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In vitro anti-cancer effect of Crataegus oxyacantha berry extract on hormone receptor positive and triple negative breast cancers via regulation of canonical Wnt signalling pathway

Salini K (salinik1@hotmail.com)
University of Madras - Guindy Campus https://orcid.org/0000-0002-3621-2879
Niranjali Devaraj Sivasithamparam
University of Madras - Guindy Campus https://orcid.org/0000-0001-6371-8552

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In vitro anti-cancer effect of *Crataegus oxyacantha* berry extract on hormone receptor positive and triple negative breast cancers via regulation of canonical Wnt signalling pathway

Salini Kombiyil[a] and Niranjali Devaraj Sivasithamparam[a]
[a]Department of Biochemistry, University of Madras, Guindy campus, Chennai, 600025, India

Corresponding author: Niranjali Devaraj Sivasithamparam
E-mail ID: niranjali@yahoo.com; profniranjali@gmail.com

ORCID ID: Salini K- https://orcid.org/0000-0002-3621-2879
S. Niranjali Devaraj: https://orcid.org/0000-0001-6371-8552

Abstract

Breast cancer treatment strategy depends mainly on the receptor status. Our aim was to identify a herbal preparation, effective against breast cancer, irrespective of hormone sensitivity, and to understand its molecular mechanism. The rich antioxidant composition of Hawthorn (*Crataegus oxyacantha*) makes it a promising anti-cancer drug candidate. Polyphenol-rich methanolic extract of *C. oxyacantha* berry (M.Co) was found to be cytotoxic on hormone receptor positive (MCF-7) and triple negative (MDA-MB-231) breast cancer cell lines, at a dose (75 μg/ml) safe on normal cells. It could effectively inhibit tumor cell proliferation and arrest cell cycle at G1/S transition in both cell lines. Molecular targets were selected from different levels of canonical Wnt signalling pathway (such as autocrine and antagonistic ligands, receptor, effector, cytoplasmic components, downstream targets and pathway antagonist), since they are frequently found dysregulated in all breast cancers and their aberrant activation is associated with cancer stem cell expansion. M.Co could significantly downregulate the expression of Wnt pathway agonists and upregulate that of Wnt antagonists at transcriptional and translational levels, in both cell lines. To conclude, *C. oxyacantha* berry extract is effective against breast cancer irrespective of its hormone dependency and cancer growth inhibition at stem cell level can be expected.

Key words:

ER+ve breast cancer, TNBC, *Crataegus oxyacantha* berry, canonical Wnt signalling pathway

Introduction

Breast cancers are classified based on receptor status. This classification helps to decide on the type of therapy targeting the specific type of breast cancer. Estrogen receptor positive (ER+ve) breast cancer accounts for about 75% [1] and triple negative breast cancers (TNBCs) account for 10-20% of all types of breast cancers [2]. ER+ve and TNBCs have different clinical, pathological and molecular features. TNBCs are more aggressive, highly invasive, often produce distant metastases and require more intense treatment, show poorer prognosis and shorter duration of survival [3]. ER+ve breast cancers can be treated with tamoxifen, aromatase inhibitors, competitive estrogen antagonists and phytoestrogens like quercetin [4, 5]. Current treatment strategies against TNBCs include chemotherapy agents, such as anthracyclines, taxanes, ixabepilone, and platinum agents, as well as selected biological agents [6]. Targetted therapy trials with anti-epidermal growth factor receptor (anti-EGFR), EGFR-Tyrosine kinase inhibitors are going on [7]. Recently approved targeted therapies for TNBCs include PARP inhibitors olaparib and talazoparib and checkpoint inhibitor, atezolizumab in combination with nab-paclitaxel [8]. A potent chemical/herbal preparation, which shows inhibitory effect on ER+ve and triple negative breast cancers, can be developed as an efficient drug against breast cancer, irrespective of its hormone sensitivity.

Hawthorn, our herb of interest, is best-known for treating heart conditions in western herbalism, Chinese medicine, Ayurveda and Homeopathy [9, 10]. Majorly studied as a cardioprotective agent [11–13], different preparations of hawthorn were also found to exhibit hypolipidemic [14, 15] anti-hyperglycemic [16, 17] anti-oxidant [18, 19] anti-inflammatory [20–22], cytotoxic [23–25], immunomodulatory [26], hepatoprotective [27] activities. Its

chemopreventive effect on skin tumor formation in mouse epidermal cell line has also been reported [28]. Hawthorn constitutes a wide range of exciting phytochemicals that are responsible for its excellent pharmacological properties. Major phytocompounds include flavonoids, proanthocyanidins, phenolic carboxylic acids, cardiotonic amines, saponins, Vitamin C, Vitamin B etc. [9, 19, 29, 30]. The most commonly used species for medicinal purposes is *Crataegus oxyacantha* (common hawthorn) because of its wide availability and the high quantity of hawthorn's chemical constituents [31].

Wnt signalling studies have offered new insights in biomedical research, especially in tumorigenesis, cancer stem cells and drug discovery [32]. Its aberrant constitutive activation leads to expansion of stem cell lineages, promoting carcinogenesis [33]. Wnt signalling is associated with mammary gland development and tissue remodelling during pregnancy [34]. Wnt signalling through β-catenin i.e, the canonical Wnt signalling is the primary pathway for Wnt-mediated oncogenesis in the mammary gland [35, 36]. It is frequently found dysregulated in primary human breast tumors and breast cancer cell lines, regardless of the receptor status [37–39]. Different studies have demonstrated the inactivation of negative regulators and amplification of positive regulators of the Wnt signalling pathway in breast cancer [36, 40]. Many plant polyphenols are known to employ regulation of Wnt/β-catenin pathway as one of their anti-carcinogenic mechanisms [41–44]. Crude extracts rich in polyphenols are also found to inhibit canonical Wnt signalling pathway [45, 46].

Our aim was to evaluate the anti-carcinogenic potential of the methanolic extract of *C. oxyacantha* berry [M.Co] on breast cancer cell lines MCF-7 (ER+ve) and MDA-MB-231 (TNBC) and to study its effect on regulating molecular markers representing different levels of the canonical Wnt signalling pathway in both cell lines.

Materials and Methods

Chemicals

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), TMB (3, 3', 5, 5'-tetramethylbenzidine), PI (propidium iodide) and crystal violet from Sigma Aldrich (St. Louis, MO, USA); BSA, TRI reagent, random hexamer, deoxy nucleoside triphosphate (dNTPs), PCR Master Mix (2X), DNA 100bp ladder from Genei™ (India); Cell culture media and Fetal Bovine Serum (FBS) from Gibco life technologies (USA); trypsin and antibiotic solution from Himedia Laboratories (India); Vectashield from Vector Laboratories (Peterborough). Primers from Eurofins Genomics India Pvt.Ltd. (Bangalore, India), Primary antibodies from Abcam (USA) & Cell Signalling Technology® (USA) and secondary antibodies from Thermo Fisher Scientific (USA). All other chemicals, solvents and reagents were from Sisco Research Laboratories Pvt. Ltd. (India) and SD Fine Chemicals (India).

Extract preparation

Dried berries of *C. oxyacantha* obtained from Hering Pharma (Pondichery, India), were washed, dried under shadow and powdered finely. Methanolic extract of *C. oxyacantha* berries (M.Co) was prepared from 10 g of powdered berry using 100ml of 70% methanol by soxhlation at 65ºC for 6 h. Methanol was removed by rotary evaporation and the remaining aqueous portion was concentrated using lyophilization. Yield was calculated using the following formula (mass of the extract/mass of the dried raw plant material) X100.

Cell line maintenance

Mammary carcinoma cell lines – MCF-7 (RRID: CVCL_0031) and MDA-MB-231 (RRID: CVCL_0062) were obtained from National Centre for Cell Sciences (NCCS, Pune, India). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from healthy volunteers using ficoll-hypaque [47]. MCF-7 cell lines were cultured in high glucose Dulbecco's Modified

Eagle Medium (DMEM), MDA-MB-231 cells in Leibovitz L-15 medium and PBMCs in RPMI 1640 medium, all supplemented with 10% FBS and 1X antibiotic, antimycotic solution (10,000 U Penicillin, 10mg Streptomycin and 25µg Amphotericin B per ml in 0.9% normal saline). MCF-7 cell lines and PBMCs were maintained at 37°C, in presence of 5% CO_2 and MDA-MB-231 cell lines at 37°C in CO_2 free environment. A split ratio of 1:2 was maintained for both cell lines. Medium was changed once in three days.

Cytotoxicity assay

MCF-7, MDA-MB-231 and PBMCs were seeded in a 96 well plate, at a cell density of 1x 10^6 cells/ well and incubated with different concentrations of M.Co dissolved in DMSO, for 48 h. Control and treated cells were then incubated with 10µl of MTT in dark for four hours at 37 $^{\circ}$C and purple formazan crystals formed were dissolved in 100µl DMSO. The plate was read on an ELISA plate reader (DNM-9602 microplate reader, Beijing Perlong New Technology Co., Ltd) at 570nm. Percentage viability was calculated by the formula [(mean absorbance of sample/ mean absorbance of control) ×100] and IC_{50} of M.Co was determined [48].

Clonogenic assay

MCF-7 and MDA-MB-231 cells were seeded in a six-well plate at a concentration of 1000 cells per well. After overnight incubation, cells were incubated in incomplete medium with or without M.Co, for 24 h and 48 h. After that, the medium was removed and both control and treated cells were maintained in the growth medium for 1- 3 weeks with periodic media change. Once visible colonies were formed, the growth media was removed and washed gently with 1X phosphate buffered saline (PBS). The colonies were fixed with 1% formaldehyde for 10 min and stained in 0.05% crystal violet for 30 min [49]. Excess stain

was washed off with PBS and the plate was allowed to dry. The stained colonies were then photographed.

Cell cycle analysis

MCF-7 and MDA-MB-231 cells were treated with M.Co for 24h and 48 h. Control and treated cells were trypsinized and suspended in 100µl 1X PBS. Cells were fixed by adding the suspension drop wise into 70% ice-cold ethanol and incubated at 4° C overnight. Cell pellet obtained by centrifugation at 1500 rpm for 10 min was suspended in 500µl of the hypotonic staining solution (Sodium Citrate- 0.1gm, Triton-x-100- 0.3ml, PI - 0.01gm, RNase- 0.01gm in 100ml distilled water). It was then incubated in dark at 37°C for 30 minutes. Stain was then washed off and cells were suspended in 250µl of 1X PBS [50]. Cell cycle analysis was performed using FACS Calibur (BD Biosciences, USA). The data were analyzed using Multicycle software (Phoenix Flow Systems, USA).

Molecular studies

Targets for molecular studies were selected from different stages of canonical Wnt signalling pathway such as ligands [Wnt3A, Wnt5A]; receptor [phosphorylated lipoprotein receptor-related protein-6 (pLRP6)]; cytoplasmic components [adenomatous polyposis coli (APC), phosphorylated glycogen synthase kinase 3β (pGSK3β)]; effector molecule [β- catenin] and downstream targets- c-myc, cyclin D1, Peroxisome proliferator-activated receptor δ (PPARδ), Cyclooxygenase-2 (COX-2)] and antagonist [E-cadherin].

Semi quantitative reverse transcriptase (RT) PCR

Control and M.Co treated MCF-7 and MDA-MB-231 cells were lysed in phenol–guanidinium thiocyanate-based Tri Reagent. RNA was isolated from the lysate [51] and converted to cDNA [52]. Semi quantitative RT PCR was carried out [53] using the following primers- β- actin (F: 5' GATGAGATTGGCATGGCTTT 3'and R: 5' GAGAAGTGGGGTGGCTT 3');

APC (F: 5'AAACGAGCACAGCGAAGAAT 3'and R: 5' GCTTTCTGCCACTCCTTGA 3'); β – catenin (F: 5' TATGGAAGCTGAGGGAGCCA 3'and R: 5' GGTCCATACCCAAGGCATCC 3'); cyclin- D1 (F: 5' CTCACACGCTTCCTCTCCAG 3'and R: 5' GGGACTGTCAGTGGAGCAC 3'); c-myc (F: 5' ATGCCCCTCAACGTTAGCTT 3'and R: 5' GTGTGACCGCAACGTAGGA 3'); COX-2 (F: 5' CAGAGTTGGAAGCACTCTATGG 3'and R: 5' CTGTTTTAATGAGCTCTGGATC 3'); PPAR δ (F: 5' TTGAGCCCAAGTTCGAGTTTGCTG 3'and R: 5' ATTCTAGAGCCCGCAGAATGGTGT 3'). The RT-PCR products were visualized on a 2% agarose gel stained with ethidium bromide, using UV transilluminator (Bioworld, Bengaluru, India).

Immunocyto fluorescence staining

MCF-7 and MDA-MB-231 cells were treated with M.Co for 48 h. Both control and treated cells were fixed in ice-cold methanol for 15 min at room temperature and permeabilized with 0.1% Triton-X-100 for 10 min. Non- specific binding was blocked with 0.5% Bovine Serum Albumin (BSA) for 45 min. Excess BSA was washed off and cells were incubated with primary antibodies [Rabbit polyclonal antibodies against Wnt 3A (Abcam Cat# ab28472, RRID:AB_2215308), Wnt 5A (Abcam Cat# ab229200, RRID:AB_2890100), pLRP 6 (CST cat# 2568, RRID:AB_2139327), Cyclin D1 (CST Cat# 2922, RRID:AB_2228523), pGSK3β (Abcam cat #ab107166, RRID:AB_11143750) and Mouse monoclonal antibodies against β-catenin (Abcam Cat# ab22656, RRID:AB_447227) and E- cadherin (CST Cat# 14472, RRID:AB_2728770)] diluted 1:150 with 0.5% BSA for 1 h. Cells were washed in PBS and incubated with fluorescein isothiocyanate (FITC) conjugated secondary antibodies [anti-rabbit (Thermo Fisher Scientific Cat# PA1-84922, RRID:AB_931578) and anti-mouse (Thermo Fisher Scientific Cat# PA1-74422, RRID:AB_2540458)] at a dilution of 1:300 for 1 h in dark. Cells were counterstained in PI (1:1000 dilution) for 2 min, in dark, washed in PBS and mounted using Vectashield. Slides were viewed and photographed using Leica SP2 Confocal Microscope [54].

Indirect Enzyme Linked Immunosorbent Assay (ELISA)

MCF-7 and MDA-MB-231 cells were treated with M.Co for 48 h. Both control and treated cells were harvested and lysed with lysis buffer [50mM Tris (pH- 7.4), 150mM NaCl,1% Triton-X-100, 0.1% SDS], freeze- thawed twice and centrifuged at 12,000 rpm for 20 min. Supernatant was used to study the expression of Wnt3A and Wnt5A and lysate for that of pLRP-6, pGS3Kβ, β-catenin, cyclin- D1 and E-cadherin. Protein estimation was done by Lowry's method [55]. Samples were diluted to get a final protein concentration of 25µg/ml in bicarbonate-carbonate coating buffer (pH- 9.6). The microtitre plate was then coated with 50 µl of the sample and incubated overnight at 4°C. The remaining protein binding sites in the coated wells were blocked by incubation with 1% BSA for 2 h at room temperature. Primary antibodies, at a dilution of 1:500 in 1% BSA, were added to the respective wells, incubated for 2 h at room temperature and excess antibody was washed off. The corresponding secondary antibodies [HRP conjugated anti-rabbit (Thermo Fisher Scientific Cat# PA1-86143, RRID:AB_933610) and anti-mouse (Thermo Fisher Scientific Cat# PA1-84406, RRID:AB_933651)] at a dilution of 1:1000 in PBS were added to the wells and incubated for 1 h at room temperature. The wells were then washed with PBS. 100 µl of TMB reagent along with 10 µl of 30% H_2O_2 was added to the wells and incubated for 10-15 min. After sufficient colour development, 100 µl of stop solution (2N H_2SO_4) was added to the wells and kept for 5 min. The absorbance was read at 450 nm [56].

Statistical analysis

All the results were expressed as mean ± S.D for three experiments. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. Significance was set at $P< 0.01$.

Results and Discussions

Extract preparation

Berries, in general, are rich in phenolic phytochemicals, which are responsible for their anticancer effects. Berry bioactives regulate sub-cellular signalling pathways of cancer cell proliferation, apoptosis and tumor angiogenesis [57, 58]. Hydro alcoholic extracts of hawthorn excel in antioxidant properties and pharmacological efficacy. A study showed that 70% methanol extracts from hawthorn had higher concentrations of polyphenols and exhibited good antioxidant properties [59]. So, it was decided to prepare M.Co with 70% methanol. Soxhlet extraction was chosen since higher temperatures facilitate extraction of more phytocompounds. Yield of M.Co was found to be 20.8%. HPLC analysis (data not shown) revealed a good polyphenol profile of M.Co as follows- catechin (0.012mg/g), epicatechin (0.412mg/g), catechin-o-gallate (0.066 mg/g), epicatechin-o-gallate (0.082 mg/g), quercetin (0.032 mg/g), ellagic acid (0.252 mg/g) and gallic acid (5.3 mg/g).

Cytotoxicity assay

Berry extracts are reported to inhibit breast cancers irrespective of its hormone dependency. To state a few examples, extracts from freeze-dried fruits of blueberry and strawberry were effective in inhibiting the growth of MCF-7 and T47-D, irrespective of estrogen dependence[60]. Grape seed extract could inhibit the growth of ER+ve and ER-ve breast cancer cell lines- MCF-7, MDA-MB-231 and MDA-MB-468 [61]. MTT assay, which measures cellular metabolism, is widely used for studying the cytotoxic, anti- tumor and anti-proliferative effects of herbal extracts. PBMCs isolated from healthy human volunteers are used as normal cell control in cytotoxicity assays, to find out whether the drug affects normal cell metabolism also [62]. We observed that the DMSO concentration used to dissolve M.Co was non-toxic to MCF-7 and MDA-MB-231 cells. The IC_{50} of M.Co was found to be 75µg/ml in both MCF-7 and MDA-MB-231 cell lines. In case of PBMCs, IC_{50} was found to

be between 500µg/ml and 750µg/ml **(Fig 1).** This confirmed that M.Co is cytotoxic to breast cancer cell lines, regardless of their hormone dependency. Also, the M.Co concentration which was effective on breast cancer cells is safe on normal cells.

When cancer cells undergo apoptosis, upon drug treatment, marked changes occur in cell morphology. Cell shrinkage, rounding up and membrane blebbing due to breakdown of proteinaceous cytoskeleton by caspases; anoikis, i.e, detachment of monolayer adherent cells from their basal membrane resulting in floating cells are some of them [63]. Morphological changes caused by M.Co in MCF-7 and MDA-MB-231 cells, such as cell shrinkage, rounding up, anoikis and membrane blebbing, are clearly shown in **Fig 2**, thereby confirming its cytotoxic effect.

Cell proliferation assay

Clonogenic assay or colony formation assay is based on the ability of a single cell to proliferate into a colony and it essentially tests every cell in the population for its ability to undergo "unlimited" division [64]. Clonogenic assays (colony formation assay) are widely being used to determine the effect of cytotoxic drugs on different tumors, since it has shown reliable clinical prediction [65]. In the present study, crystal violet stained plates in **Fig 3** clearly depict that MCF-7 and MDA-MB-231 control cells have prominent clonogenic property, which was reduced after 24 h of treatment with M.Co. The number and size of colonies were decreased significantly after 48 h of treatment with M.Co. Thus it can be concluded that M.Co can inhibit breast cancer cell proliferation, irrespective of its hormone dependence.

Cell cycle analysis

One reason for targeting the cell cycle in anticancer therapy is the high frequency of mutations of their key molecules in human malignancy [66]. Moreover, induction of cell cycle arrest in cancer cells leads to apoptosis, thus inhibiting their uncontrolled proliferation. Many cell cycle specific (CCS) drugs- including plant-derived compounds- are currently being used for cancer therapy. Phytocompounds have been reported to inhibit cell cycle at different stages. Vinca alkaloids (vincristine, vinblastin, vinorelbin and vindesin) blocked cancer cell cycle at the M phase [67]. The flavonoid Silibinin induced cell-cycle arrest at G2/M transition in colon cancer [68]. Resveratrol could block the cell cycle in MCF-7 at S phase, but did not affect the cell cycle in MDA-MB-231 cells [69].

Flow cytometric analysis of MCF-7 and MDA-MB-231 cells treated with M.Co for 24 h and 48 h is displayed in **Fig 4**. In case of both cell lines, the number of cells getting accumulated in Go/G1 phase had significantly increased after 48h treatment with M.Co. A significant decrease in the number of cells entering S phase was observed after 24 h, which was further reduced after 48 h treatment with M.Co. A corresponding increase in the number of cells entering apoptosis was observed after 24h and 48h treatment with M.Co, in both cell lines. This clearly suggests that M.Co could arrest G1/S transition of breast cancer cell lines, irrespective of its receptor status.

Molecular studies

Since canonical Wnt signalling is tightly controlled at multiple cellular levels, it offers ample targets for cancer drug development. Autocrine Wnt signalling is found to be responsible for the drug resistance of ER+ve breast cancer cell lines [70]. Also, aberrant Wnt/β-catenin signalling is found to have a role in the proliferation and metastasis of TNBCs [39, 71]. This makes canonical Wnt pathway and its components the most attractive targets for developing effective breast cancer therapy irrespective of the hormone dependence.

In brief, Wnt ligands (such as Wnt 3A) bind to low density lipoprotein receptor-related protein (LRP6) and transmembrane receptors of the Frizzled (Fz) family. In the absence of Wnt ligands, the key molecule β-catenin is marked for ubiquitination and proteasomal degradation, by a multi-protein β-catenin degradation complex, consisting of tumor suppressor APC, scaffold protein Axin, GSK-3β and casein kinase 1. Activation of receptors by Wnt ligand binding leads to phosphorylation of GSK-3β, causing the dissociation of β-catenin degradation complex. This failure to degrade β-catenin, leads to the stabilization and accumulation of cytosolic β-catenin, which then gets translocated to the nucleus and activates Wnt target genes by binding to transcription factors of the T-cell factor and the lymphoid enhancing factor (TCF/LEF) family. A number of Wnt/β-catenin target genes have been identified, which include those that regulate cell proliferation, embryonic development and tumor progression such as c- myc, cyclin D1, PPAR- δ, COX-2, Matrix metalloproteinases (MMP)-7, MMP-2, MMP-9, Axin-2, CD-44 *etc* [72]. Non-canonical ligand, Wnt-5a enhances β-catenin/E-cadherin complex formation via a Ca^{2+}-dependent mechanism in human breast epithelial cells [73] and thereby antagonizes canonical Wnt signalling by inhibiting the downstream transcriptional activity of β-catenin [74].

A comprehensive expression analysis of Wnt signalling molecules in immortalized human mammary epithelial cells and six breast cancer cell lines (including ER+ve and TNBCs), showed that redundant expression of Wnt ligands such as Wnt3A, frizzled receptors, co-receptors and LEF/TCF transcription factors was maintained in breast cancer cell lines. In contrast, the expression of non-canonical Wnt pathway ligands WNT5A was usually down regulated [40]. Several studies have observed elevated level of nuclear and/or cytoplasmic β-catenin, c-myc and cyclin D1 in breast cancer cell lines, but not in normal breast cells [75, 76]. Different studies have also demonstrated the inactivation of negative regulators of the pathway, such as APC, axin, axin-2, secreted Frizzled related protein-1 (sFRP-1) [36, 77].

At transcriptional level, molecular markers such as APC, β-catenin, cyclin D1, c-Myc, COX-2 and PPARδ were selected. Semi quantitative reverse transcriptase PCR assay **(Fig 5)** revealed that M.Co could significantly downregulate mRNA levels of effector molecule β-catenin and downstream targets cyclin D1, c-myc, COX-2 and PPARδ in both the cell lines. This supports the anti-proliferative and cell cycle arresting effect of M.Co. The cytoplasmic component APC, which is an essential part of β-catenin destruction complex and a negative regulator of the pathway, is found to be upregulated upon M.Co treatment in both cell lines.

Effect of M.Co on translational level expression of markers Wnt3A, Wnt5A, pLRP6 (active form of the receptor), pGSK3β (which indicates an active pathway), β- catenin, cyclin D1 and E-cadherin was visualized using immunofluorescence staining and quantified by indirect ELISA. It is clear from the confocal microscopic images **(Fig 6)** that, the expression of autocrine Wnt ligand Wnt3A was suppressed and that of antagonistic ligand Wnt5A was upregulated by M.Co treatment in ER+ve and TNBC cell lines. Cell surface expression of Wnt co-receptor pLRP6 was very clear in both the control cells, which was found to be reduced in M.Co treated ones. Cytoplasmic expression of pGSK-3β was higher in control cells when compared to treated cells. Cytoplasmic and nuclear accumulation of β–catenin was clearly visible in MCF-7 and MDA-MB-231 control cells. M.Co treatment showed reduced β–catenin expression in MDA-MB-231 cells and complete inhibition of its nuclear translocation was visible in MCF-7 cells. M.Co induced downregulation of the downstream target cyclin-D1 in both the cell lines, which is supported by the G1/S transition arrest caused by M.Co. M.Co treatment induced upregulation of E-cadherin, which upon activation by Wnt 5A ligand forms a complex with β–catenin, making it unavailable for canonical Wnt pathway. Indirect ELISA results **(Fig 7)** also supported the observations made in immune staining. In both MCF-7 and MDA-MB-231 cell lines, M.Co treatment significantly downregulated the expression levels of autocrine ligand Wnt 3A, active receptor pLRP6,

active cytoplasmic component pGSK-3β, effector molecule β–catenin and downstream target cyclin D1. Meanwhile, the expression of canonical Wnt pathway antagonists Wnt5A and E-cadherin were significantly upregulated upon M.Co treatment, in both cell lines.

Many polyphenolic phytocompounds use regulation of irregular Wnt/β-catenin signalling pathway as one of their anti-carcinogenic mechanisms. Some examples are: epigallocatechin-3-gallate (EGCG) [78], quercetin [79], ellagic acid [80], genistein [81], curcumin [42], resveratrol [82], lycopene [83] and compound Kushen [84]. Polyphenol rich crude extracts such as total extract of white and green tea [45], *Panax notoginseng* extract [85], pomegranate extract [86], Ethanolic extracts of *Angelica koreanae* radix, *Cannabis sativa* semen, *Ephedrae intermedia Schrenk* radix, and *Vitis rotundifolia* fruit [46] and *Antrodia camphorate* [87] are also shown to regulate canonical Wnt signalling pathway. It is reported for the first time that the regulatory effect of *C. oxyacantha* berry extract on canonical Wnt signalling pathway is one of the mechanisms behind its anti-carcinogenic property on ER+ve (MCF-7) and triple negative (MDA-MB-231) breast cancer cell lines.

Conclusion

The methanolic extract of *C. oxyacantha* berry exhibits commendable *in vitro* anti-cancer effect against breast cancers, irrespective of their hormone dependency. The extract could effectively regulate canonical Wnt signalling pathway, at different stages, and it is found to be one of the mechanisms behind the cytotoxic, anti-proliferative and cell cycle arresting properties exhibited by the extract. Since canonical Wnt pathway regulation is involved, the extract can be looked forward to have anti-inflammatory and anti-metastatic properties. Canonical Wnt pathway regulation may enable the extract to inhibit cancer at stem cell level, which needs to be confirmed further. Hence, *C. oxyacantha* berry extract is a promising

candidate to be developed as an effective drug against breast cancers, regardless of its receptor status.

Declarations

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Availability of data and material: Yes. Data have been generated as part of the routine work.

Ethics approval: Not applicable

Consent for publication: The authors have their consent to publish their work

Figure captions

Fig 1: Study of Cytotoxic effect of M.Co on PBMCs, MCF-7 and MDA-MB-231 cell lines by MTT assay. All values are expressed as the mean ± SD of three measurements

Fig 2: Morphological changes in control and M.Co treated MCF-7 and MDA-MB-231 cells. Phase contrast microscopic images (**a**) MCF-7 control cells (**b**) MCF-7 cells treated with M.Co for 24 h (**c**) MCF-7 cells treated with M.Co for 48 h (**d**) M.Co treated MCF-7 cells undergoing apoptosis (arrow) (**e**) MDA-MB-231 control cells (**f**) MDA-MB-231 cells treated with M.Co for 24 h (**g**) MDA-MB-231 cells treated with M.Co for 48 h (**h**) M.Co treated MDA-MB-231 cells undergoing apoptosis (arrow)

Fig 3: Study of anti-proliferative effect of M.Co on MCF-7 and MDA-MB-231 cell lines by clonogenic assay. Colonies stained with crystal violet (**a**) MCF-7 control cells (**b**) MCF-7 cells treated with M.Co for 24 h (**c**) MCF-7 cells treated with M.Co for 48 h (**d**) MDA-MB-231 control cells (**e**) MDA-MB-231 cells treated with M.Co for 24 h and (**f**) MDA-MB-231 cells treated with M.Co for 48 h

Fig 4: Study of effect of M.Co on cell cycle of MCF-7 and MDA-MB-231 cell lines by flow cytometry (PI staining). **(a)** control MCF-7 cells (**b**) MCF-7 cells treated with M.Co for 24 h (**c**) MCF-7 cells treated with M.Co for 48 h (**d**) graph depicting percentage of cells in each phase of cell cycle in control and M.Co treated MCF-7 cells (**e**) control MDA-MB-231 cells (**f**) MDA-MB-231 cells treated with M.Co for 24 h (**g**) MDA-MB-231 cells treated with M.Co for 48 h and (**h**) graph depicting percentage of cells in each phase of cell cycle in control and M.Co treated MDA-MB-231cells. Each value is expressed as mean ± SD of three

experiments. Statistical significance set at p<0.01. Comparisons are made as a- control Vs M.Co (24h) and b- control vs M.Co (48h)

Fig 5: Study of the effect of M.Co on transcriptional level expression of APC, β catenin, Cyclin D1, c-Myc, COX-2 and PPARδ in control and M.Co treated MCF-7 and MDA-MB-231 cells by Semi quantitative reverse transcriptase PCR. **(a)** RT-PCR products of APC, β catenin, Cyclin D1, c-Myc, COX-2, PPARδ and β- actin **(b & c)** Respective densitometry values normalized with β-actin and presented as "expression in arbitrary units"(using ImageJ software) of MCF-7 and MDA-MB-231 cell lines respectively. All values are expressed as the mean± SD of three measurements. Statistical significance set at p<0.01. Comparisons are made as a*- control cells vs M.Co treated cells

Fig 6: Study of the effect of M.Co on translational level expression of Wnt 3A, Wnt 5A, pLRP6, pGSK3, β catenin, Cyclin D1 and E-cadherin by immunofluorescence staining. **(a)** Control and M.Co treated MCF-7 cells (**b)** Control and M.Co treated MDA-MB-231 cells

Fig 7: Study of effect of M.Co on translational level expression of Wnt 3A, Wnt 5A, pLRP6, pGSK3, β catenin, Cyclin D1 and E-cadherin by indirect ELISA. Values are represented as absorbance at 450 nm. **(a)** Control and M.Co treated MCF-7 cells (**b)** Control and M.Co treated MDA-MB-231 cells. Each value is expressed as mean ± SD of three experiments. Statistical significance set at P<0.01. . Comparisons are made as a*- control cells vs M.Co treated cells

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Figures

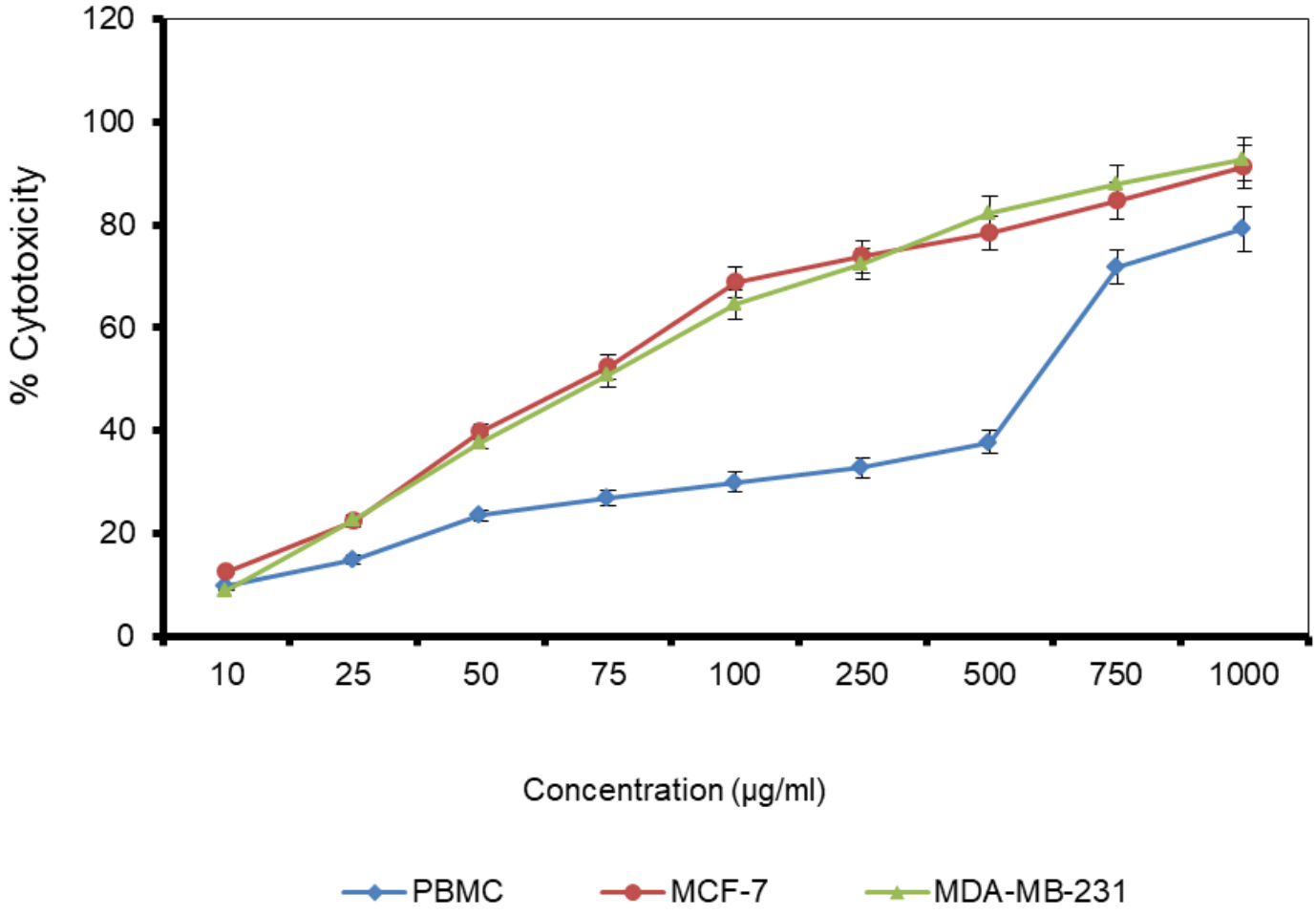

Figure 1

Study of Cytotoxic effect of M.Co on PBMCs, MCF-7 and MDA-MB-231 cell lines by MTT assay. All values are expressed as the mean ± SD of three measurements

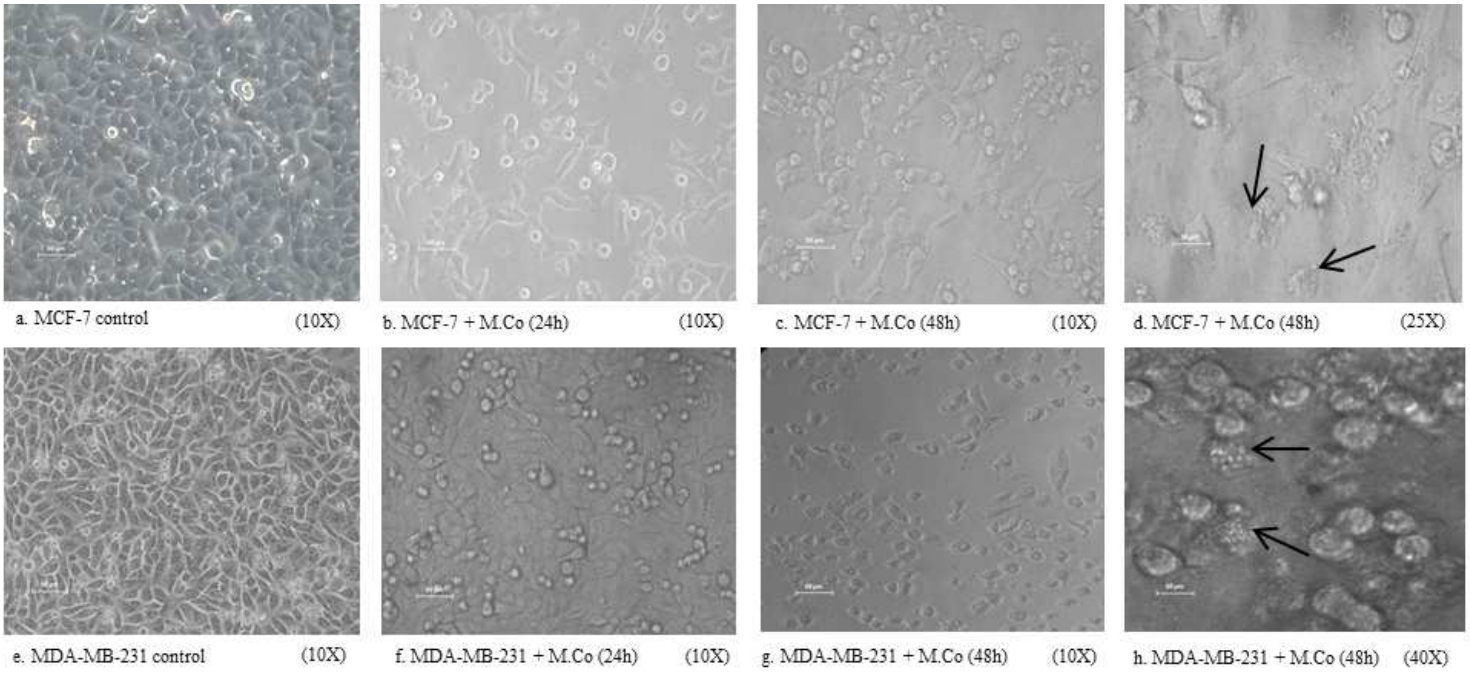

Figure 2

Morphological changes in control and M.Co treated MCF-7 and MDA-MB-231 cells. Phase contrast microscopic images (a) MCF-7 control cells (b) MCF-7 cells treated with M.Co for 24 h (c) MCF-7 cells treated with M.Co for 48 h (d) M.Co treated MCF-7 cells undergoing apoptosis (arrow) (e) MDA-MB-231 control cells (f) MDA-MB-231 cells treated with M.Co for 24 h (g) MDA-MB-231 cells treated with M.Co for 48 h (h) M.Co treated MDA-MB-231 cells undergoing apoptosis (arrow)

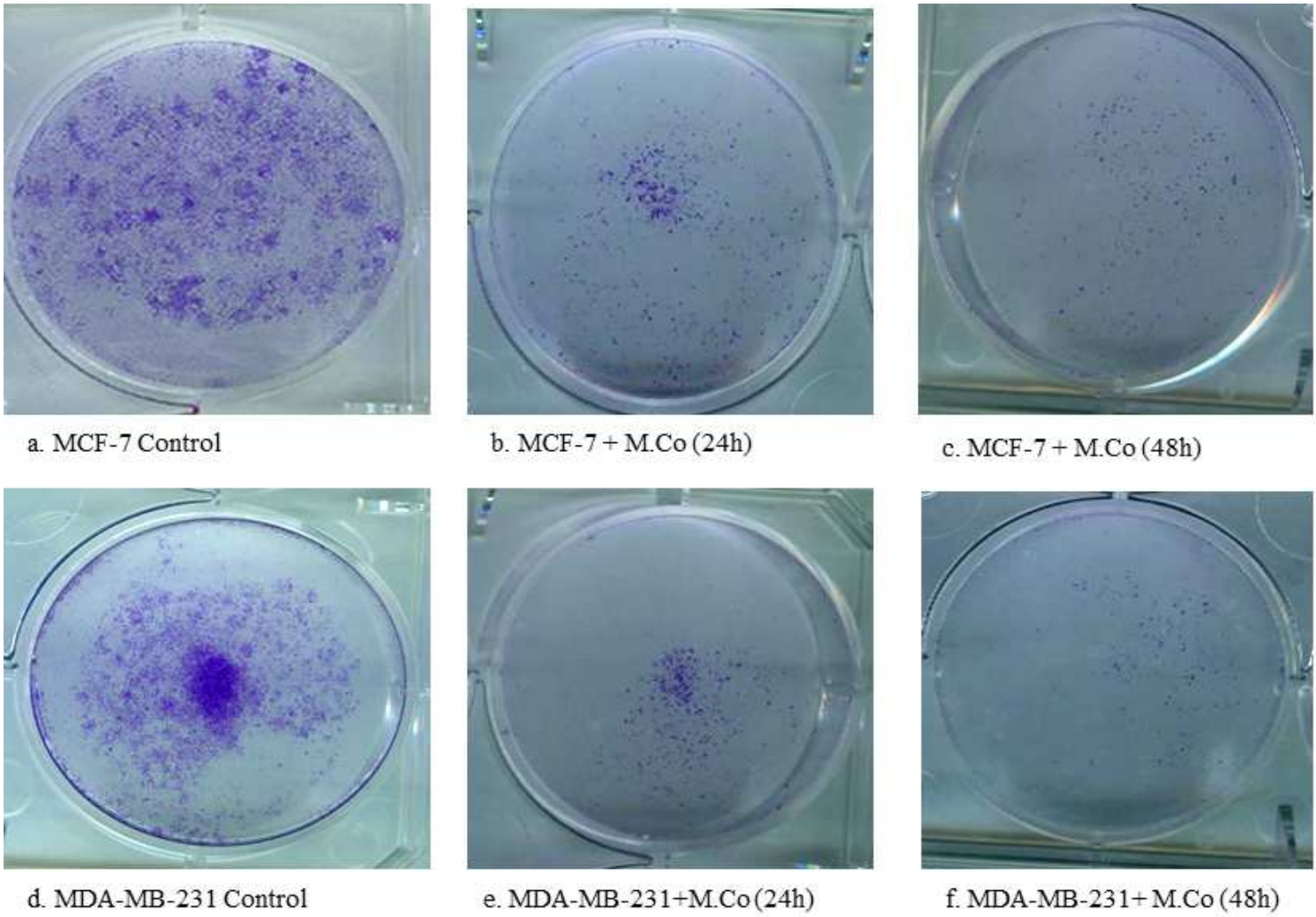

Figure 3

Study of anti-proliferative effect of M.Co on MCF-7 and MDA-MB-231 cell lines by clonogenic assay. Colonies stained with crystal violet (a) MCF-7 control cells (b) MCF-7 cells treated with M.Co for 24 h (c) MCF-7 cells treated with M.Co for 48 h (d) MDA-MB-231 control cells (e) MDA-MB-231 cells treated with M.Co for 24 h and (f) MDA-MB-231 cells treated with M.Co for 48 h

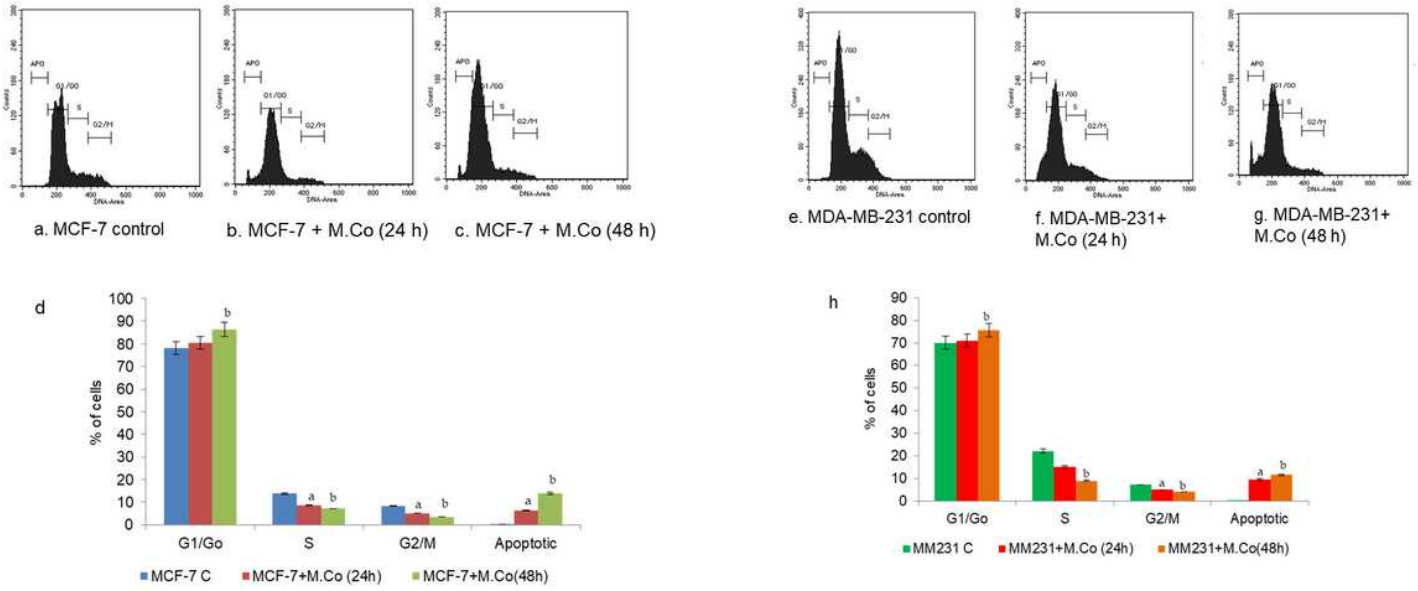

Figure 4

Study of effect of M.Co on cell cycle of MCF-7 and MDA-MB-231 cell lines by flow cytometry (PI staining). (a) control MCF-7 cells (b) MCF-7 cells treated with M.Co for 24 h (c) MCF-7 cells treated with M.Co for 48 h (d) graph depicting percentage of cells in each phase of cell cycle in control and M.Co treated MCF-7 cells (e) control MDA-MB-231 cells (f) MDA-MB-231 cells treated with M.Co for 24 h (g) MDA-MB-231 cells treated with M.Co for 48 h and (h) graph depicting percentage of cells in each phase of cell cycle in control and M.Co treated MDA-MB-231cells. Each value is expressed as mean ± SD of three experiments. Statistical significance set at $p<0.01$. Comparisons are made as a- control Vs M.Co (24h) and b- control vs M.Co (48h)

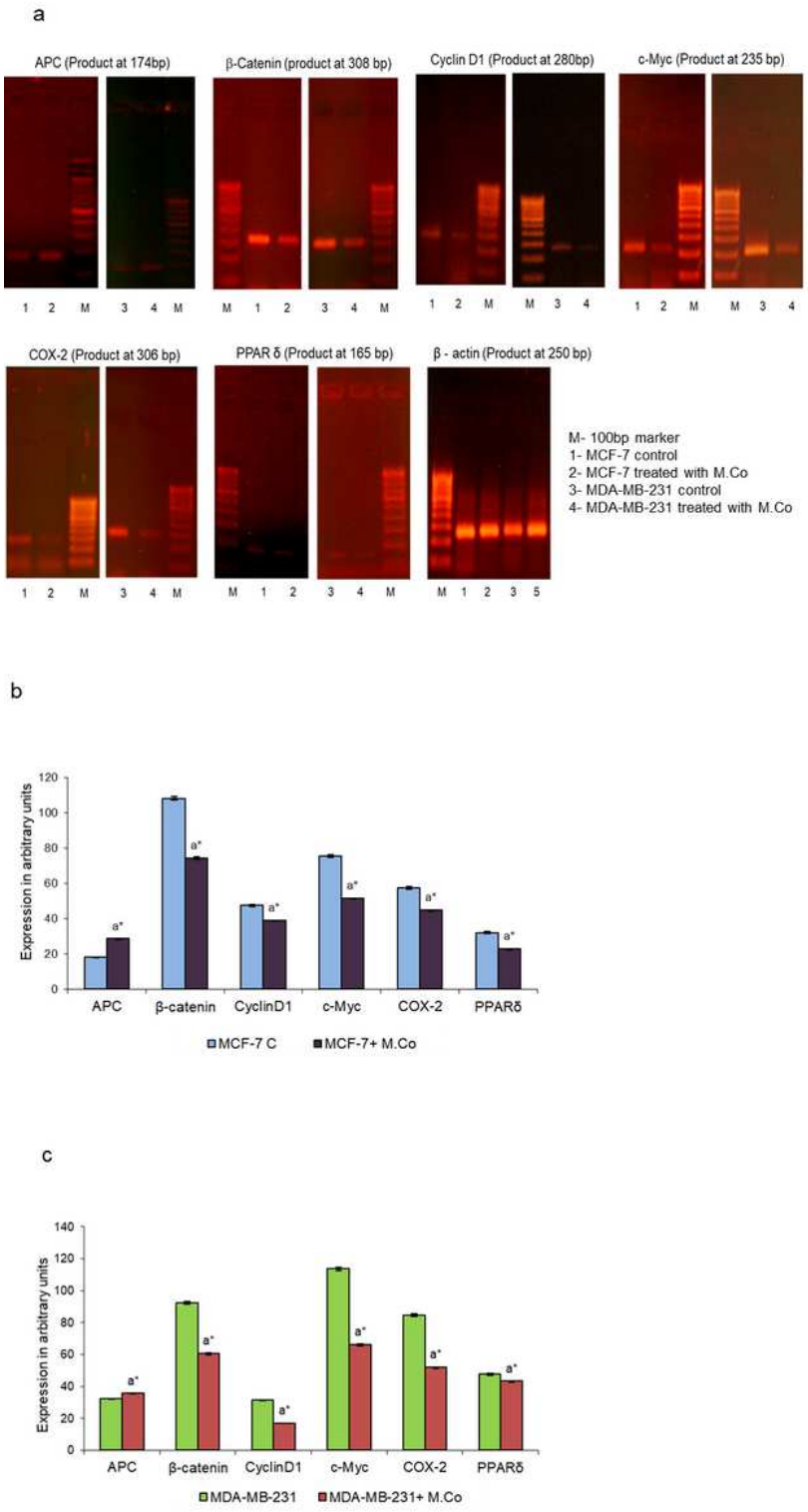

Figure 5

Study of the effect of M.Co on transcriptional level expression of APC, β catenin, Cyclin D1, c-Myc, COX-2 and PPARδ in control and M.Co treated MCF-7 and MDA-MB-231 cells by Semi quantitative reverse transcriptase PCR. (a) RT-PCR products of APC, β catenin, Cyclin D1, c-Myc, COX-2, PPARδ and β- actin (b & c) Respective densitometry values normalized with β-actin and presented as “expression in arbitrary units”(using ImageJ software) of MCF-7 and MDA-MB-231 cell lines respectively. All values are expressed

as the mean± SD of three measurements. Statistical significance set at p<0.01. Comparisons are made as a*- control cells vs M.Co treated cells

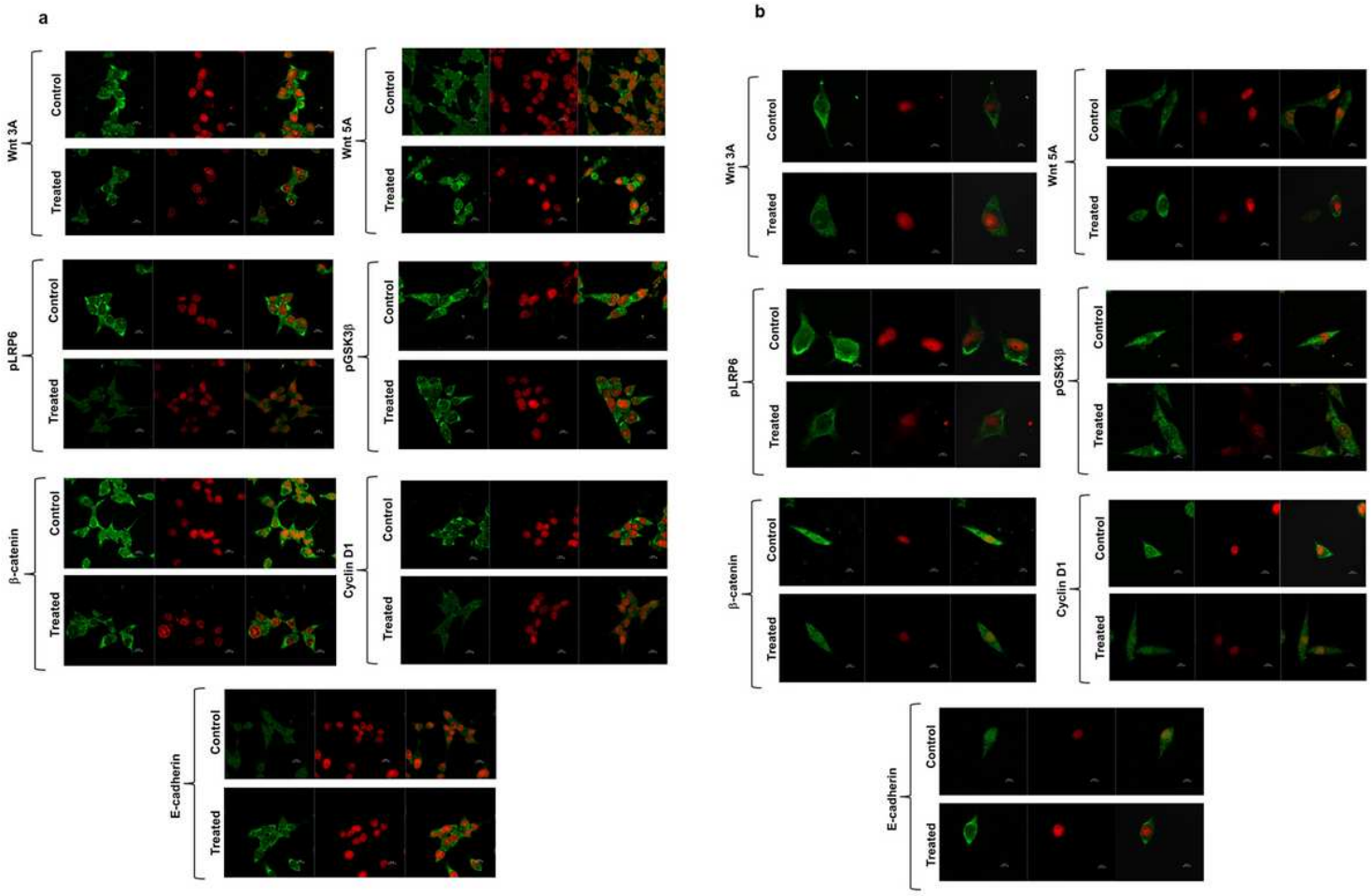

Figure 6

Study of the effect of M.Co on translational level expression of Wnt 3A, Wnt 5A, pLRP6, pGSK3, β catenin, Cyclin D1 and E-cadherin by immunofluorescence staining. (a) Control and M.Co treated MCF-7 cells (b) Control and M.Co treated MDA-MB-231 cells

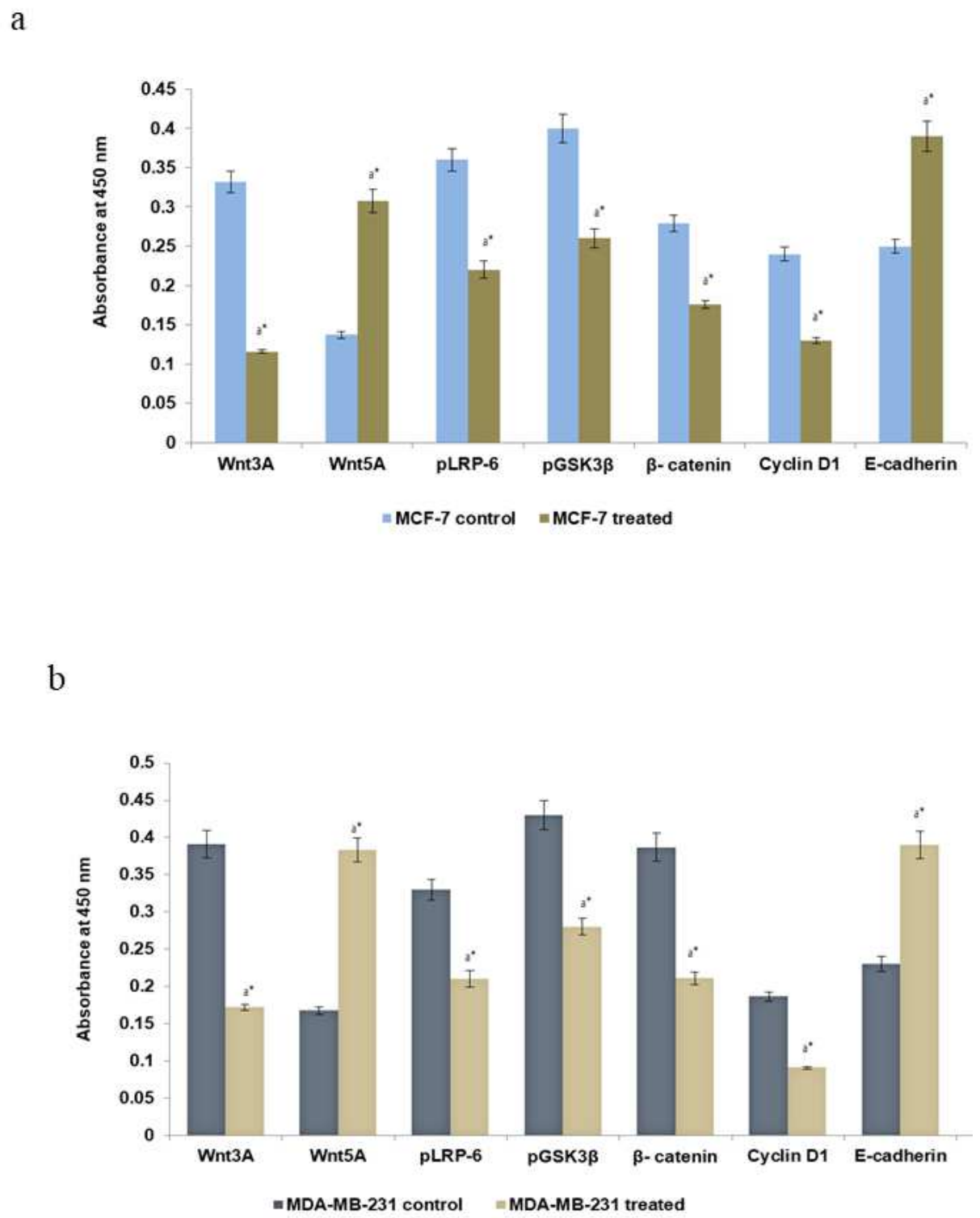

Figure 7

Study of effect of M.Co on translational level expression of Wnt 3A, Wnt 5A, pLRP6, pGSK3, β catenin, Cyclin D1 and E-cadherin by indirect ELISA. Values are represented as absorbance at 450 nm. (a) Control and M.Co treated MCF-7 cells (b) Control and M.Co treated MDA-MB-231 cells. Each value is expressed as mean ± SD of three experiments. Statistical significance set at $P<0.01$. . Comparisons are made as a*- control cells vs M.Co treated cells