Effect of Procyanidin-rich Extract from Natural Cocoa Powder on Cellular Viability, Cell Cycle Progression, and Chemoresistance in Human Epithelial Ovarian Carcinoma Cell Lines

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ABSTRACT

Background: Over the last 400 years, cocoa and chocolate have been described as having potential medicinal value, being consumed as a beverage or eaten as food. Concentration-dependant, antiproliferation, and cytotoxic effects of some of their polyphenolic constituents have been demonstrated against various cancers. Such an effect remains to be demonstrated in ovarian cancer. Objective: To investigate the effect of cocoa procyanidins against ovarian cancer in vitro using OAW42 and OVCAR3 cell lines. Materials and Methods: Cocoa procyanidins were extracted and enriched from non alkalized cocoa powder. The polyphenolic content and antioxidant activity were determined. Effect on cell viability was determined after the treatment with ≤1000 μg/mL cocoa procyanidin-rich extract on OAW42 and OVCAR3 and normal human dermal fibroblasts. Similarly, chemosensitization effect was determined by pretreating cancer cell lines with extract followed by doxorubicin hydrochloride treatment. The effect of treatment on cell cycle and P-glycoprotein (P-gp) expression was determined using flow cytometry. Results: The cocoa extract showed high polyphenolic content and antioxidant activity. Treatment with extract caused cytotoxicity and chemosensitization in OAW42 and OVCAR3 cell lines. Normal dermal fibroblasts showed an increase in cell viability post treatment with extract. Treatment with extract affected the cell cycle and an increasing percentage of cells in hypodiploid sub-G1 phase was observed. Treatment of OVCAR3 with the extract caused reduction of P-gp expression. Conclusion: Cocoa procyanidins were found to be selectively cytotoxic against epithelial ovarian cancer, interfered with the normal cell cycle and sensitized cells to subsequent chemotherapeutic treatment. Chemosensitization was found to be associated with Pgp reduction in OVCAR3 cells. Key words: Chemosensitization, cocoa procyanidins, epithelial ovarian cancer, P-glycoprotein

SUMMARY

• Among the naturally occurring flavonoids, procyanidins have been shown to be effective against cancers.
• Non alkalized cocoa powder is one of the richest sources of procyanidins.
• Cocoa procyanidin-rich extract (CPRE) caused cytotoxicity and chemosensitization in ovarian carcinoma cell lines OAW42 and OVCAR3.
• CPRE induced normal cell cycle progression.
• CPRE also downregulated P-glycoprotein, which mediates chemoresistance in multidrug-resistant OVCAR3 cell line.

INTRODUCTION

Ovarian cancer is a common and highly aggressive gynecological malignancy in women associated with a high mortality[1,2] and low 5 years survival rates, with epithelial ovarian cancer being the leading cause.[3] Current treatment strategies include a combination of surgical removal and chemotherapy using platinum-based drugs and taxanes. However, the disease has a poor prognosis with a high chance of relapse,[4] mainly attributed to the development of acquired resistance to chemotherapeutic drugs.[5] P-glycoprotein (P-gp), a membrane protein belonging to the ABC transporter family, is an efflux pump involved in the removal of chemotherapeutic drugs out of the cell and is implicated to be the main cause of resistance to the drugs. P-gp mediated drug resistance is common among a variety of cancers and the downregulation of P-gp is being targeted as an approach to counteract the acquired resistance in cancer cells. As conventional chemotherapeutics not only pose the problem of acquired resistance but also nonselctive cytotoxicity toward normal cells, use of naturally occurring compounds such as
Polyphenols in plants is being explored as an alternative approach in cancer treatment.[6] Polyphenols, especially flavonoids, are a widely distributed class of secondary metabolites in plants with varied health benefits, specifically as anticancer agents.[16,19] Among flavonoids, procyanidins have garnered attention for their anticancer, antiangiogenic, anti-inflammatory, and cardioprotective effects, owing to their high antioxidant and pro-oxidant activity.[10,11] They have also been proven to be potent P-gp inhibitors in immortalized cell lines and the blood-brain barrier.[20] Rich sources of procyanidins include many regularly consumed foods such as apples, cocoa and cocoa products, berries, and grapes.[21] Among these, cocoa (Theobroma cacao L., Sterculiaceae) and its products, such as non alkaliized cocoa powder, are considered to be one of the richest sources of catechins and procyanidins.[13,14] Cocoa, native to South America, was cultivated more than 1500 years ago by the Mayas.[15] Consumption of cocoa-derived products has been shown to offer a wide range of health benefits such as cardioprotection, reduction of chronic inflammation, anti-inflammatory, and cancer prevention as evident through various human intervention and cohort studies.[16,19]

In this study, non alkaliized cocoa powder was used to extract and enrich procyanidins using solvent extraction. The cocoa procyanidin-rich extract (CPRE) was screened for phytochemicals, polyphenolic content, and antioxidant activity. The effect on cell viability was evaluated in vitro using OAW42[21] and multidrug refractory OVCAR3[13] epithelial ovarian cancer cell lines. The effect of extract cotreatment with doxorubicin was determined in order to establish chemosensitizing effect. Effect of the extract on the cell cycle was evaluated. Furthermore, the involvement of P-gp expression in the development of acquired drug resistance was assessed using flow cytometry.

MATERIALS AND METHODS

Source material
Non alkalized cocoa powder was purchased from Morde Foods Pvt. Ltd, Mumbai, India. As per the Certificate of Analysis (No. FRM/QC/026) provided by the manufacturer, the cocoa powder met the required standards of appearance, flavor, aroma, total fat (11.2%), and moisture content (21.4%), particle size (200 mesh), total (7.78%), and acid insoluble ash content (0.22%). The powder was stored in vacuum packaged polyethylene pouches at room temperature until use. All experiments were performed using only one batch of obtained material.

Chemicals and reagents
4-dimethylaminocinnamaldehyde (DMAC), diphénylpicrylhydrozayl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and propidium iodide (PI) were purchased from Sigma Aldrich, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from HiMedia Laboratories Pvt. Ltd., France. Gallic acid was purchased from Loba Chemie Pvt. Ltd., India, (+) catechin from Natural Remedies Pvt. Ltd, India, and procyanidin B2 from Santa Cruz Biotechnology, USA. Doxorubicin hydrochloride API was a generous gift from Veeda Clinical Research Pvt. Ltd., Mumbai, India. As per the Certificate of Analysis (No. FRM/QC/026) the product was a brown dry powder expected to contain a high quantity of procyanidins, and the extraction yield was calculated. This powder was labeled as CPRE and stored at room temperature for further analysis.

Evaluation of antioxidant activity
The original method of Blois[27] was slightly modified and used for the determination of scavenging activity of DPPH free radical. CPRE was dissolved and diluted in methanol, ranging from 15 to 40 μg/mL. 400 μL of 0.9 mM DPPH prepared in methanol was added to each tube. To set up a reaction mixture, 20 μL each of various standard dilutions and extract were diluted with 2.38 mL of methanol. About 2.4 mL of methanol was taken in the blank tube. To each of the tubes, 100 μL of 2% DMAC (prepared in chilled 1:1 6N H2SO4:methanol, v/v) was added and incubated at room temperature for 15 min. The absorbance was measured at 640 nm. The results were expressed as mg gallic acid equivalents or mg GAE/g of extract.

Phytochemical screening
Phytochemical screening using standard methods. The preliminary tests were carried out for the detection of carbohydrates (Molisch test), proteins (Biuret test), flavonoids (NaOH test and lead acetate test), tannins, and phenols (Ferric chloride test) and alkaloids (Dragendorff’s test, Mayer's test, and Wagner’s test).

Determination of total phenol and total procyanidin content
Total phenol content of CPRE was determined using a modified folin-Ciocalteau method.[28] Gallic acid (20–100 μg/mL) in methanol was used as standard. CPRE (1 mg/mL, 1:5 v/v diluted) was used as the test sample. The final volume was kept at 1 mL. A blank containing 1 mL of methanol was maintained. About 5 mL of folin-ciocalteau reagent (1:10 v/v in distilled water) was added to each tube. After 5 min, 4 mL of 7.5% sodium carbonate was added and allowed to react for 15 min at room temperature. The absorbance was measured at 760 nm using ultraviolet (UV) visible spectrophotometer (Lambda 25, Perkin Elmer, Inc., USA). The results were expressed as mg gallic acid equivalents or mg GAE/g of extract. Total procyanidins in CPRE were determined by colorimetric reaction with DMAC.[26] Procyanidin B2 (10–60 μg/mL) in methanol was used as a standard. CPRE (1 mg/mL) was diluted 1:5 with methanol. To set up a reaction mixture, 20 μL each of various standard dilutions and extract were diluted with 2.38 mL of methanol. About 2.4 mL of methanol was taken in the blank tube. To each of the tubes, 100 μL of 2% DMAC (prepared in chilled 1:1 6N H2SO4:methanol, v/v) was added and incubated at room temperature for 15 min. The absorbance was measured at 516 nm. The results were expressed as mg procyanidin B2 equivalents or mg PB2E/g of extract.

Extraction of polyphenols and enrichment of procyanidins from non alkalized cocoa powder
Non alkalized cocoa powder (25 g) was defatted in n-hexane (cocoa powder: n-hexane, 1:4, w/v) on a shaker for 1 h. The solvent was decanted, and the powder was allowed to dry overnight. Defatted material was extracted with 70% aqueous acetone using ultrasonication (3 cycles × 250 mL, 30 min per ultrasonication cycle).[22] The extracts were pooled and filtered through Whatman paper No. 1. An equal volume of ethyl acetate was added to the extract; the mixture was transferred to a separating funnel, shaken vigorously, and allowed to stand to enable separation. The lower aqueous layer was re-extracted with an equal volume of ethyl acetate. Liquid-liquid extraction with ethyl acetate[23] was repeated one more time. The upper organic layer fractions were pooled and dried to give pale brown dry powder expected to contain a high quantity of procyanidins, and the extraction yield was calculated. This powder was labeled as CPRE and stored at room temperature for further analysis.
The radical scavenging activity was calculated as follows:

\[
\text{% Scavenging activity} = \left(\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{untreated}}}{{\text{Absorbance}}_{\text{control}}}\right) \times 100.
\]

Results were represented as IC\(50\) in µg/mL.

Antioxidant activity of CPRE was determined by ferric reducing power assay, modified from the original method.\(^{29}\) Various concentrations of the extract (20–100 µg/mL) in methanol were mixed with 0.2M sodium phosphate buffer and 1% potassium ferricyanide (2.5 mL each). This mixture was kept at 50°C for 20 min. About 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. The upper layer of solution was mixed with distilled water, and 0.5 mL of freshly prepared 0.1% ferric chloride solution was added to it. The absorbance was measured at 700 nm. Control was prepared in similar manner wherein extract/standard was replaced with methanol. The increase in reducing power is indicated by an increase in absorbance value. The reducing power was represented as EC\(50\) (effective concentration having 0.5 absorbance value) L-ascorbic acid was used as a standard for all the anti-oxidant activity assays.

Thin layer chromatography

Thin layer chromatography (TLC) was carried out on precoated silica gel F254 plates (0.2 mm, Merck, Darmstadt, Germany) as stationary phase and ethyl acetate: Glacial acetic acid: Formic acid: Methanol (7.5:0.2:0.3:1, v/v) as mobile phase. Plates were derivatized using 1% DMAC in 3M hydrochloric acid\(^{30}\) that is specific for proanthocyanidins (+) catechin was used as standard. Antioxidant bioautography\(^{31}\) using 0.1% DPPH as spray reagent was carried out. High-performance TLC (HPTLC) study was carried out for confirming the presence of (+) catechin and procyanidin B2 reported to be present in cocoa powder.\(^{32}\) HPTLC fingerprinting was performed on precoated silica gel F254 plates at room temperature. Solutions of standards and sample were applied to the plates using the Camag (Muttenz, Switzerland) linomat V sample applicator equipped with a 100 µL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed using the same mobile phase as that used in aforementioned TLC, in a Camag glass twin trough chamber saturated with mobile phase vapor for 25 min. After development, the plates were dried and then scanned using a Camag TLC scanner with WINCAT software (Camag, Switzerland).

Cell culture and conditions

OAW42 and OVCAR3 cell lines were a generous gift from Dr. Sharmila Bapat from National Centre for Cell Sciences, Pune, India. OAW42 and OVCAR3 were maintained and propagated in Dulbecco’s Modified Eagle’s Medium and Roswell Park medium Institute 1640 and streptomycin (100 µg/mL medium) and incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\).

Assessment of in vitro effect of cocoa procyanidin-rich extract on cell viability and chemosensitization

Effect of CPRE on cell viability of ovarian cancer cell lines was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. OAW42 and OVCAR3 were seeded in 96 well plates at 3 \times 10^3 cells/well and 1.5 \times 10^4 cells/well, respectively, and incubated for 24 h. Cells were treated with 400, 600, 800, and 1000 µg/mL of CPRE in serum free media. OAW42 was treated for 24 h and OVCAR3 for 72 h. During post treatment, medium in each well was discarded and wells were washed with DPBS (Dulbecco’s PBS). About 100 µl serum-free medium containing MTT (0.5 mg/mL) was added to each well. The plate was incubated at 37°C for 4 h. Media was removed, and the purple formazan product was dissolved by addition of 150 µl dimethylsulfoxide. Absorbance was recorded after 15 min at 570 nm against a reference wavelength of 655 nm.

The cell viability was calculated as follows:

\[
\text{% Cell viability} = \left(\frac{\text{absorbance of treated cells/absorbance of untreated cells}}{100}\right) \times 100,
\]

The effect of CPRE on the viability of human dermal fibroblasts was also evaluated similarly.

For evaluating chemosensitization, the cells were pretreated with suboptimal IC\(50\) concentrations of CPRE for OAW42 and OVCAR3 for their respective effective period, followed by doxorubicin hydrochloride (1 µg/mL) treatment for 24 h. Controls were maintained for doxorubicin or extract treatment alone. Results were reported as percentage cell viability.

Cell cycle analysis

Effect of CPRE on the cell cycle was evaluated by staining of cells with PI followed by flow cytometry\(^{32}\) OAW42 and OVCAR3 were seeded in 6 well plates at a seeding density of 0.3 \times 10^6 cells/well and 0.5 \times 10^6 cells/well, respectively, and incubated for 24 h. Cells were treated with 600, 800, and 1000 µg/mL of CPRE for 24 h in serum free media. Doxorubicin hydrochloride was used as a positive control. Post treatment, the cells were trypsinized and fixed with cold 70% ethanol. Cells were centrifuged washed with DPBS twice to remove traces of ethanol. About 0.8 µl of DNAase-free RNase was added to each tube and incubated at 37°C for 10 min. Staining was carried out by adding 500 µl of PI (30 µg/mL) to each tube. Flow cytometry data was acquired for 10,000 cells using BD FACSaria system and BD FACSDiva software (BD Biosciences, USA). The data were analyzed offline using ModFit LT 4.1 software, Verity Software House, USA.

Analysis of P-glycoprotein expression

For evaluating P-gp expression, OVCAR3 cells were seeded onto 6 well plates, at a density of 0.5 \times 10^6 cells/well and incubated for 24 h. The cells were treated with 600, 800, and 1000 µg/mL of CPRE for 24 h in respective serum free media. Doxorubicin hydrochloride was used as a positive control. After incubation, the cells were washed with DPBS, trypsinized, and centrifuged at 1200 rpm for 5 min. The cells were the resuspended in DPBS containing 10% heat-inactivated FBS, and P-gp antibody (UC2) conjugated with FITC (diluted as per manufacturer’s instructions). Mouse IgG2a-FITC was used as an isotype control. After incubation with antibody for 30 min at 37°C in the dark, the cells were washed twice with DPBS containing 10% FBS, suspended in ice-cold PBS buffer, and kept on ice until analysis. Flow cytometry data was acquired for 10,000 cells using BD FACSaria system and BD FACSDiva software (BD Biosciences, USA). Nonlabeled cells were analyzed in order to detect autofluorescence. FITC fluorescence indicated cells bound to the labeled P-gp antibody and therefore, positive for P-gp expression.

Statistical analysis

All quantitative experiments were carried out in triplicate (n = 3). Results were expressed as mean ± standard deviation. Regression analysis to calculate IC\(50\) and EC\(50\) values was done using Microsoft Excel Version 2007 (Microsoft Corporation, USA). Statistical analysis was carried out using GraphPad Prism 5.0 software (GraphPad Software, Inc., USA) using one-way and two-way ANOVA. Results at P < 0.05 were considered significant.

RESULTS

Cocoa procyanidin-rich extract was found to be rich in polyphenols and possessed high anti-oxidant activity

The qualitative phytochemical screening of CPRE indicated the presence of flavonoids, such as tannins and phenols. Alkaloids were found to be
Cocoa procyanidin-rich extract was selectively cytotoxic to cancer cells and sensitized them to doxorubicin treatment

CPRF exerted cytotoxicity on OAW42 [Figure 2.i] and OVCAR3 [Figure 2.ii] posttreatment of 24 and 72 h, respectively. The IC50 obtained for OAW42 was 863.84 ± 115.04 µg/mL and for OVCAR3 was 896.84 ± 70.01 µg/mL. No cytotoxicity was observed on normal human dermal fibroblasts. On the contrary, there was an increase in cell viability, indicated by increased formation of formazan product in treated cells as compared to untreated cells (data not shown). Successive treatment of cells with CPRF followed by doxorubicin hydrochloride caused significantly higher cytotoxicity in both OAW42 and OVCAR3 as compared to treatment with either of them alone [Figure 2.iii and 2.iv], thus demonstrating chemosensitization.

Appearance of hypodiploid sub-G1 phase in treated cells indicated possible DNA damage and cell death

CPRF was found to interfere with normal cell cycle progression in both cell lines. Treatment of OAW42 and OVCAR3 with various concentrations of CPRF showed a significant percentage of cells in sub-G1/G0 (hypodiploid) phase, which increased with increasing concentration. Significant accumulation of cells in the S phase was seen in OVCAR3 cells treated with 1000 µg/mL of CPRF. Doxorubicin hydrochloride, used as positive control, showed a significant percentage of cells arrested in S-phase and in sub-G1/G0 phase [Figure 3].

Reduced P-glycoprotein expression was observed posttreatment with cocoa procyanidin-rich extract

Effect of CPRF on P-gp expression was evaluated in OVCAR3 cells only, as OAW42 cells did not show initial P-gp expression (data not shown). P-gp expression was found to decrease post treatment with CPRF, as compared to untreated control and the results were statistically significant [Figure 4]. No nonspecific binding of the isotype control was observed (data not shown), indicating that the labeled P-gp antibody bound specifically to P-gp present on the cell membrane.

DISCUSSION

Cocoa and its products have been consumed since the Mesoamerican civilization, later spreading to Spain and Christian European countries. Over the last 400 years, many medicinal uses have been revealed for cocoa or chocolate. Cocoa polyphenols are associated with many medicinal properties such as antiangiogenic, cardioprotective, cosmetic, anti-inflammatory and anticancer. Dutching or alkalization of naturally obtained cocoa powder leads to a loss in the polyphenol content. Therefore, nonalkalized cocoa powder was used as source material to successfully extract and enrich procyanidin group of compounds, which are polymeric condensation products of catechins (flavan-3-ols). The resultant extract possessed high polyphenol content, enriched with (+)-catechin, procyanidin B2, and other related compounds. It also possessed efficient antioxidant activity. Several factors such as the choice of solvent, method of extraction influence the extraction yield, and polyphenol content. Moreover, cocoa powder from different sources may differ in polyphenol content due to cultivar, conditions of growth, sampling, and analytical procedures. The effect of CPRF on cell viability was evaluated in vitro on OAW42 and OVCAR3 cell lines using the MTT assay. Reduced cellular viability was observed in both cell lines and was measured in terms of IC50 value. CPRF was found to be differentially cytotoxic to OAW42 and OVCAR3.
Figure 2: Percentage viability of treated (i) OAW42 and (ii) OVCAR3 cells by MTT assay. Chemosensitization assay for (iii) OAW42 and (iv) OVCAR3 represented as percentage viability versus treatment given to the cells. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni’s posttest. Results were considered to be significant at $P < 0.05$. The significance amongst treatment groups is denoted as lower case alphabets placed on top of the error bars, representing the following comparisons: (a) Treated groups versus untreated group; (b) only doxorubicin versus other treatment groups; (c) 600 μg/mL versus other treatment groups; (d) 800 μg/mL versus other treatment groups (e) 600 μg/mL versus 800 μg/mL treatment group.

Figure 3: Cell cycle distribution represented as histograms for (a) OAW42 (b) OVCAR3. (c and d) graphical representation of statistical significance within each phase for OAW42 and OVCAR3, respectively. Statistical analysis was carried out using two-way ANOVA followed by Bonferroni’s posttest at significance $^*P < 0.05$ for treated cells as compared to untreated control within each phase.
μ01 contributes to the toxicity of polyphenols against cancer. demonstrated good anti-oxidant activity, which is one of the factors that extracted and enriched from natural cocoa powder. The CPRE or CPRE of its kind, to the best of our knowledge. Procyanidins were successfully ovarian cancer cell lines OAW42 and OVCAR3. This is the first report compounds enriched from non alkalized or natural cocoa powder on The study demonstrates cytotoxic effect of procyanidin group of compounds enriched from non alkalized or natural cocoa powder on ovarian cancer cell lines OAW42 and OVCAR3. This is the first report of its kind, to the best of our knowledge. Procyanidins were successfully extracted and enriched from natural cocoa powder. The CPRE or CPRE demonstrated good anti-oxidant activity, which is one of the factors that contributes to the toxicity of polyphenols against cancer.52 The extract was found to be selectively cytotoxic to ovarian cancer cells and sensitized them to doxorubicin. Chemosensitization of the cells was accompanied by decrease in P-gp levels in OVCAR3 cells treated with the extract. Both OAW42 and OVCAR3 cells showed a dose-dependant increase of cell population in the hypodiploid sub-G0 phase on treatment with CPRE, indicating irreversible DNA damage which may have contributed to its cytotoxic effect. CPRE has shown therapeutic potential against ovarian cancer in vitro, by interfering with the regular cell cycle progression and overcoming chemoresistance conferred by P-gp. Efforts to elucidate the mechanism by which CPRE exerts toxicity and to identify of the molecular components involved are currently underway.

**CONCLUSION**

The study demonstrates cytotoxic effect of procyanidin group of compounds enriched from non alkalized or natural cocoa powder on ovarian cancer cell lines OAW42 and OVCAR3. This is the first report of its kind, to the best of our knowledge. Procyanidins were successfully extracted and enriched from natural cocoa powder. The CPRE or CPRE demonstrated good anti-oxidant activity, which is one of the factors that contributes to the toxicity of polyphenols against cancer.52 The extract was found to be selectively cytotoxic to ovarian cancer cells and sensitized them to doxorubicin. Chemosensitization of the cells was accompanied by decrease in P-gp levels in OVCAR3 cells treated with the extract. Both OAW42 and OVCAR3 cells showed a dose-dependant increase of cell population in the hypodiploid sub-G0 phase on treatment with CPRE, indicating irreversible DNA damage which may have contributed to its cytotoxic effect. CPRE has shown therapeutic potential against ovarian cancer in vitro, by interfering with the regular cell cycle progression and overcoming chemoresistance conferred by P-gp. Efforts to elucidate the mechanism by which CPRE exerts toxicity and to identify of the molecular components involved are currently underway.

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**Conflicts of Interest**

There are no conflicts of interest.

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Aparna Khanna obtained her Ph.D. degree in 1995 from Jawaharlal Nehru University, New Delhi, while working at the National Institute of Immunology. Dr. Khanna was a Wellcome Trust Postdoctoral fellow (1995–1996) at the University of Glasgow, UK. She currently holds the position of Dean at the Sunand Divata School of Science, NMIMS (deemed to be) University, Mumbai, India. Dr. Khanna has been working in the area of stem cells since the year 2003, with expertise in establishment and characterization of cell cultures in vitro. Currently, she is involved in projects related to stem cell imaging and differentiation as well as evaluation of phytoextracts for hepatoprotective and anticancer activity, using cell lines as a model system.