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ORIGINAL ARTICLE

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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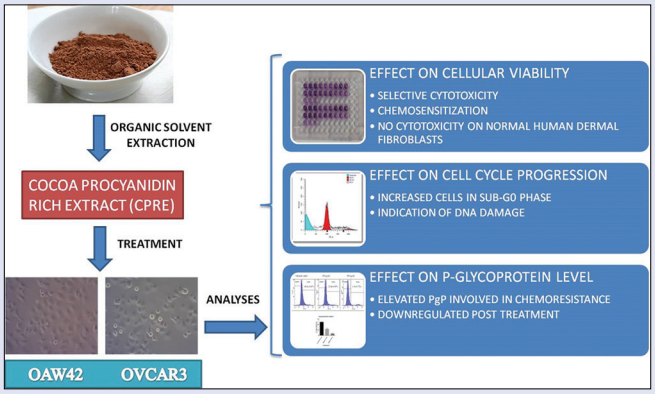
ABSTRACT

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-G_1/G_0 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 P-gp 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 affected normal cell cycle progression
- CPRE also downregulated P-glycoprotein, which mediates chemoresistance in multidrug-resistant OVCAR3 cell line.



Abbreviation used: P-gp: P-glycoprotein, CPRE: Cocoa procyanidin rich extract, DMAC: 4-dimethylaminocinnamaldehyde, DPPH: Diphenylpicrylhydrazyl, ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), PI: Propidium iodide, FITC: Fluorescein isothiocyanate, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, TLC: Thin layer chromatography, HPTLC: High-performance thin layer chromatography.

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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 nonselective cytotoxicity toward normal cells, use of naturally occurring compounds such as

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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.[7-9] Among flavonoids, procyanidins have garnered attention for their anticancer, antiageing, antihypertensive, 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.[6] Rich sources of procyanidins include many regularly consumed foods such as apples, cocoa and cocoa products, berries, and grapes.[12] Among these, cocoa (*Theobroma cacao* L., Sterculiaceae) and its products, such as non alkalized 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, antihypertensive effect, and cancer prevention as evident through various human intervention and cohort studies.[16-19]

In this study, non alkalized 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[20] and multidrug refractory OVCAR3[21] epithelial ovarian cancer cell lines. The effect of extract cotreatment with doxorubicin was determined in order to establish chemo sensitizing 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 (2.14%), 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), diphenylpicrylhydrazyl (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., India. Cell culture media and reagents were purchased from Genetix Biotech Asia Pvt. Ltd., India. P-gp monoclonal antibody (clone UIC2) and IgG2a isotype control, conjugated with fluorescein isothiocyanate (FITC), were purchased from Abcam, UK. All the other analytical chemicals and solvents were purchased from Fisher Scientific, USA.

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.

Phytochemical screening

Qualitative phytochemical screening of CPRE was performed using standard methods.[24] 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.[25] 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 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 H_2SO_4: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 procyanidin B2 equivalents or mg PB2E/g of extract.

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. A control tube containing methanol was maintained. The reaction mixture was incubated in dark at room temperature for 30 min. The absorbance was measured at 516 nm.

Radical scavenging activity of CPRE was also evaluated using the ABTS assay. The monocation of $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate.[28] The $ABTS^{\bullet+}$ was produced by allowing 7 mM ABTS stock solution to react with 2.45 mM potassium persulfate (final concentration) in the dark for 14–16 h before use, at room temperature. The $ABTS^{\bullet+}$ solution was diluted with phosphate buffered saline (PBS, pH 7.4) to an absorbance value of 0.70 (±0.02) at 734 nm. To 20 µl of CPRE or standard diluted in methanol, 1 mL of this solution was added. Concentrations of the extract ranged from 100 to 500 µg/mL. Absorbance was measured at 734 nm.

The radical scavenging activity was calculated as follows:

% Scavenging activity = ([Absorbance$_{control}$ − Absorbance$_{test}$)/ Absorbance$_{control}$] × 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 $EC_{0.5}$ (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 procyanidins (+) 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.[13] 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 3×10^3 cells/well and 1.5×10^4 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:

% Cell viability = (absorbance of treated cells/absorbance of untreated cells) *100. IC_{50}, i.e., the concentration toxic to 50% of the cell population was calculated. 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 0.3×10^6 cells/well and 0.5×10^6 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 DNAse-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 (50 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 0.5×10^6 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 (UIC2) 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 $EC_{0.5}$ 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

absent. The percentage yield was 3.53%. The total polyphenolic content was found to be 143.04 ± 3.41 mg GAE/g of extract, and the total procyanidin content was 65.99 ± 9.12 mg PB2E/g of extract. TLC showed the presence of flavonoids characterized by quenching of short UV radiation at 254 nm [Figure 1a]. The ethyl acetate fraction showed the enriched presence of procyanidin group of compounds on derivatization with 1% DMAC reagent as highly specific green colored bands [Figure 1b] that are characteristic to procyanidin group of compounds.[26] HPTLC fingerprinting of the extract confirmed the presence of (+) catechin (Rf value −0.68) and procyanidin B2 (Rf value − 0.62) by comparison of the spectral scan of extract with those of the two standards [Figure 1c].

TLC autography using 0.1% DPPH showed maximum antioxidant activity in the ethyl acetate fraction as compared to the aqueous and crude extract, with antioxidant constituents appearing as yellow bands against a purple background [Figure 1b].[33] Thus, the radical scavenging activity of this fraction was evaluated using DPPH and ABTS assays, and the IC_{50} values were 31.75 ± 0.94 μg/mL and 294.15 ± 38.4 μg/mL, respectively. The fluorescence recovery after photobleaching $EC_{0.5}$ value for the extract was 266.72 ± 14.85 μg/mL. The difference in values might be due to the different mechanisms and reaction characteristics of each assay.[34]

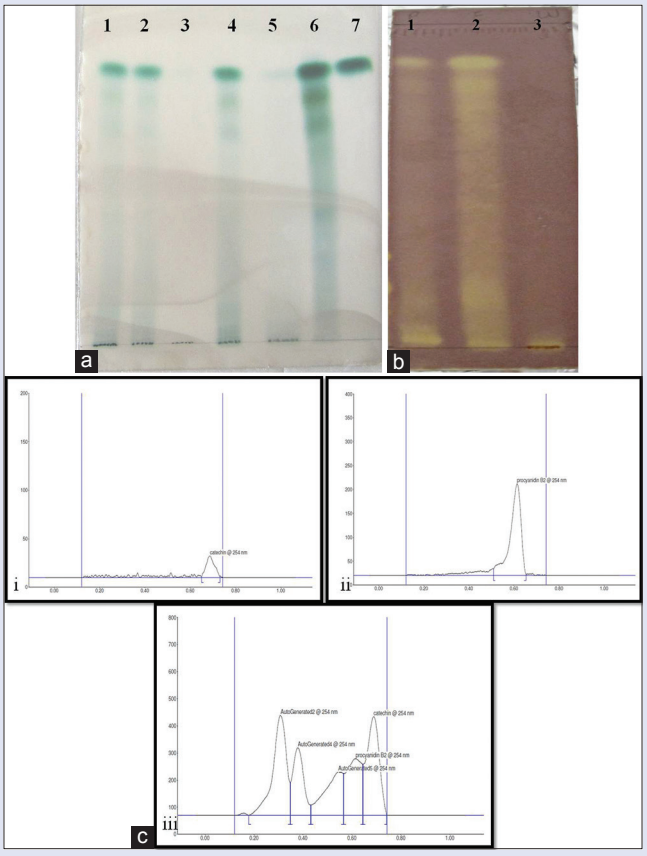

Figure 1: Thin layer chromatography of extract at various extraction stages. (a) Derivatized with acidified 1% 4-dimethylaminocinnamaldehyde. Lane 1: Extraction cycle 1, Lane 2: Extraction cycle 2, Lane 3: Extraction cycle 3, Lane 4: Pooled crude extract, Lane 5: Aqueous fraction, Lane 6: Ethyl acetate fraction and Lane 7: (+)-catechin. (b) Thin layer chromatography bioautography of cocoa extracts with 0.1% diphenylpicrylhydrazyl. Lane 1: Pooled extract, Lane 2: Ethyl acetate extract, Lane 3: Water fraction of extract. (c) Ultraviolet spectra analysis of standards and extracts after high-performance thin layer chromatography. (i) (+)-catechin, (ii) procyanidin B2 and (iii) ethyl acetate extract of cocoa powder

Cocoa procyanidin-rich extract was selectively cytotoxic to cancer cells and sensitized them to doxorubicin treatment

CPRE exerted cytotoxicity on OAW42 [Figure 2.i] and OVCAR3 [Figure 2.ii] posttreatment of 24 and 72 h, respectively. The IC_{50} 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 CPRE 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-G_1/G_0 phase in treated cells indicated possible DNA damage and cell death

CPRE was found to interfere with normal cell cycle progression in both cell lines. Treatment of OAW42 and OVCAR3 with various concentrations of CPRE showed a significant percentage of cells in sub-G_1/G_0 (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 CPRE. Doxorubicin hydrochloride, used as positive control, showed a significant percentage of cells arrested in S-phase and in sub-G_1/G_0 phase [Figure 3].

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

Effect of CPRE 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 CPRE, 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.[35] Over the last 400 years, many medicinal uses have been revealed for cocoa or chocolate.[36] Cocoa polyphenols are associated with many medicinal properties such as antiangiogenic,[37] cardioprotective,[38] cosmetic,[39] anti-inflammatory,[40] and anticancer.[41] Dutching or alkalization of naturally obtained cocoa powder leads to a loss in the polyphenol content.[42] Therefore, non alkalized 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.[43] Moreover, cocoa powder from different sources may differ in polyphenol content due to cultivar, conditions of growth, sampling, and analytical procedures.[14,44]

The effect of CPRE 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 IC_{50} value. CPRE was found to be differentially cytotoxic to OAW42

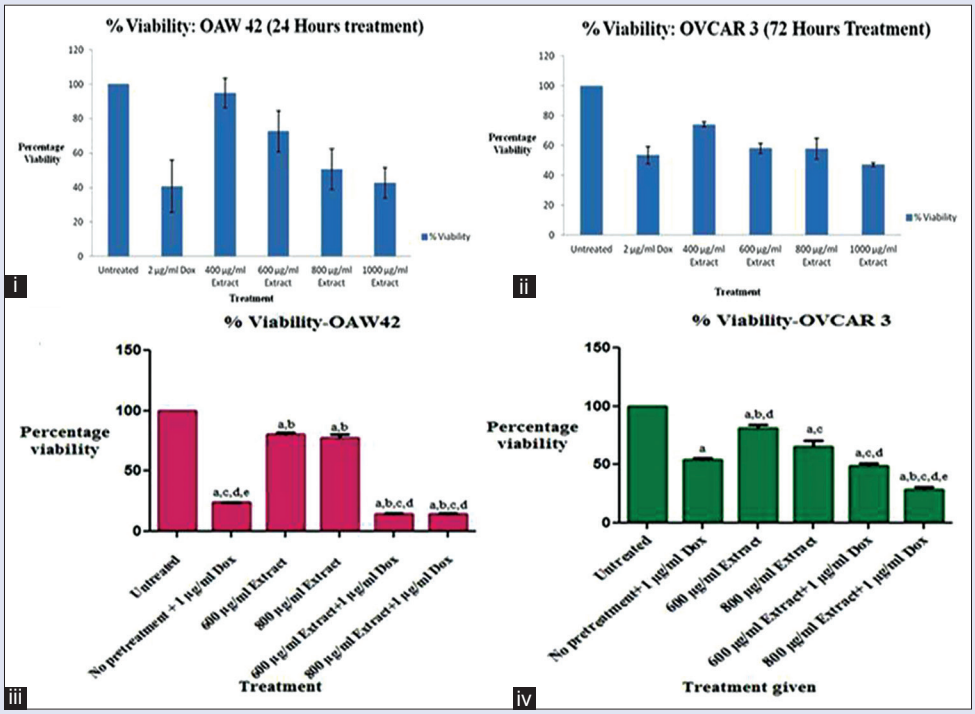

Figure 2: Percentage viability of treated (i) OAW42 and (ii) OVCAR3 cells by MTT assay. Chemosensitization assay for (iii) OAW42 and (iv) OVCAR3 represented as percentage viability versus treatment given to the cells. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's posttest. 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

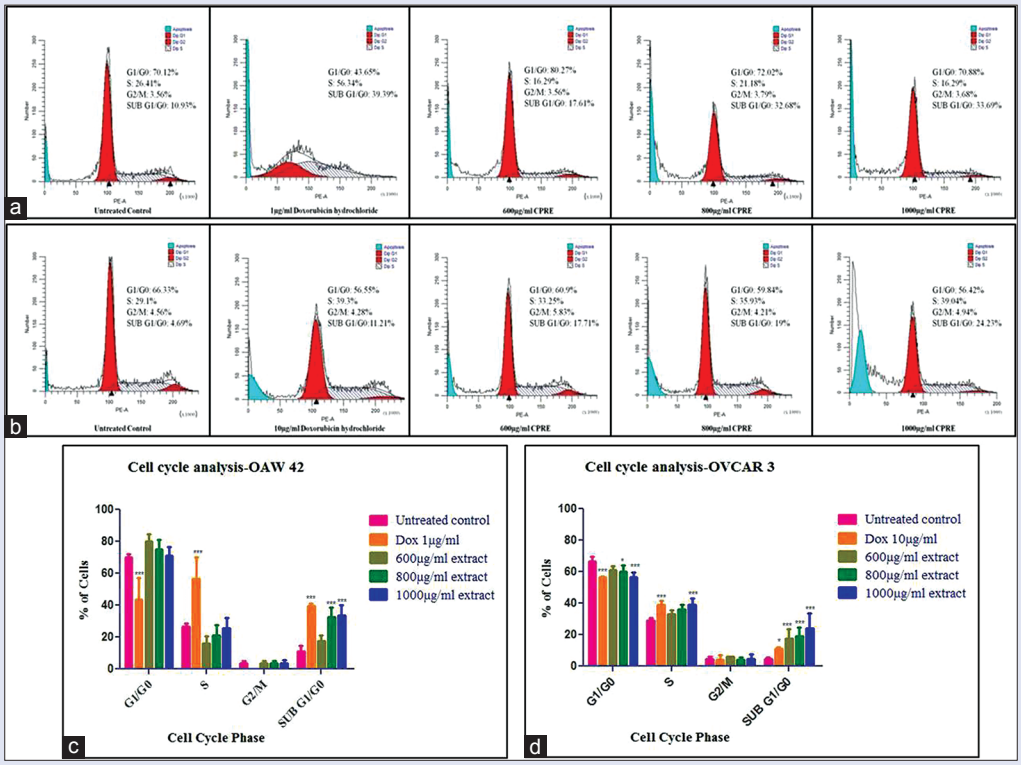

Figure 3: Cell cycle distribution represented as histograms for (a) OAW42 (b) OVCAR3. (c and d) graphical representation of statistical significance within each phase for OAW42 and OVCAR3, respectively. Statistical analysis was carried out using two-way ANOVA followed by Bonferroni's posttest at significance $*P < 0.05$ for treated cells as compared to untreated control within each phase

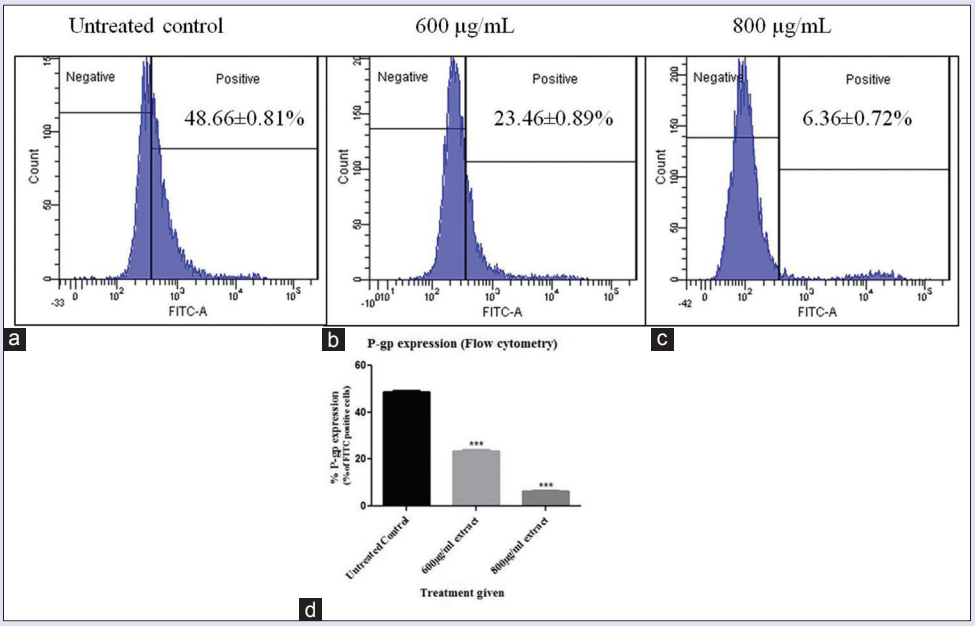

Figure 4: P-glycoprotein expressing (fluorescein isothiocyanate positive) OVCAR3 cells represented as histograms for (a) untreated cells (b) 600 µg/mL extract treatment (c) 800 µg/mL extract treatment and (d) graphical representation of statistical significance. Statistical analysis was carried out using one-way ANOVA followed by Dunnett's posttest, and results were significant at $P < 0.05$ as compared to the untreated control

(IC_{50} = 863.84 ± 115.04 µg/mL, 24 h treatment) and OVCAR3 (IC_{50} = 896.84 ± 70.01 µg/mL, 72 h treatment). OVCAR3 not only has a higher doubling time than OAW42 but is also drug resistant.[45,46] This difference in duration of treatment could be attributed to the fact that OAW42 divides more rapidly than OVCAR3. Previously, procyanidins from cranberry have been shown to be more cytotoxic to rapidly dividing cells.[47] CPRE treatment was found to be nontoxic to normal human dermal fibroblasts. Conversely, it caused an increase in viability of human dermal fibroblasts by virtue of the antioxidant properties of polyphenolic constituents.[48] Treatment with CPRE demonstrated selective cytotoxicity of the extract toward cancer cells, leaving the normal cells unharmed.

Targeting the cell cycle is one of the approaches in cancer therapy.[49] CPRE treatment on both, OAW42 and OVCAR3, interfered with the normal cell cycle progression. An increasing trend was observed in the appearance of the hypodiploid sub-G_1/G_0 phase, with increasing CPRE concentration, indicative of DNA damage and cell death.[32]

Pretreatment of OAW42 and OVCAR3 with CPRE augmented the cytotoxic effect of subsequent doxorubicin hydrochloride treatment. A previous report has similarly demonstrated chemosensitizing effect of procyanidins on another ovarian cancer cell line, SKOV3.[50] OVCAR3 is a multidrug-resistant cell line having acquired resistance to clinically relevant concentrations of doxorubicin, cisplatin, and melphalan.[21] Acquired multidrug-resistance, though governed by a variety of cellular mechanisms,[51] is largely linked to the over-expression of P-gp. OVCAR3 cells treated with extract showed reduced P-gp levels as compared to those of untreated cells, indicative of lowered resistance and subsequent chemosensitization of cancer cells.[6]

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-G_0 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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