Indian Gooseberry (*Emblica officinalis* Gaertn.) suppresses cell proliferation and induces apoptosis in human colon cancer stem cells independent of p53 status via suppression of c-Myc and cyclin D1

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ABSTRACT

Indian Gooseberry, also known as Amla, a widely consumed fruit in South Asia, was evaluated for its anti-proliferative and pro-apoptotic mechanisms on human colon cancer stem cells (HCCSC). Amla extracts suppressed proliferation and induced apoptosis independent of p53, a tumour suppressor gene, in HCCSCs. Further, amla extracts suppressed cell proliferation by targeting the Wnt/ β -catenin signaling pathway as seen by decreased nuclear translocation of β -catenin. Additionally, this led to suppressed expression of c-Myc and cyclin D1, key proteins involved in cell proliferation. Inhibition of stem-ness of HCCSCs by amla may be due to its effect on the Wnt/ β -catenin signaling. These results indicate that amla suppresses HCCSC proliferation and induces apoptosis independent of p53 status via potentially targeting Wnt/ β -catenin signaling pathway. Amla is therefore a promising functional food for preventing colon cancer and might be a novel resource for the food industry.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

Trigonelline (PubChem CID: 5570); Naringin (PubChem CID: 442428); Kaempferol (PubChem CID: 5280863); Catechin (PubChem CID: 73160); Quercetin (PubChem CID: 5280343); Colchicine (PubChem CID: 6167); Luteolinidin (PubChem CID: 441701); Naringenin (PubChem CID: 932); Apigeninidin (PubChem CID: 159360).

KEYWORDS

Colon cancer, cancer stem cells, Amla (*Emblica officinalis* Gaertn.) seed and pulp, proliferation, apoptosis, stem-ness, Wnt/ β -catenin signaling

1. INTRODUCTION

Emblica officinalis Gaertn. or *Phyllanthus emblica* L. commonly known as Indian gooseberry or amla, is a widely consumed fruit in India and other South Asian countries for centuries. Amla fruit and its parts (seed or pulp) are used in culinary to prepare pickles, chutneys, candies and vegetable dishes. Amla is highly nutritious functional food and rich source of bioactive molecules with many pharmacological properties (Baliga & Dsouza, 2011; Mishra et al., 2015). Recently, it has been shown that amla can inhibit the growth of SW620 (colorectal) cells *in vitro*, and carcinogen induced skin cancer, stomach cancer, and liver cancer in rodent models (Ngamkitidechakul et al., 2010; Chen et al., 2011; Krishnaveni & Mirunalini, 2012). However, there are no studies to the best of our knowledge, demonstrating the anti-cancer efficacy of amla specifically against cancer stem cells (CSCs), one of the primary drivers of carcinogenesis.

Colon cancer is one of the most commonly diagnosed cancers in both men and women, posing a serious demographic and economic burden worldwide; it is also the third leading cause of cancer deaths in the US. In 2015, an estimated 69,090 men and 63,610 women were diagnosed with colorectal cancer, while 26,100 men and 23,600 women died due to the disease (Siegel et al., 2015). Colon cancer is characterized by mutational inactivation of tumour suppressor genes and activation of oncogenic genes, resulting in the formation of adenomatous polyps, ultimately developing into an advanced adenoma with high-grade dysplasia that progress to an invasive cancer (Markowitz & Bertagnolli, 2009). In recent years, accumulated experimental evidence has suggested that cancers, including colon cancer, have a hierarchal organization regulated by a small number of self-renewing cancer cells, called CSCs (Ajani et al., 2015). Finding strategies to target CSCs has immediate therapeutic consequences as cancer growth is sustained by CSCs.

CSCs are mutated stem cells having ability to self-renew and regenerate tumour mass. CSCs have been proposed as the driving force of tumourigenesis and the seeds of metastases (Medema, 2013). CSCs mimic the functionality of normal adult stem cells maintaining their undifferentiated state while dividing non-symmetrically. The CSCs have shown to be resistant to conventional chemotherapeutic regimens that target homogeneous populations of rapidly growing differentiated tumour cells, for e.g., CD133-positive colon CSCs were shown to be resistant to the conventional cytotoxic drug 5-fluorouracil (FU) and the resistance was shown recently to be dependent on Wnt signaling (Deng et al., 2010). The Wnt signaling pathway proteins are a group of intracellular signaling molecules that constitute the principal driving force behind the biology of the crypt and play an important role in the maintenance and proliferation of stem cell reservoirs. The loss or alteration of function of Wnt signaling proteins (mutations in APC, Axin, β -catenin) is linked to the pathogenesis of colon cancer (Anastas & Moon, 2013).

Wnt signaling is crucially important for maintaining stem-ness (self-renewal) in normal colon stem cells and is a common pathway that is deregulated in most colon cancers (Holland et al., 2013). Furthermore, intestinal stem cells with aberrant Wnt signaling or nuclear accumulation of β -catenin considered oncogenic (CSCs; Qiu et al., 2010). Because Wnt/ β -catenin signaling plays a key role in maintenance of stem-ness, and survival/proliferation of cancer stem cells, targeting the Wnt/ β -catenin signaling is a good strategy for cancer prevention. Furthermore, p53, one of the critical tumour suppressor genes is mutated in 50–75% of colon cancer cases, and marks transition to metastasis (Haupt et al., 2003; Markowitz & Bertagnolli, 2009). Even FU is shown to function via p53-dependent pathways, and thus chemopreventive agents that work even in the absence of p53 need to be identified and tested.

Due to the long latency period of colon cancer, there is an opportunity to prevent colon cancer through dietary modulation. Accumulated evidence has shown that the dietary plant extracts, especially fruits, vegetables, grains and herbs, have preventive/protective effect against colon cancer (Ricciardiello et al., 2011; Pistollato et al., 2015). These foods are rich in biologically active components such as polyphenols, glucosinolates, and carotenoids among others. These components individually or sometimes in combination in the food have been linked to inhibition of one or more hallmarks of cancer (Hanahan & Weinberg, 2011). Furthermore many of the bioactive compounds have also shown to target cancer stem cells, specifically in the colon (Pistollato et al., 2015). Thus, dietary intervention is emerging as an alternative to prevent the progression of colon cancer (Kasdagly et al., 2014) mainly due to its potency and reduced toxicity.

The aim of the present study was to characterize the bioactive profile, and the anti-proliferative and pro-apoptotic activities of the amla extracts (seed and pulp) on HCCSCs. To

test the effectiveness of amla extract against colon cancer, we utilized HCCSC and human colon cancer cells (HCT116) and treated them with amla extracts. We were able to demonstrate that amla suppresses HCCSC proliferation and elevates apoptosis via suppressing Wnt/ β -catenin signaling pathways and induction of mitochondrial mediated apoptosis, respectively.

2. MATERIALS AND METHODS

2.1. Chemicals.

Fetal bovine serum (FBS) was purchased from HyClone (Pittsburgh, PA, USA). BrdU Cell Proliferation Assay Kit was obtained from Cell Signaling Technology (Danvers, MA, USA). Gallic acid, Trolox (($\pm$)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), Folin-Ciocalteu reagent and all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of amla extracts.

Indian gooseberry (Amla) fruits collected from botanical garden of Yogi Vemana University campus, Kadapa, Andhra Pradesh, India and voucher specimen with a number YVUH-1990, have been deposited at the Herbarium, Yogi Vemana University, Kadapa – 516003, A.P., India. The seed and pulp of the amla fruit were separated, air-dried at room temperature and ground into a fine powder in a blender. Amla seed or pulp powder was extracted in two ways; **1)** serially in a continuous sequence with hexane, acetone, methanol, ethanol and water, or **2)** extracted directly with individual solvents with the sample to solvent ratio 1:10 (1 g in 10 mL solvent), by vortexing for 2 min and incubating at 4 °C overnight in a shaking incubator. After incubation, the extracts were collected and centrifuged at 3250 xg for 10 min. Supernatant was collected, filtered

through 0.45 µm PTFE syringe filter (Tisch Scientific, North Bend, OH, USA), and concentrated in a nitrogen evaporator (Organomation Associates Inc, Berlin, MA, USA) to reduce the volume by 90% using graduated tubes. The concentrated extracts were used for quantification of total phenolics and antioxidant ability. Extracts were aliquoted and stored at -80 °C until analysis.

2.3. Total phenolics in amla extracts.

Total phenolic content of the amla seed and pulp extracts was determined using a modified Folin-Ciocalteu colourimetric method as described earlier (Singleton et al., 1999; Madiwale et al., 2011). The phenolic content was expressed as milligrams of gallic acid equivalents per gram of air-dried sample of amla seed or pulp (mg GAE/g).

2.4. Antioxidant activity of amla extracts.

The antioxidant activity was measured using a modified 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, ABTS) assay using our in-house protocol (Madiwale et al., 2011). The antioxidant activity of the samples was expressed as milligrams of Trolox equivalents per gram of air-dried sample of amla seed or pulp (mg TE/g).

2.5. LC-MS/MS analysis.

Methanolic extract (direct extraction, 1 µL) of amla seed or pulp was injected on a Waters Acquity UPLC system. Separation was performed using a Waters Acquity UPLC T3 column (1.8 µm, 1.0 x 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (acetonitrile, 0.1% formic acid). Injections were made in 100% A, which was held for 1 min, a 12 min linear gradient to 95% B was applied, and held at 95% B for 3 min. Then the gradient

was returned to starting conditions over 0.05 min and held for 3.95 min. Flow rate was kept constant at 200 μ L/min for the duration of the run. The column was held at 50 $^{\circ}$ C, samples were held at 5 $^{\circ}$ C. Column eluent was infused into a Waters Xevo G2 Q-TOF MS fitted with an electrospray source. Data was collected in positive ion mode, scanning from 50 - 1200 Da at a rate of 0.2 seconds per scan, alternating between MS and data dependent acquisition (DDA) MS/MS mode. Collision energy was set to 6 V for MS mode and ramped from 15-30 V for MS/MS mode. Calibration was performed prior to sample analysis via infusion of sodium formate solution with mass accuracy within 1 ppm. The capillary voltage was held at 2200 V, the source temperature at 150 $^{\circ}$ C, and the desolvation temperature at 350 $^{\circ}$ C with a nitrogen desolvation gas flow rate of 800 L/h. Raw data files were converted to mzXML format using massWolf, and the MS/MS spectra were extracted using XCMS and written to msp format using a custom script in the statistical program R (Broeckling et al., 2013). Later msp files were submitted to Mass Bank database (<http://www.massbank.jp/?lang=en>) to obtain the data for identification of tentative compounds present in amla pulp or its seed. These tentative compounds were identified based on high scores according to Sumner et al (2007). The phenolic compounds were identified by comparing their retention time, and mass spectra with those obtained from standard compounds, when available. HPLC was used to quantify some of the identified compounds in the extracts. Absolute content of individual compounds was calculated based on the standard curves and reported as μ g per gram dry weight.

2.6. Cell lines.

HCCSCs, positive for cancer stem cell markers CD133, CD44, CD34, Aldehyde Dehydrogenase, Telomerase, Sox2, cKit, and Lin28, were obtained from Celprogen Inc. (San Pedro, CA, USA).

To maintain the cells in their undifferentiated state, HCCSCs maintenance media (10% FBS) and specially coated cell culture flasks (Celprogen) were used. Cells were maintained at 37 °C and 5% CO₂. Cell cultures at approximately 80% confluence were used for all *in vitro* experimental procedures.

Human colon cancer cell lines HCT116 p53^{+/+} and HCT116 p53^{-/-} were a gift from Dr. Bert Vogelstein (School of Medicine, Johns Hopkins University, Baltimore, MD, USA). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ as described earlier (Radhakrishnan et al., 2011).

2.7. Lentiviral shRNA-mediated knockdown of p53 in HCCSCs.

HCCSCs were infected with lentiviral particles encoding shRNA targeting p53 (Santa Cruz Biotechnology, Paso Robles, CA, USA) according to the supplier's protocol. Protein extract from these cells were run on a gel to confirm complete suppression of p53 as described earlier (Massey et al., 2014).

2.8. Cell proliferation assay.

The BrdU cell proliferation assay kit was obtained from Cell Signaling Technology (Danvers, MA, USA) and used for the detection of 5-bromo-2'-deoxyuridine (BrdU) incorporated into cellular DNA during cell proliferation. Briefly 20,000 cells (HCCSC or HCT116) were seeded per well in a 96 well plate, after 24 h, cells were treated with amla extracts (amla seed, AS or amla pulp, AP) in serum free CSC media at 25, 50, 100 or 200 µg GAE/mL, respectively. The treatments were added in triplicate at a volume of 150 µL per well and then allowed to incubate for 20 h at 37°C. After incubation, BrdU was added at a concentration of 10 µM per well, and the

plate was incubated at 37 °C for an additional 4 h to allow incorporation of BrdU into cellular DNA (24 h total). At the end of 4 h incubation, the medium was removed, and BrdU incorporation was assayed according to the manufacturer's protocol. The experiment was performed in triplicate, and the data was expressed as the mean $\pm$ S.E.

2.9. TUNEL assay

The TUNEL (terminal transferase dUTP nick end labeling) assay was performed to determine the effect of methanol extracts of amla seed or amla pulp on apoptosis. Cells (4×10^4) were seeded in four-chambered glass slides and after treatment for 24 h with amla extracts (AS or AP) in serum free media at 25, 50, 100 or 200 μ g GAE/mL, the slides were prepared and analyzed for TUNEL staining according to the manufacturer's protocols (Roche Diagnostics, Indianapolis, IN, USA). For analysis, 10 images from each well were taken, containing at least 50 cells per image. The cells were counted, and the data were expressed as the percentage of TUNEL positive cells to total cells (percent apoptosis).

2.10. Colony formation assay.

Ability of amla extracts to alter the stem-ness of colon CSCs was evaluated through colony formation assay (Ottinger et al., 2012) by counting the number of colonies that can form after treatment. Briefly, 150,000 HCCSCs were seeded per well in a 6-well plate and incubated for 24 h in complete growth media. After 24 h, growth media was removed and cells were treated with methanol extract of amla seed or pulp in serum free media at 25, 50, 100 or 200 μ g GAE/mL for 24 h. Cells were collected by trypsinization. After proper dilution of cells, 100 cells were seeded into each well of a new 6-well plate and incubated for 10 days in complete growth media. Media

was removed and cells were fixed using 3.7% paraformaldehyde in 70% ethanol for 10 min. The cells were stained with 0.05% Coomassie blue for 20 min and then rinsed with PBS. Stained colonies were counted under a dissecting microscope.

2.11. Western blot analysis.

HCCSCs were seeded at a density of 2.0×10^6 cells per culture plate in HCCSC growth media for 24 h. After 24 h incubation, the cells were incubated with FU (IC₅₀ 570 μ M or 75 μ g/mL) or methanol extract of amla seed (AS, IC₅₀ - 78 μ g GAE/mL) or amla pulp (AP, IC₅₀ - 140 μ g GAE/mL) in HCCSC serum free media for 24 h. Protein was extracted using NE-PER Nuclear and Cytoplasmic protein extraction kit (Thermo Fisher Scientific, Rockford, IL, USA).

Cytoplasmic or nuclear protein extracts (40 μ g) were incubated at 98 °C for 5 min and separated by 4-12% Bis-Tris precast gels (Bio-rad Laboratories, Hercules, CA) at 120 V for 2 h in MOPS running buffer (Bio-rad) and electrophoretically transferred to PVDF Immobilon-FL membrane (Millipore, Billerica, MA, USA) at 35 V for 95 min in TBS transfer buffer. PVDF membranes were incubated in superbloc solution (Thermo Fisher Scientific) for 2 h at room temperature.

Membranes were incubated with either PARP rabbit monoclonal antibody, Bax rabbit monoclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Bcl2 antibody, rabbit polyclonal anti-p53 antibody, mouse polyclonal anti- β -catenin antibody, mouse polyclonal anti-DNA topoisomerase II β antibody, mouse polyclonal anti-cyclin D1 antibody, mouse polyclonal anti-c-Myc antibody, goat polyclonal anti- β -catenin antibody or goat polyclonal anti- β -actin antibody (all 1:500, Santa Cruz Biotechnology, Paso Robles, CA, USA) for 2 h at room temperature. Membranes were subsequently washed with TBS with 0.1% Tween 20 and then probed with IR dye conjugated secondary antibodies, Donkey anti-goat, goat anti-

rabbit or goat anti-mouse IR dye 800 or IR dye 680 obtained from LICOR Biosciences, Lincoln (1:100,000). All antibody dilutions were made in superblock solution. The membranes were scanned in an Odyssey infrared image system (LICOR Biosciences, Lincoln, NE, USA) and the band intensities were quantified using the Odyssey software. Protein intensities were normalized to β -actin, a loading control for cytosolic proteins or to DNA topoisomerase II β , a loading control for nuclear proteins.

2.12. Statistical analysis.

Data were represented as means $\pm$ standard error (SE). Statistical significance was determined by one-way ANOVA, followed by the post-hoc Fisher least significant differences test for multiple means comparisons. All statistical analyses were performed using SPSS version 21 (IBM, Armonk, NY, USA). Means not sharing the same letter are statistically significant from each other ($p < 0.05$).

3. RESULTS

3.1. Methanol is the best solvent for extraction of polyphenols from amla seed or amla pulp with high anti-proliferative activities.

For extraction of total phenolics from amla seed and pulp, we began serial solvent extractions starting with low to high polarity solvents in the order of increasing polarity (hexane, acetone, methanol, ethanol and water). We observed that highest levels of phenolics were extracted in methanol compared to other solvents. In parallel with the phenolics data, we also observed an elevated antioxidant activity in the methanol extracts in both amla seed and pulp compared to other solvents (**Figure 1A**). Anti-proliferative activity of amla extracts was tested against

HCCSCs and was compared against the standard cytotoxic drug FU. Surprisingly, FU elevated cell proliferation in HCCSCs compared to control at low concentrations (25 $\mu\text{g/mL}$). Our results are similar to an earlier study where the authors observed that FU at lower concentrations (1 -10 $\mu\text{g/mL}$) promoted cell proliferation in CD133+ colon cancer cells (CD133 is marker for stem cells, Deng et al., 2010). However, FU at higher concentration (100 $\mu\text{g/mL}$), resulted in suppressed proliferation (**Figure 2**). The acetone and the methanol fraction exhibited significantly higher cell growth inhibition (37 – 47%) compared to control and FU. The methanol fraction had the highest anti-proliferative activity compared to other solvents (**Figure 1B**). Anti-proliferative activity was not observed in hexane extractions of amla, possibly due to very low extraction of phenolic compounds in hexane.

The amla seed and pulp powder were also extracted separately with the individual solvents - acetone, methanol, ethanol and water. Similar to serial solvent extraction, the methanol solvent extracted the highest level of phenolics as compared to the other solvents ($p < 0.05$, **Figure 1C**). Our results demonstrate that methanol was the best solvent that extracted highest level of phenolics and the methanol extracts had elevated antioxidant activity. The methanol extracts also exhibited greater anti-proliferative activities compared to acetone, ethanol and water extractions ($p < 0.05$, **Figure 1D**). Although the acetone and ethanol extracts had significantly higher total phenolics and antioxidant potential compared to water extract, interestingly, the anti-proliferative effects were not significantly higher. Thus, the direct methanol extract of amla seed (AS) or amla pulp (AP) was used for further studies.

3.2. LC-MS/MS analysis

Methanol extracts of amla seed and pulp were analyzed by LC-MS/MS for the identification of the major bioactive compounds in the extracts. We found different classes of compounds in the amla extracts including phenolic acids, flavonoids, alkaloids, and anthocyanins (Table 1, AS or AP spectra presented in supplementary data Figure S1 and S2). The tentative compounds identified were trigonelline, naringin, kaempferol, embinin, catechin, and quercetin based on high score according to Sumner et al., (2007) in methanol extract of amla seed (AS). Methanol extract of amla pulp (AP) showed kaempferol, quercetin, trigonelline, naringin, and isorhamnetin as the major bioactive constituents. We used analytical standards to confirm the presence of these compounds in the extracts. Based on retention time and mass spectral matching, we confirmed the presence of trigonelline, naringin, catechin, quercetin, kaempferol, and luteolinidin in the amla seed and pulp extracts. Further, we determined their absolute contents by HPLC and compared with standard references and shown the data in Table 1. Data revealed that the catechin constituent was present in higher levels in both amla seed and amla pulp as compared to other bioactive compounds. In addition, we also confirmed the presence of apigeninidin in the amla seed and colchicine in the amla pulp extracts.

3.3. Amla suppresses proliferation in human colon cancer stem cells (HCCSC) & human colon cancer cells (HCT116) in a p53-independent manner

To investigate dose dependent anti-proliferative activities of methanol extracts of amla seed (AS) or amla pulp (AP), HCCSC and HCT116 cells were treated with increasing concentrations (25, 50, 100 or 200 µg GAE/mL) of amla extracts, AS and AP, for 20 h and BrdU assay for anti-proliferative activity was conducted as described in the methods. FU was included in the study as a positive control. Our results demonstrated that amla extracts (AS or AP) suppressed cell

proliferation in both cell lines in a dose dependent manner (**Figure 2A, B**). IC₅₀ of methanol extract of amla seed (AS) against HCCSCs and HCT116 were 78±5 µg GAE/mL (78±5 µg GAE/0.86 mg dried AS powder) and 94±8 µg GAE/mL (94±8 µg GAE/1.05 mg dried AS powder), respectively. For amla pulp (AP), the IC₅₀ against HCCSC and HCT116 were 140±10 µg GAE/mL (140±10 µg GAE/1.0 mg dried AP powder) and 164±10 µg GAE/mL (164±10 µg GAE/1.17 mg dried AP powder), respectively. Further, to determine if anti-proliferative efficacy is retained even in the absence of p53, HCCSCs with shRNA-attenuated p53 and HCT116 p53^{-/-} cells were treated with increasing concentrations of AS or AP (25, 50, 100 or 200 µg GAE/mL). A significant suppression (p<0.05) of proliferation was observed in both these cell lines even in the absence of p53 (**Figure 2A, B**). In addition, the anti-proliferative efficacy, even in the absence of p53, showed dose-dependency. As expected, the FU treatment showed minimal suppression of cell proliferation in the absence of p53 in both the cell lines.

3.4. Amla induced apoptosis in a p53 independent manner.

The ability of methanol extract of amla seed (AS) or pulp (AP) to induce apoptosis in HCCSCs and HCCSCs with shRNA-attenuated p53 was measured by PARP cleavage, the hallmark of apoptosis, using western blotting (Yang et al., 2004). **Figure 3** shows the induction of PARP cleavage as accumulation of cleavage fragment (89 kDa) in amla treated cells. Amla extracts (AS & AP) induced apoptosis in HCCSCs independent of p53 was supported by increased PARP cleavage (**Figure 3A & B**). PARP cleavage method of determining apoptosis was validated using the *In situ* cell death detection using the TUNEL assay (DNA fragmentation, Supplementary Figure S3).

3.5. *Amla induces apoptosis by elevating Bax/Bcl-2 ratio.*

To evaluate the mechanism implicated in the amla-induced apoptosis, we measured proteins Bax and Bcl-2, pro- and anti-apoptotic proteins in the intrinsic apoptotic signaling pathway in HCCSC. Methanol extract of amla seed (AS) (IC₅₀: 78 µg GAE/mL) or amla pulp (AP) (IC₅₀: 140 µg GAE/mL) treated HCCSCs showed increased Bax protein levels in cytoplasm with decreased Bcl-2 protein and increased Bax/Bcl-2 ratio (**Figure 4**).

3.6. *Amla suppresses the stem-ness of HCCSCs via suppressed Wnt/β-catenin signaling.*

To test the effect of amla extracts on the stem-ness (self-renewal) of colon cancer stem cells, the HCCSCs were treated with increasing concentrations (25, 50, 100, or 200 µg GAE/mL) of methanol extracts of amla seed (AS) or pulp (AP) for 24 h and colony formation assay was performed as described in the methods. Amla extracts significantly inhibited the formation of colonies (stem-ness) of HCCSCs in a dose-dependent manner (p<0.05). The methanol extract of amla seed (AS) completely inhibited the formation of colonies at 200 µg/mL (**Figure 5A, B**). Further, we tested the efficacy of amla extracts on the regulation of Wnt/β-catenin signaling, the key signaling pathway involved in self-renewal (stem-ness) and proliferation of cancer stem cells. We observed a significant increase of β-catenin levels in cytoplasm with decreased transport to nucleus. We also observed decreased expression of Wnt target proteins, c-Myc, and cyclin D1 in the nucleus (**Figure 5C**). These findings indicate amla suppresses the stem-ness property of HCCSC by targeting the Wnt/β-catenin signaling pathway.

4. DISCUSSION

Epidemiological studies have shown the correlation of fruit and vegetable consumption and the reduced risk of various cancers (Benetou et al., 2008; Oyeboode et al., 2014). We and others have shown that these phytochemicals affect a myriad of intracellular targets, and this quality makes amla (AS/AP) as a desirable chemotherapeutic agents against cancer (Reddivari et al., 2007; Radhakrishnan et al., 2011; Madiwale et al., 2011; Kasdagly et al., 2014; Chen et al., 2014; Pistollato et al., 2015; Ramakrishna et al., 2015). Indian gooseberry is an antioxidant-rich fruit with containing polyphenols such as trigonelline, naringin, kaempferol, embinin, catechin, isorhamnetin and quercetin (**Table 1**) (Mishra et al., 2015). In the present study, we tested extraction efficacy of different solvents and identified methanol as the best solvent for extracting highest levels of bioactive compounds from amla seed or pulp (**Figure 1**). Further, we investigated the anti-proliferative and pro-apoptotic effects of methanolic extract of amla seed (AS) or pulp (AP) against HCCSCs and HCT116 cells. Amla demonstrated dose-dependent anti-proliferative properties in human colon cancer stem cells (HCCSC) and colon cancer cells (HCT116) and pro-apoptotic properties in HCCSCs independent of p53 status of the cells (**Figures 2 and 3**). We also observed increased expression of pro-apoptotic protein Bax (and Bax/Bcl-2 ratio) in HCCSCs (**Figure 4**). The pro-apoptotic Bcl-2 protein, Bax resides in the cytosol but translocate to mitochondria following death signaling, where it forms a heterodimer with Bcl-2, and functions as an apoptotic activator by increasing the opening of the mitochondrial voltage-dependent anion channel. This promotes the release of cytochrome c and further leads to activation of effector caspases (Ola et al., 2011). This study reveals that amla was able to induce intrinsic mitochondrial apoptotic signaling pathway in HCCSCs. P53 is referred as the guardian of the genome as it plays a critical role in anti-tumour activity by inducing pathways such as growth arrest, apoptosis, and senescence, as well as blockade of angiogenesis. The p53

tumour suppressor protein can activate either extrinsic or intrinsic apoptotic pathway through transcriptional upregulation of a number of genes. However, p53 is mutated in over 50% of human cancers (Haupt et al., 2003), thus making drugs such as FU, that work via p53-dependent mechanisms less efficacious. Our results, that effectiveness of amla in HCCSCs (and HCT116 cells) independent of p53 status, are crucial for future translational studies against colon cancer. Our findings warrant further testing of amla extracts *in vivo* for their anti-cancer effects against p53 knockout models of colon cancer.

Amla extracts inhibit the stem-ness property (colony formation) of colon CSCs in a dose-dependent manner (**Figure 5**). Consistent with these findings, we previously demonstrated that phenolic-rich extract from the dermal layer of sweet sorghum stalks and triphala also suppressed colony formation in a dose-dependent manner (Massey et al., 2014; Ramakrishna et al., 2015). Thus, in addition to anti-proliferative and pro-apoptotic activities, amla also alters the stem-like properties by inhibiting colon cancer stem cell self-renewal. Wnt/ β -catenin signaling is crucially important in maintaining stem-ness in normal colon stem cells through nuclear translocation of β -catenin that activates a specific subset of the TCF/LEF family in order to promote a progenitor-like gene expression signature (*c-Myc* and *cyclin D1*). This pathway is one of the most commonly deregulated pathways in colon cancer (Chen et al., 2014). Our data showed that the anti-proliferation and inhibition of self-renewal (colony formation) capacity by amla could be due to the inhibition of Wnt/ β -catenin signaling pathway (**Figure 5C**). Treatment with amla extracts significantly decreased nuclear translocation of β -catenin and suppressed c-Myc and cyclin D1 protein levels ($p < 0.05$, **Figure 5C**). We observed that amla contains high content of flavonoids; catechin, naringin, trigonelline, quercetin, and kaempferol and these compounds can promote anti-proliferative activities by effecting Wnt/ β -catenin signaling pathway at different

levels, from the ligand receptor interaction to the methylation of genes expressing pathway components (Tarapore et al., 2012; Amado et al., 2014). Quercetin was shown to interact with β -catenin and block binding between β -catenin and TCF (Park et al., 2005). Kaempferol suppressed β -catenin/TCF transcriptional activity in SW480 colon cancer cells, which has inactivating mutation of the *APC* gene and shows a constitutively active transcriptional activity of β -catenin/TCF. This can further suppress c-Myc and cyclin D1 (Park & Choi, 2010). Naringin also has shown to target β -catenin in breast cancer cells (Li et al., 2013). EGCG (epigallocatechin gallate), a major catechin of tea effectively inhibited carcinogenesis by affecting Wnt/ β -catenin signaling pathway both in *in vitro* and *in vivo* studies (Singh et al., 2011). Kim et al (2006) noticed suppression of Wnt signaling and target a gene (*c-Myc*) by catechin of tea in invasive breast cancer cells. As amla is a mixture of these compounds, we believe that the anti-cancer effect of amla is due to additive/synergistic effects of the polyphenols.

In conclusion, we have shown amla extracts (seed and pulp) suppressed cancer stem cell proliferation independent of p53 status and induced apoptosis in HCCSCs. Amla extract suppressed Wnt/ β -catenin signaling by suppressing nuclear transport of β -catenin and downregulated β -catenin target proteins c-Myc and cyclin D1, which explains suppressed HCCSC proliferation and stem-ness (Amendola et al., 2009). Furthermore, efficacy of amla extracts was similar to cytotoxic drug FU. Our results warrant pre-clinical evaluation of amla in animal models to investigate its potential use for chemoprevention or as an adjunct to conventional chemotherapy in the management of colon cancer.

ABBREVIATIONS

PARP – Poly ADP ribose polymerase; BrdU – Bromodeoxyuridine; Bax – Bcl-2 associated X protein; Bcl-2 –B cell lymphoma 2 protein, Wnt – Wingless/Integrated; FU – 5-florouracil. AS – Methanol extract of amla seed; AP – Methanol extract of amla pulp.

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Competing interests

The authors declare that they have no competing interests

Authors' contribution

RV performed the experiments in this study under guidance of JV. LR and JV conceived the study and participated in its design and coordination. RV and SR wrote the manuscript. SR and

EKKH helped in confirmation of bioactive constituents of the extracts using LC-MS/MS. JV was the Principal Investigator of the study. All authors read and approved the final manuscript.

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Tables

Table 1: Tentative compounds determined by LC-ESI-QTOF-MS/MS in methanol extract of amla seed (AS) and amla pulp (AS).

S. No.	m/z	RT ^a	Score ^b	Tentative identified molecule ^c	Massbank ID	Molecular formula	Exact mass	Area under curve	Absolute content (µg/gdw) [#]
Methanol extract of amla seed (AS)									
1	138.06	0.42	0.689	Trigonelline*	PR100409	C7H7NO2	137.05	467409	2.65 ± 0.06
2	315.07	2.91	0.786	Naringin*	PB000804	C27H32O14	580.18	351497	182.20 ± 0.29
3	291.09	3.35	0.684	Catechin*	PB006302	C15H14O6	290.08	155872	1110.82 ± 3.9
4	465.1	4.34	0.975	Quercetin*	CE000171	C15H10O7	302.04	99784	2.70 ± 0.02
5	287.06	4.96	0.957	Kaempferol*	PB005704	C15H10O6	286.05	344920	46.32 ± 0.49
6	339.12	5.51	0.601	Colchicine*	BML00544	C22H25NO6	399.17	178898	-
7	307.1	5.74	0.603	Noscapine	CO000310	C22H23NO7	413.15	41120	-
8	411.26	7.8	0.601	Geniposide	TY000059	C17H24O10	388.14	12991	-
9	616.18	8.15	0.717	Hypaconitine	TY000048	C33H45NO10	615.3	1818	-
10	463.26	9.38	0.618	Peonidin-3-O-beta-D-glucoside	PR020073	C22H23O11	463.12	3698	-
11	607.26	11.11	0.871	Embinin	TY000131	C29H34O14	606.19	242233	-
12	609.27	11.36	0.609	Neodiosmin	PR020040	C28H32O15	608.17	3668	-
13	609.27	11.64	0.841	Reserpine	CE000152	C33H40N2O9	608.27	3668	-
14	625.26	11.73	0.68	Isorhamnetin-3-O-rutinoside	PR020050	C28H32O16	624.17	70844	-
15	593.28	12.13	0.747	Acaciin	PR020027	C28H32O14	592.18	65564	-
16	1185.6	12.17	0.784	Fortunellin	PR020026	C28H32O14	592.18	34744	-
17	615.26	12.18	0.676	Amentoflavone	CE000045	C30H18O10	538.09	35740	-
Methanol extract of amla pulp (AP)									
1	138.06	0.43	0.704	Trigonelline*	KOX00886	C7H7NO2	137.05	285692	2.20 ± 0.01
2	265.04	1.45	0.702	Apomorphine	PB005947	C17H17NO2	267.13	9220	-
3	305.03	2.43	0.833	Naringin*	PB000804	C27H32O14	580.18	80434	92.52 ± 3.54
4	303.02	4.27	0.897	Quercetin*	PB006206	C15H10O7	302.04	332708	4.22 ± 0.12
5	287.05	4.45	0.811	Kaempferol*	PB005704	C15H10O6	286.05	720747	60.56 ± 0.50
6	463.09	4.72	0.626	Isorhamnetin	TY000220	C16H12O7	316.06	41076	-
7	540.31	6.04	0.587	Amentoflavone	CE000045	C30H18O10	538.09	483	-
8	557.18	6.35	0.7	Fukugetin	TY000181	C30H20O11	556.1	2323	-
9	677.37	9.33	0.723	Icariin	TY000037	C33H40O15	676.24	13852	-
10	593.28	12.12	0.742	Acaciin	PR020027	C28H32O14	592.18	29463	-
11	607.3	13.18	0.774	Embinin	TY000131	C29H34O14	606.19	35059	-
12	805.55	13.52	0.611	Loganin	TY000062	C17H26O10	390.15	32547	-
13	463.36	14.68	0.719	Scoparin	TY000252	C22H22O11	462.12	55784	-
14	611.5	16.55	0.67	Hesperidin	PR020046	C28H34O15	610.19	37450	-
15	158	18.66	0.611	Sotalol	EA017013	C12H20N2O3S	272.12	1785	-
16	184.97	18.7	0.627	Mecamylamine	KOX00784	C11H21N	167.17	124	-
17	96.96	19.54	0.669	Carbanilide, N,N'-dimethyl-	UF009613	C15H16N2O	240.13	771	-
18	291.09	3.35	0.148	Catechin*	PB006302	C15H14O6	290.08	6068	533.06 ± 8.72

^aThe retention times (in minutes) are according to the LC MS/MS system. ^bSimilarity score of retrieved spectrum to a query data. ^cTentative molecules were identified based on high score (Sumner et al., 2007). For details, please check materials and methods. *indicates confirmed metabolites by comparison of retention time and mass spectra against reference standards. [#]gdw = gram dry weight of amla seed/pulp. Quantification (mean ± SD) was done using HPLC standard curves.

Figures and their legends

Figure 1. Total phenolics, antioxidant activity, and anti-proliferative efficacy of serial solvent (**A and B**) or direct solvent extractions (**C and D**) of amla pulp and amla seed. Total phenolics and antioxidant activity (**A, C**) were determined by Folin-Ciocalteu Reagent and ABTS assay, respectively, as explained in the materials and methods. Anti-proliferative efficacy (**B, D**) was determined using BrdU assay by treating the HCCSCs with solvent control - DMSO, FU - 5-Flourouracil (25 or 50 µg/mL), and serial or direct solvent extracts of amla pulp or seed at 25, 50, or 100 µg GAE/mL for 20 h. Results were expressed as mean ± SE for three experiments at each time point. GAE – Gallic acid equivalents, TE – Trolox equivalents. The error bars with the same letter indicates no significance at $p < 0.05$.

Figure 2. Anti-proliferative efficacy of methanol extracts of amla seed (AS) or amla pulp (AP) on HCCSCs with functioning p53 and shRNA-attenuated p53 (A) and p53^{+/+} & p53^{-/-} cells of HCT116 (B). Cells were treated with 5-florouracil (25, 50, or 100 µg/mL), or methanol extracts

amla seed (AS) or amla pulp (AP) (25, 50, 100, or 200 μg GAE/mL) for 20 h and BrdU assay was performed as described in the methods. Results were expressed as mean $\pm$ SE for three experiments at each time point. The error bars with the same letter indicates no significance at $p < 0.05$.

Figure 3. Effect of methanol extracts of amla seed and pulp on apoptosis of HCCSCs (A) and HCCSC p53-shRNA cells (B). Cells were treated with IC₅₀ of 5-fluorouracil (75 $\mu\text{g}/\text{mL}$), methanol extract of amla seed (78 μg GAE/mL) or amla pulp (140 μg GAE/mL) and apoptosis was analyzed by measuring cleaved PARP using western blotting. Data are representative of three individual experiments, a representative blot is presented. C – Control, FU – 5-fluorouracil, AS – methanol extract of amla seed, and AP – methanol extract of amla pulp. Results were expressed as mean $\pm$ SE. The error bars with the same letter indicates no significance at $p < 0.05$.

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628 **Figure 4.** Effect of methanol extract of amla seed (AS) or pulp (AP) on pro- and anti-apoptotic
 629 protein expression. HCCSC cells were treated with IC₅₀ of methanol extract of AS (78 μ g
 630 GAE/mL) or AP (140 μ g GAE/mL) or 5-fluorouracil (75 μ g/mL). Cytoplasmic proteins were
 631 extracted by using NE-PER cytoplasmic extraction kit (Pierce) and then analyzed using western
 632 blotting for pro-apoptotic protein – Bax, and anti-apoptotic protein – Bcl-2. Data are
 633 representative of three individual experiments, a representative blot is presented. Results were
 634 expressed as mean $\pm$ SE. C- Control, FU – 5-fluorouracil, AS – methanol extract of amla seed,
 635 and AP – methanol extract of amla pulp. The error bars with the same letter indicates no
 636 significance at $p < 0.05$.

Figure 5. Amla suppresses the stem-ness of HCCSC via suppressed Wnt/β-catenin signaling. (A & B) Effect of methanol extracts of amla (AS or AP) on the stem-ness of HCCSCs. Cells were treated with 5-florouracil (FU, 25, 50, or 100 μg/mL) or methanol extracts of AS or AP (25, 50, 100, or 200 μg GAE/mL) for 24 h and colony formation assay was performed as described in methods. Representative images taken from the colony forming assay (A). (C) Effect of methanol extracts of amla (AS or AP) on Wnt/β-catenin signaling in HCCSCs. HCCSC cells were treated for 24 h with IC50 of methanol extracts of AS (78 μg GAE/mL) or AP (140 μg GAE/mL) or 5-florouracil (FU, 75 μg/mL). Cytoplasmic and nuclear proteins were extracted by using NE-PER cytoplasmic extraction kit and then analyzed by western blot for β-catenin, actin, c-Myc, cyclin D1, and DNA topoisomerase IIβ. Data are representative of three individual experiments, a representative blot is presented. Results were expressed as mean ± SE. C-

649 Control, FU – 5-Fluorouracil, AS – methanol extract of amla seed, and AP – Methanol extract of
650 amla pulp. The error bars with the same letter indicates no significance at $p < 0.05$.

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