications in rheumatism, sprains and bruise, sciatica and chest affections. The seeds possess high medicinal value because they are reported to contain array of therapeutically major classes of phytochemicals such as triterpenoids, flavones, aglycones, glycosides and a range of fatty acids and esters. Seed extracts yield compounds structurally related to the flavanolignans[6,8] namely hydnowightin, hydnocarpin, neohydnocarpin and luteolin. Even though all the five flavanolignans demonstrated antidiabetic, antioxidant activities and moderate cytotoxicity against few cancer cell lines[9,10,] hydnocarpin proved to be the

[a.] 1.Chemical Sciences & Technology Division (CSTD), Organic Chemistry Section, CSIR- National Institute for Interdisciplinary Science & Technology (CSIR-NIIST), Industrial Estate, Pappanamcode, Thiruvananthapuram- 695019, Kerala, India
[b.] 2. Academy of Scientific and innovative research (AcSIR), New Delhi.

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most promising agent[11,4] with significant cytotoxicity against cancer cells of various origins.

Given their phenomenal concoction assorted qualities and novel modes of action, natural products keep on working as lead compounds in many drug discovery programs. However, to keep up with the on-growing demand for new therapeutic agents, new strategies for enhancing their therapeutic potential are needed. Semi-synthetic modifications of natural products aimed at enhancing their biological properties or the total synthesis of analogues serve as great starting points for generating molecules with greater bio-availability, bio-distribution and rapid bio-response. Molecular transporters containing octaguanidine residues enhanced the cell penetrating ability most effectively and delivered a variety of cargos[12] As of date our group developed a new guanidinium rich poly- (propylene imine) dendron based molecular transporter[13], which was observed to be non-toxic with higher cellular uptake efficiency contrasted with Arg-8-mer in various cancer cells. The high cell penetrating ability of the G8-dendron encourages us to develop a new hydnocarpin appended guanidium rich dendron (Hy-G8). The new synthetic derivative demonstrated excellent water solubility and improved cytotoxicity via caspase mediated apoptosis in cancer cells. The systematic approach adopted in the current study was illustrated in fig 1.
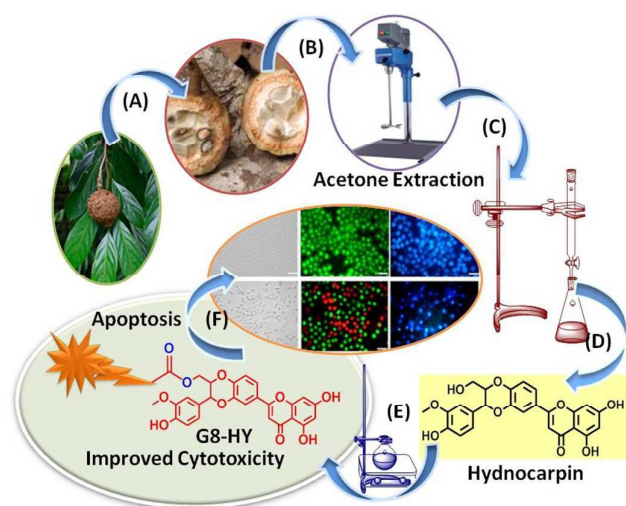

Fig 1: Thematic representation of Hy-G8 derivative synthesis. (A) Separation of seeds from *Hydnocarpus wightiana* Blume, (B) acetone extraction of seed, (C) column chromatographic separation, (D) isolation and purification of Hydnocarpin, (E) synthetic transformation with G8-PPI Dendron and (F) biological evaluation of Hy-G8.

Experimental

2.1. Materials and methods: IR spectra were recorded on Bruker Alpha FT-IR spectrometer.^{1}H NMR spectra were recorded at 500 MHz using deuterated methanol (CD_3OD) as the solvent on Bruker AV 500 spectrometer. Tetramethylsilane (TMS) was used as the internal standard and chemical shift

values are expressed in δ-scale in units of parts per million (ppm) and coupling constants (J) in Hz. Mass spectra were recorded using Thermo Scientific Exactive mass spectrometer under ESI/HRMS at 61800 resolutions. Analytical thin layer chromatography was performed on Merck 60 F_{254} silica gel plate (0.25mm thickness), visualization was done with UV light (254nm and 365nm) and column chromatography using Merck silica gel (100-200 mesh). Chromatography was carried out using varying polarities of hexane-ethyl acetate mixtures as solvent. Other chemicals were purchased from Sigma Aldrich and Merk.

2.2. Preparation of extracts: The seeds of *Hydnocarpus wightiana* Blume were thoroughly cleaned and dried in air oven maintained at 40^0C for three days. It was then thoroughly powdered and weighed approximately 750g. The powdered material was first extracted using hexane in a mechanical stirrer (1.5L) three times at room temperature. Further it was extracted using chloroform (1.5L) three times and finally with acetone (1.5L) three times at room temperature. The total extracts were concentrated under reduced pressure using Buchii rotary evaporator. This yielded about 400g of the crude hexane extract, 25g of chloroform and 45g of acetone extract. Based on the observation in TLC, the acetone extract was selected for the isolation of compounds.

2.3 Antioxidant Activity: Earlier studies revealed that the hexane and chloroform extracts of *Hydnocarpus wightiana* Blume does not have any appreciable free radical scavenging activity and acetone extract posses anti oxidant activity against selected free radicals[14]. This encouraged us to examine the free radical scavenging property of acetone extract against experimentally generated free radicals by established antioxidant assays.

2.3.1 DPPH radical scavenging assay: The radical scavenging effects of the acetone extract on a 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical were estimated[15]. Various concentrations of samples and standards (3 mL) were mixed with 1 mL of 0.1mM of DPPH and the mixture were shaken vigorously and allowed to incubate at room temperature (25 ± 3^0C) for 30 min. The scavenging activity was quantified spectrophotometrically at 507 nm against 95% ethanol as blank. Ascorbic acid was used as the standard and the percent DPPH scavenging effect was expressed as percent inhibition from the given formulae:

% inhibition of DPPH radical = $[A_0 - A_1/A_0] \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the sample/standard.

2.3.2 FRAP (Ferric reducing antioxidant power) assay: This assay measures the total antioxidant capacity of the compound and the procedure was conducted according to Benzie and Strain[16] followed by modification. The working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 m M 2, 4, 6-tripytidyl-s-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio prior to use and heated to 37^0C in a water bath. Three milli litre (3 mL) of FRAP reagent was added to 100 µL of various concentrations of samples and standards. Absorbance was measured at 593 nm at 0 min and

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again after 90 min. Ascorbic acid was used as the standard and changes in the absorbance after 90 min from initial reading were compared and percent inhibition was calculated as described.

2.3.3 Hydroxyl radical scavenging assay: Hydroxyl radical scavenging activity was measured comparing deoxyribose and acetone extract for hydroxyl radical generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction)[17]. The reaction mixture contained (1 mL) 100 µL 2-deoxy-2-ribose (28mM in 20mM phosphate buffer, pH 7.4), 500 µL of different concentrations of samples and controls, 200 µL 1.04 mM $FeCl_3$, 100 µL 1 mM H_2O_2 and 100 µL ascorbic acid, was incubated at 37^0C for 1 hr. Thiobarbituric acid (1%) and trichloroacetic acid (2.8%) were added and incubated at 100^0C for 20 min, after rapid cooling the absorbance was measured at 532 nm. Alpha tocopherol was used as the standard and the percentage inhibition was calculated.

2.3.4 Superoxide radical scavenging assay: The scavenging activity towards superoxide anion radicals was measured by the method of Liu et al[18]. Superoxide anions were generated in a non-enzymatic phenazinemethosulfatenicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitrobluetetrazolium (NBT). Superoxide anion was generated in 3 mL of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 mL of NBT (300µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 mL of various concentrations of samples and standards. The reaction was initiated by adding 0.75 mL of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature (25 ± 3^0C), the absorbance at 560 nm was measured. Alpha tocopherol was used as the positive control and percent inhibition was calculated.

2.4 Total Phenolic Content (TPC): Total phenolic content[19,20] (TPC) of the acetone extract was determined using the Folin-Ciocalteu reagent. Briefly, 0.5 mL of freshly prepared Folin-Ciocalteu reagent in distilled water (1:2), was added to 1.0 mL extracts of different concentrations (200-1000 µg/mL) and mixed thoroughly and then neutralized with 1 mL saturated sodium carbonate solution and made up to 10 mL with distilled water. After 2h, the absorbance of the resulting solution was measured at 764 nm using a spectrophotometer. The TPC was determined by plotting the Gallic acid calibration curve (20 to100 µg/mL) and expressed as Gallic acid equivalents (mg GAE / 100g dry weight of sample).

2.5 Total Flavonoid Content (TFC): About 1 mL of acetone extract (1mg/mL) was made up to 5 mL using distilled water and 0.3 mL of 5% (w/v) $NaNO_2$ was added to it and kept for 5 min. After 5 min. 0.3 mL of 10 % (w/v) $AlCl_3$ was added to form a flavonoid- aluminum complex. After 6 min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL using distilled water. The solution was mixed well again and the absorbance was measured against a reagent blank, at 415 nm. Blank was prepared by adding all reagents except $AlCl_3$. Distilled water was added in place of $AlCl_3$ in the blank. The TFC was determined by plotting the quercetin calibration curve

(20 to100 µg/mL) and expressed as milligrams of quercetin equivalents (mg QE/ 100 g of dry weight of sample)[21]

2.6 Isolation of compounds and its chemical modification: Based on the observation in TLC, acetone extract was used for the isolation of compounds. A fatty acid rich fraction was isolated from hexane extract. The acetone extracts which showed good anti-oxidant activity, total phenolic and good flavonoid content was selected for further in depth studies. It was subjected to column chromatography using silica gel (100-200 Mesh), the column was eluted with chloroform and increasing amount of methanol which yielded 574 different fractions. From 354-407 fractions, we have isolated our target compound hydnocarpin in good yield

2.6.3 Hydnocarpin (Hy):IR (KBr, υmax, cm^{-1}):3186,2961, 1664, 1625, 1518, 1437, 1383, 1271, 1164, 895, 800; ^{1}H NMR (500 MHz, CD_3OD):δ 8.65 (s, 1H), 7.55 (d, J= 8.2, 1H), 7.09 (d, J= 8.7, 1H), 7.05, (s, 1H), 6.94 (d, J= 8.7, 1H), 6.88 (d, J = 8.0, 1H), 6.66 (s, 1H), 6.49 (s, 1H), 6.25 (s,1H), 4.18-4.16 (m, 1H), 3.90 (s, 3H), 3.76 (d, J =10.8, 1H), 3.54 (dd, J1 = 12.1, J2 = 3.95, 1H), 3.46(s,1H)(SI Fig 1).; ^{13}C NMR(125 MHz, CD_3OD) δ181.7, 164.2, 162.8, 161.3, 157.3, 147.6, 147.1, 147.0, 143.6, 126.9, 123.6, 120.5, 119.8, 117.5, 115.3, 114.7, 111.6, 103.8, 103.7, 98.9, 94.0, 77.9, 76.3, 60.0, 55.6; HR-MS (m/z): 465.1183 (M+1 peak).

2.6.5 Hypnocarpin Guanidium derivative (Hy-G8): To the solution of hydnocarpin (15 mg, 0.3232 mmol) in dry DMF (3 mL) Boc protected G8- PPI (92 mg, 0.03232 mmol, prepared using previous reported procedure from our group (SI Section 2)[13], catalytic amount of DMAP (base) and EDC (7.33 mg, 0.03879 mmol) were added. The mixture was stirred at room temperature for 24 h. Completion of the reaction was monitored through TLC and the volatile components were removed under rotary evaporator and the Boc protected product formed was purified by column chromatography. Ethyl acetate (4mL) saturated with gaseous HCl was added to a solution Hy-Boc-G8 in ethyl acetate (1mL). The reaction mixture was stirred for 4h then the solution was concentrated and the residue was washed with ethyl acetate to remove less polar impurities. The residue was dried and purified using MPLC on Supelclean[TM] LC-18 reverse-phase silica gel (MeOH /H_2O). The purity of product was checked by HPLC (SI Fig 3) and then dissolved in de-ionised water yielding Hy-G8. The compound was identified by spectroscopic techniques (SI Fig 2). IR (KBr, υmax, cm^{-1}): 3473, 2922, 2817, 2579,2377,1747,1642, 1501, 1260, 1180, 1164; ^{1}H NMR (500 MHz, CD_3OD):δ1.19-1.55 (m, 22H), 2.49 (m, 20H), 2.52 (m, 16H), 3.08 (m, 16H), 3.36 (m, 8H), 3.52 (m, 2H), 3.56 (m, 6H), 3.68 (m, 4H). 3.74 (s, 3H,), 4.20 (d J=5Hz, 2H), 4.26 (m, 1H), 5.25 (d J=10Hz, 1H), 6.29 -7.57-(m, 9H). MALDI TOF MS Calculated 1720.2400 and found 1721.3258

2.7 Cell lines: The human cancer cell line A549 (lung adenocarcinoma) was obtained from American Type Culture Collection (ATCC, USA). A375 (malignant melanoma) cells were obtained from obtained from NCCS, Pune, India. Fibroblast-like mouse pre-adipocyte cell line 3T3L1 was obtained from Inter University Centre for Genomics and Gene Technology,

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University of Kerala, Thiruvananthapuram, India. The cells were maintained in DMEM media with 10% fetal bovine serum and 5 % CO_2 incubator at 37°C.

2.8 In vitro cytotoxicity assays: The growth inhibition capacity of Hydnocarpin (Hy) and Hydnocarpin-G8 (Hy-G8) was evaluated in cancer cell lines using the 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay as previously reported[22]. This assay measures cell viability by assessing the cleavage of tetrazolium salt by mitochondrial dehydrogenase. The absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek Power Wave XS). The inhibitory rate on the cells were calculated using the following formulas:

Proliferation rate (PR) % = [Abs sample/Abs control] x 100;
Inhibitory rate (IR) % = 100 − PR.

MTT assays were performed on A375, A549 and 3T3-L1 with various concentrations of Hy and Hy-G8 ranging from 2.5 μg/mL to 80 μM for 24 and 48 h.

Cytotoxicity of Hy-G8 was further confirmed with LDH assay (Pierce LDH Cytotoxicity Assay Kit-88953, USA) as per protocol given in the kit using respective controls. Finally the absorbance at 490nm and 680nm is measured using a plate-reading spectrophotometer to determine LDH activity.

% Cytotoxicity = Compound treated LDH activity −Spontaneous
LDH activity x 100

Maximum LDH activity − Spontaneous LDH activity

2.9 Apoptotic assays: Evaluation of the mode of cytotoxicity exhibited by the Hy-G8 was performed on cancer cells with various apoptotic assays after administration of Hy-G8 (20 μM) for 24 h. Morphological evaluation for apoptotic changes were performed under phase contrast microscope (Olympus 1X51, Singapore) under suitable magnification. Observation of nucleus for any changes was done with Hoechst staining and the cells were observed under an inverted fluorescent microscope using a DAPI filter (Olympus 1X51). Acridine orange-ethidium bromide dual staining is the most commonly used method to detect apoptosis and is based on the differential uptake of two fluorescent DNA binding dyes by viable and nonviable cells, respectively. Assessment of apoptosis using the acridine orange-ethidium bromide dual staining procedure was performed as described earlier[23]. The cells were observed under an inverted fluorescent microscope, using a FITC filter (Olympus 1X51, Singapore) to view the apoptotic or non-apoptotic cells. Furthermore, TUNEL assay (Dead End[TM] fluorometric TUNEL system G3250, Promega, USA) was used to detect the incorporation of the fluorescein-12-dUTP in the fragmented DNA of apoptotic cells, using the terminal deoxynucleotidyl transferase recombinant (rTdT) enzyme as per the manufacturer's instruction using propidium iodide as counter stain.

2.10 Caspase assay: The effect of both initiator caspases (caspases 8, 9 and 2) and executioner caspases (caspase 3) was determined by using Apo Alert[TM] Caspase Profiling kit (Clontech, CA, USA) as per the kit protocol. Cells were treated with 20 μM Hy-G8 for 24 h and finally, samples were transferred to 96-well plates for fluorimetric reading

(excitation: 380 nm; emission: 460 nm), and the OD obtained was recorded under a spectrofluorometer (FLx800, BioTek).

2.11 Wound healing assay: The scratch wound healing assay was performed to evaluate the anti-metastatic potential of Hy-G8 on A375 and A549 cells, as described previously[19,20]. The cancer cells have inherent potential for invasion and metastasis which makes them escape from the cytotoxic agent and establishes a tumor at a new niche. Hence evaluation of the anti-metastasis potential of any cytotoxic agent holds special attention. Briefly, cells were seeded into six-well plates at a density of 1.0×10^5/well until they reached 80% confluence. A scrape was made through the confluent monolayer with a sterile plastic pipette tip of 1mm diameter. Afterwards, the dishes were washed twice with PBS and incubated at 37°C in fresh DMEM complemented with 10% fetal bovine serum in the presence or absence of the indicated concentrations of Hy-G8. At the bottom side of each dish, three arbitrary places were marked where the width of the wound was measured with an inverted microscope (objective x 10). The wells were rinsed with PBS three times to remove floating cells and debris. The culture plates were incubated at 37 °C and in 5% CO_2. Wound closure was expressed as the average ± SEM of the difference between the measurements at time zero and the 12-24 h time period considered.

3 Results and discussion:

3.1 Isolation of crude extracts from *Hydnocarpous wightiana* Blume seeds: The seeds were subjected to extraction using hexane and acetone. The acetone extract was selected to screen for antioxidant activity and further fractionalization.
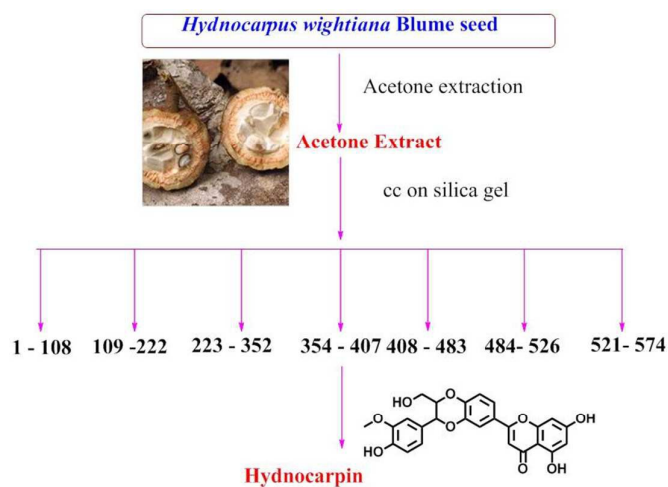

Fig 2: Isolation of compounds from Acetone extracts of *Hydnocarpous wightiana* Blume seeds.

Thin layer chromatography of the acetone extract was carried out using solvents of polarity starting from 100 % chloroform to 10 % methanol in chloroform, after analysing TLC the extract was subjected to column chromatography. From the acetone extracts 574 fraction pools were collected and further

sub column of 374-407 yielded the isolation of hydnocarpin in good yield (fig 2).

3.2 Antioxidant action of acetone extract: Screening of acetone extract against a wide array of experimentally generated free radicals revealed the astonishing antioxidant property[26] of the extract. The DPPH radical was considered to be a model of lipophilic radical, a chain reaction in lipophilic radicals was initiated by lipid autoxidation[27]. The radical scavenging activity of acetone extract against experimentally generated DPPH ions is shown in Fig. 3 A, which showed excellent scavenging activity even at low concentrations. The scavenging activity increases in a concentration dependent manner. The IC_{50} value of acetone extract was found to be 262.7 ± 1.3 µg/mL and for ascorbic acid it was 77.5 ± 1.5 µg/mL. FRAP assay directly measures total antioxidants or reductants in a sample. The antioxidant capacity of acetone extract based on the ability to reduce ferric ions was calculated as percent of inhibition as shown in Fig. 3 B. There is a gradual increase in antioxidant activity with an increase in concentration up to 500 µg/mL with an IC_{50} of 70 ± 1.8 µg/mL, whereas the standard ascorbic acid showed relatively stable activity even from lower concentrations with an IC_{50} less than even 1 µg/mL. Hydroxyl radicals attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule it contacts and is known to be capable of abstracting hydrogen atoms from membrane lipids and brings about peroxidic reaction of lipids. Acetone extract was not found to be a good scavenger of hydroxyl radical with a maximum inhibition of 40 % at 500 µg/ mL whereas alpha tocopherol showed good scavenging effect with an IC_{50} at 64µg/mL (Fig. 3 C). The scavenging ability of acetone extract against superoxide ions generated in the PMS-NADH-NBT system was shown in (Fig. 3 D). Acetone extract exhibited a dose dependent increase in scavenging activity with an IC_{50} at 299 ± 2.8µg/mL whereas the positive control alpha tocopherol generated IC_{50} at 61.5± 1.7 µg/ mL. The radical scavenging activity of the acetone extract is promising and might be helpful in preventing or reducing the progress of various oxidative stress induced diseases including cancer and thereby beneficial for human health. From the earlier studies it was proved that the isolated compounds from the acetone extract hydnocarpin and isohydnocarpin did not exhibit much antioxidant activity on DPPH test model, the strong radical scavenging activity of the acetone extract might be due to the presence of luteolin in substantial amounts. Hence use of whole extract is more adequate than its isolated compounds for free scavenging assays[14].

3.3 Total phenolic and flavonoid content: The Estimation of total phenolic content revealed that the acetone extract contains 642 ± 27 mg Gallic acid equivalents in 100 g extract. Total flavonoid content was determined by plotting the quercetin calibration curve (20 to100 µg/mL) and expressed as milligrams of quercetin equivalents (mg QE/ 100 gram of dry weight of sample). The study revealed that the acetone extract of *Hydnocarpus wightiana* Blume seed contains 135 ± 9 mg Quercetin equivalents in 100 g acetone extract. The

concentration and type of phenolic substances are mainly responsible for the biological activities of the extract and high phenolic contents often attributes to higher free radical scavenging properties[28]. Flavonoids are stable and effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals[29] implicated in several diseases. The higher phenolic and flavonoid contents of acetone extract could be highly responsible for the observed superior free radical scavenging effects.

3.4 Synthetic modification of hydnocarpin: The flavanolignan hydnocarpin (Hy) was isolated from acetone extract, structure is mostly similar to silybin and it can be characterized as a small, highly functionalized molecule with alternating carbo and hetero-cycles. Preliminary screening for the cytotoxicity of Hy revealed moderate effects on cancer cells. The cytotoxic nature of Hy demanded us to investigate further structural modification in order to improve its bio availability, water solubility and transformed Hy as an excellent anti-cancer agent. Our group developed an excellent cell penetrating carrier with octa-guanidium scaffold which is showing higher cellular uptake compared to Arg-8-mer, the well-known cell penetrating peptide. It is having excellent selectivity towards lysosome and the transporter alone (G8-PPI-FL) is found to be non-toxic[13]. The primary alcoholic group at C-23 position of Hy can be readily esterified and oxidized to a carboxylic acid.
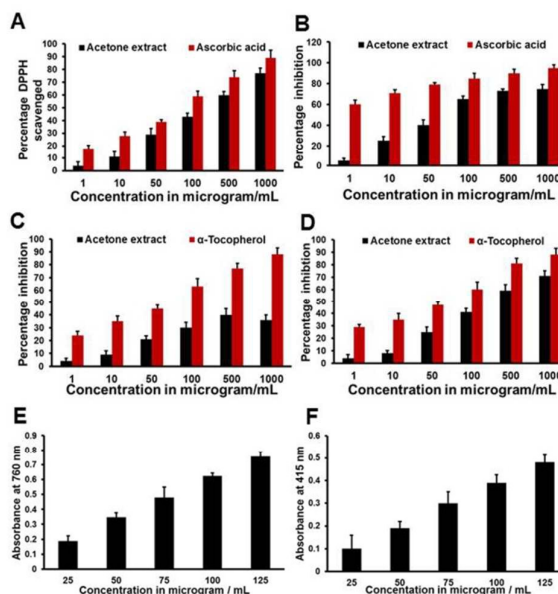

Fig 3: Antioxidant activity of Acetone extract (A) DPPH radical scavenging assay using ascorbic acid as the standard, (B) FRAP assay using ascorbic acid as the standard, (C) Hydroxyl radical scavenging assay using alpha tocopherol as the standard and (D) Superoxide radical scavenging assay using alpha tocopherol as the standard. (E) Total phenolic content (absorbance value of Gallic acid at 760 nm, expressed as Gallic acid equivalent in mg/g dry weight of sample) and (F) total flavanoid contet (absorbance value of Quercetin at 415 nm, expressed as Quercetin equivalent in mg/g dry weight of sample.

So we have conjugated the primary OH of Hy with acid terminal of molecular transporter Octa-guanidinium-poly-(propylene imine) hybrid Dendron (G8-Boc - PPI) by ester

coupling using carboxyl activating agent EDC in combination with DMAP (Scheme 1). Finally all the Boc groups of guanidine moieties were deprotected by treating with ethyl acetate saturated with HCl gas reagent. The final compound Hy-G8 was characterized by HPLC, NMR spectroscopy and MALDI-TOF mass spectrometry.
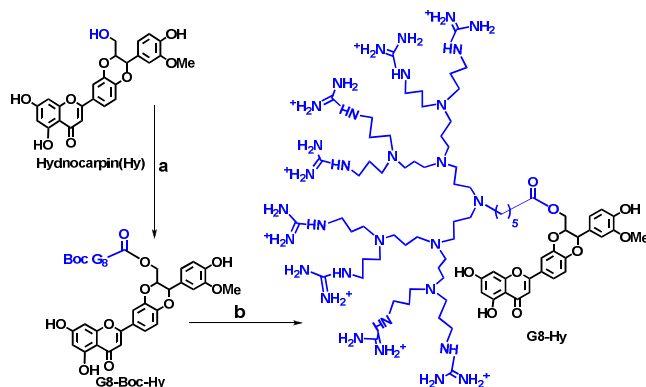
Scheme 1: Synthesis of hydnocarpin-G8 derivative; a) EDC, DMAP, dry DMF,N_2atm, 36 h.; b) EtOAc saturated with HCl (g), EtOAc

3.5 Evaluation of cytotoxic potential: Evaluation of cytotoxicity is the major step for any lead compounds aimed with human therapeutic application. Cytotoxic potential was evaluated with Hy, Hy-G8 and the carrier G8 on A375, A549 and 3T3-L1 for 24 and 48 h from a wide dosage range of 2.5 to 80 µM by MTT assay. Hy-G8 executed superior cytotoxicity than Hy in all dosages and incubation time on A375 cells. Hy also executed significant cytotoxicity whereas the carrier G8 was found to be non-toxic (Fig. 4A, Supplementary Fig. 4A, B). A375 cells were arrested by Hy-G8 with an IC_{50} value of 22.7 ± 0.9 and 14.4 ± 0.7 at 24 and 48 hrs but Hy could generate an IC_{50} at relatively higher concentrations of 57.6 ± 1.3 and 41.5 ± 1.7 at 24 and 48 hrs respectively. A similar trend in cytotoxicity was observed with A549 cells, where Hy produced an IC_{50} of 72.7 ± 1.4 at 48 hrs but failed to generate IC_{50} at 24 hrs. A marked increase in cytotoxic potential was showed by Hy-G8 (Fig. 4B, Supplementary Fig. 4C, D) with IC_{50}s of 33.7 ± 0.9 and 24 ±1.2 at 24 and 48 h. The cytotoxic potential of Hy and Hy-G8 increased in a dose-dependent manner in both the cancer cell lines. Absence of IC_{50} on normal fibroblast cell line by both Hy and Hy-G8 highlights the cancer cell oriented cytotoxicity by the agents. The enhanced cytotoxic potential of Hy-G8 in comparison with Hy evidenced on cancer cells was not observed on 3T3-LI (Fig. 4C, Supplementary Fig. 4E, F). Even though both Hy and Hy-G8 produced a maximum cytotoxicity of 18 and 17 % on 3T3-L1 at 80 µM at 48 hrs, lower dosages produced only negligible cytotoxicity; G8 proved to be non-toxic towards non-cancer cells also. The increased cytotoxicity of Hy-G8 than Hy on cancer cells was further tested with the quantification of lactate dehydrogenase (LDH) with 24 and 48 hrs of compound administration. Hy-G8 exhibited a higher cytotoxicity on A375 (Fig. 4D) and A549 (Fig. 4E) cells with marginal cytotoxicity on 3T3-L1 cells (Fig. 4F). The results

obtained with LDH assay highly correlated with the cytotoxicity evaluation with MTT assay.

The cytotoxicity of Hy and Hy-G8 evaluated on lung adenocarcinoma, malignant melanoma and fibroblast-like mouse pre-adipocyte cell line deciphers a cancer cell oriented cytotoxicity profile. Even though Hy alone was found to be good cytotoxic agent, the G8 coupled construct (Hy-G8) executes superior cytotoxicity on both the cancer cells, but a similar increase in cytotoxicity potential was not found on normal cells. Plasma membrane damage due to cytotoxic agents releases LDH into the cell culture media which could be quantified for determining the percentage of cytotoxicity caused by the agent. Improved cytotoxicity of Hy-G8, which could be attributed to the enhanced water solubility of Hy-G8 in contrast to Hy was confirmed with MTT and LDH assay.

3.4.Hy-G8 induced anticancer effects through apoptosis: To investigate the cell death mechanism induced by Hy-G8 in cancer cells, various apoptotic assays were conducted on A375 and A549 cells after the administration of 20 µM compound for 24 h. Morphological changes evaluated with phase contrast microscopy revealed a decrease in cell number, which was accompanied by salient morphological features of apoptosis, such as distorted shape, membrane blebbing, and the presence of apoptotic bodies compared to the control group
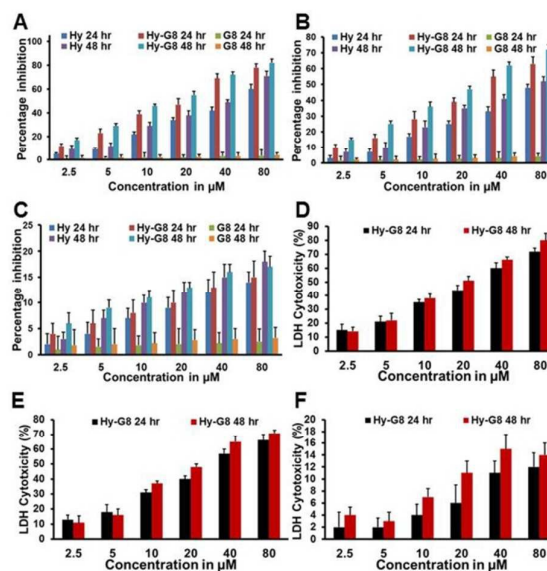

Figure 4: Assay for cytotoxicity in cancer cells and normal cells treated with Hy, Hy-G8 and G8 after 24 and 48 hrs of administration with MTT assay. (A) A375 cells; (B) A549 cells; (C) 3T3-L1 cells. Assessment of cytotoxicity of Hy-G8 by LDH assay on (D) A375 cells; (E) A549 cells and (F) 3T3-L1 cells.

on both A375 (Fig. 5A a) and A549 (Fig. 5B a) cells. In comparison with the respective controls, acridine orange-ethidium bromide staining displayed a change in colour from green to yellow/red, which is associated with other apoptotic features such as the presence of apoptotic bodies, damaged cell membrane and nuclear condensation in Hy-G8-treated cells. Significant changes in fluorescence were observed for A375 (Fig. 5A b) and A549 (Fig. 5B b) cells. Cells undergoing

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apoptosis demonstrated nuclear condensation and DNA fragmentation, which was detected by Hoechst 33342 nuclear staining. The percentage of chromatin condensation after Hy-G8 treatment was significantly higher in A375 (Fig. 5A c) and A549 (Fig. 5B c) cells as compared with the respective controls. The mechanism of cell death induced by Hy-G8 was further confirmed with the TUNEL assay in which propidium iodide was used as counter stain. Hy-G8 treated cells displayed green colour indicating TUNEL positivity, but control cells are largely TUNEL negative indicating the targeted apoptotic induction of compound on both A375 (Fig. 6A)and A549 cells (Fig. 6B). One of the mechanistic features of apoptotic cell death is the activation of caspases, a class of cysteine proteases and many anticancer drugs was reported to execute apoptosis through caspase dependent pathway [30]. Since caspases are the effective mediators of apoptosis, the expression of caspases 3, 8, 9 and 2 was estimated by using fluorimetry to thoroughly substantiate the cell death mechanisms induced by Hy-G8. Both A375 and A549 presented a significant ($P < 0.001$) increase in the expression of caspases 2, 3 and 8 (Fig. 6C). Caspase 3 was the most over expressed one with 2.4 and 2.6 fold increases in A375 and A549 cells respectively in comparison with their controls. The expression pattern of caspase 9 was not that significant in comparison with other caspases in both the cells.

Apoptosis is the most predominant mode of cell death, and several naturally occurring compounds have shown to induce apoptosis in cancer cell lines[31]. The presence of irregular bulges in the plasma membrane of the cell due to localized decoupling of the cytoskeleton from the plasma membrane is a hall mark of apoptosis. Although the budding phenomenon of apoptotic cells lasts for only a few minutes, the formation of apoptotic bodies remains visible for a few hours[32]. The enhanced cytotoxicity of the Hy-G8 compared with Hy is found to be executed through caspase mediated apoptosis.
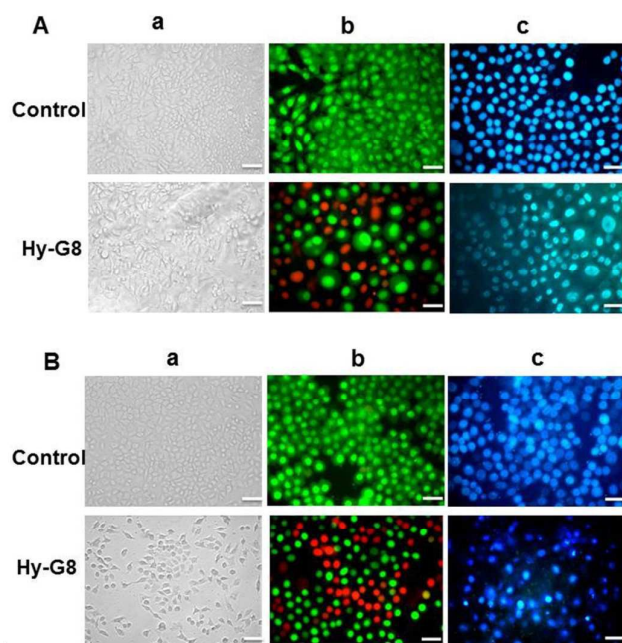
Figure 5: Morphological evaluation of apoptosis by the 24 hr administration of Hy-G8 (20 μM) on (A) A375 and (B) A549. (a) represents Phase contrast images, (b) represents Acridine orange-ethidium bromide and (c) represents Hoechst staining. Scale bar corresponds to 50 μm.

3.5 Anti-metastatic potential: The wound healing assay allows the researcher to study cell migration, cell interactions and metastatic potential of any agent under investigation. Anti-metastatic potential of Hy-G8 was evaluated on both A375 and A549 cells. It was clearly observed that Hy-G8 could hinder the migration of cells in a time dependent manner in both A375 (Fig.7A) and A549 cells (Fig.7B) whereas the untreated cells attains confluency within 24 h. The cell free scratch zone of the treated wells are large even after 24 h but the control wells displayed almost closure of the zone even after 12 h in both the cell lines. The in vitro scratch assay is an easy, low-cost and well-developed method to measure cell migration study. Compared to other methods, the in vitro scratch assay is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration, mimic cell migration during wound healing in vivo and are compatible with imaging of live cells during migration to monitor intracellular events if desired[33]. Tumor invasion and metastasis is a multistep process involving cell adhesion, proteolytic degradation and migration through the ECM, angiogenesis and many more. Common chemotherapeutic agents involved in cancer treatment aims at blocking cell cycle progression, inducing cell death and inhibiting tumor migration and thereby metastasis. Several natural compounds[34,35] have been reported to execute cancer chemoprevention through blocking call migration.
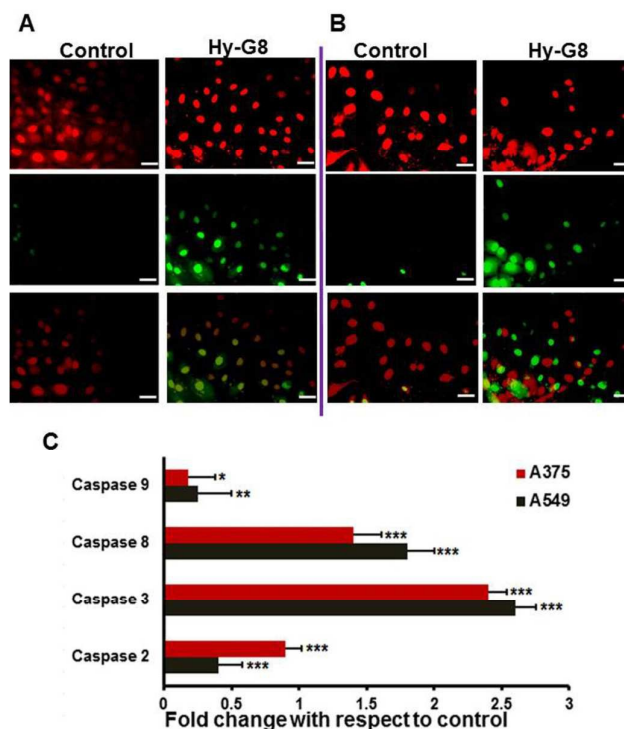
Figure 6: Morphological evaluation of apoptosis by TUNEL assay after 24 hr administration of Hy-G8 (20 µM) on (A) A375 and (B) A549 cells. Left panel represents images with PI filter, middle panel represents FITC filter and right panel represents merged images. Scale bar corresponds to 50 µm. (C) Caspase activity profiling after 24 h administration of Hy-G8 (20 µM) on A375 and A549 cells. Results are expressed as mean± SD.
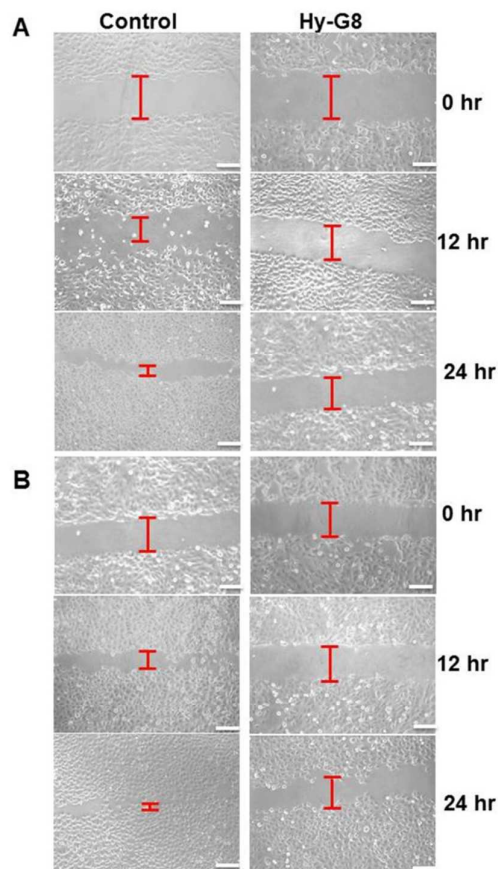

Figure7: Representative wound healing images at 0, 12, and 24 hours. Wounds were made with a pipette tip in confluent monolayers on (A) A375 and (B) A549 cells after administration of Hy-G8 (20 µM). Red lines indicated the cell free scratch zone. Scale bar corresponds to 50 µm.

Conclusions:

Phytochemical investigations on the popularly used medicinal plant *Hydnocarpus wightiana* Blume (seed) acetone extract decipher the excellent free radical scavenging property with high total phenolic and flavonoid content. The most abundant Hydnocarpin (Hy), which was isolated, purified and characterised from the extract flashes out moderate cytotoxic potential on cancer cells. In an attempt to increase the anticancer potential of Hy, chemical conjugation with a highly efficient, non-toxic cell penetrating guanidinium rich poly-(propylene imine) dendron (G8) was carried out via EDC coupling. The hybrid molecule (Hy-G8) executed excellent cytotoxicity preferentially on lung adenocarcinoma and malignant melanoma cells mediated through caspase dependent programmed cell death and also demonstrated

anti-metastatic potential. The superior anticancer potential of Hy-G8 over hydnocarpin warrants the need of detailed investigations into its molecular mechanistic mode of action both in cell lines and in animal models.
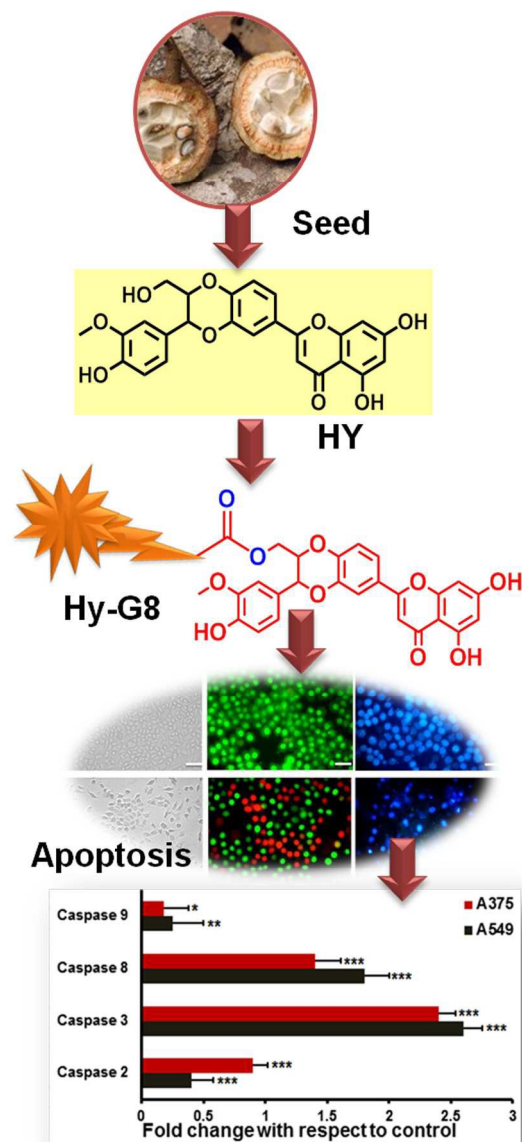
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