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Extract from *Butea monosperma* inhibits β-catenin/Tcf signaling in SW480 human colon cancer cells

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ABSTRACT

The colorectal cancer (CRC) is the fourth leading cause of cancer death in worldwide. It has been found that > 90% of CRC is caused by aberrant activation of canonical Wnt/β-catenin signaling pathway. Continuous activation of this pathway believes to be an initiating event in colorectal carcinogenesis. There are growing evidences suggest that traditional herbal plant medicines are being raised as a complementary alternative treatment for cancer. In this series, *Butea* (*B*) *monosperma* has been illustrated as a valuable traditional medicinal plant with > 45 medicinal traits. Therefore, by targets this pathway using *n*-butanol fraction of *B. monosperma* flower extract (NBF-BMFE) could be a better therapeutic strategy for treating CRC. In this present study, we evaluate the inhibitory effect of NBF-BMFE against over activated Wnt signaling mediated colon cancer cells (SW480). Interestingly, the *in vitro* finding described that the NBF-BMFE had good antiproliferative effect against SW480 human colon cancer cells. Moreover, it showed significant level of down regulated expression in Wnt signaling proteins such as β-catenin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), cyclin D1 and *c-myc* in time-dependent manner. Further, the *in silico* results of NBF-BMFE derived compounds have shown good binding interaction with target sites of β-catenin, APC and GSK-3β protein. In conclusion, NBF-BMFE may be used as an effective inhibitor for Wnt signaling targeted combined chemotherapeutic agents against CRC.

1. Introduction

The colorectal cancer (CRC) is diagnosed as third most common form of cancer in men (6,63,000 cases, 10% of the total cancers) and second in women (5,70,000 cases, 9.4% of the total cancers) (Jemal et al., 2011). Aberrant activation of Wnt/β-catenin signaling pathway is a hallmark of the majority of CRCs, results in increased stability of protein β-catenin (Barker and Clevers, 2006). Continuous activation of Wnt/β-catenin signaling is believing to be an initiating event in colorectal carcinogenesis (Fodde et al., 2001; Giles et al., 2003).

In the absence of Wnt signaling, the destruction complex consisting of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), casein kinase 1 (CK1) and scaffolding protein axin catalyses the phosphorylation of cytoplasmic β-catenin leading to its proteosomal degradation at key amino-terminal Serine (SER) and Threonine (THR) residues (Miller et al., 1999). In the presence of Wnt, transmembrane receptors Frizzled and low density lipoprotein receptor- related protein (LRP5/6) recruit the cytoplasmic protein dishevelled (Dvl) and Axin to the receptor complex, which results in the disruption of the β-catenin destruction complex (Bilic et al., 2007). Then, it allows β-catenin to

Abbreviations: CRC, colorectal cancer; NBF-BMFE, *n*-butanol fraction of *B. monosperma* flower extract; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase 3β; CK1, casein kinase 1; LRP5/6, low density lipoprotein receptor- related protein; Dvl, dishevelled; Tcf, T-cell factor; LEF, lymphoid enhancer factor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA, dichlorodihydro-fluorescein diacetate; PVDF, polyvinylidine difluoride; HRP, horse radish peroxidase; NCCS, National Centre for Cell Science; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DAB, 3,3′-diaminobenzidine; Nacl, sodium chloride; TBS-T, Tris buffered saline – Tween 20; PDA, photodiode array; LC/MS, liquid chromatography/mass spectrometry; PDB, protein data bank; LGA, Lamarckian Genetic Algorithm; IC_{50}, half maximal inhibitory concentration; ROS, reactive oxygen species; NSAIDs, nonsteroidal anti-inflammatory drugs; ASN, asparagine; GLY, glycine; CYS, cysteine; GLU, glutamic acid; ALA, alanine; GLN, glutamine; LYS, lysine; HIS, histidine; LEU, leucine; SEP, serine phosphatase; ILE, isoleucine; MET, methionine; TYR, tyrosine; ASP, aspartic acid; TPO, thyroid phosphatase; H, hour; mg, milligram; ng, nanogram; mM, millimolar; nM, nanomolar; mL, millilitre; μL, microlitre

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accumulate in the cytoplasm and subsequently translocate to the nucleus, where its directly binds with the T-cell factor (Tcf)/lymphoid enhancer factor (LEF) family, and these interactions stimulate transcription of Wnt target genes (Mao et al., 2001).

Recently, Herbal medicines and their derived phytocompounds are being raised as a complementary treatment for cancer. In this series, *Butea monosperma* Lam. Kuntze (Fabaceae), commonly known as flame of the forest, have been illustrated as a valuable traditional medicinal plant with > 45 medicinal attributes. Among its different parts, flower is the one which is associated with several pharmaceutical effects (Burlia and Khadeb, 2007; Choedon et al., 2010). Phytochemical analysis of *B. monosperma* flowers constituents showed the presence of flavonoid glucosides such as butrin, isobutrin, butein, butin, sulfurein, coreopsin, isocoreopsin, monospermoside and isomonospermoside (Wagner et al., 1986; Gupta et al., 1970). Ethanol extract of *B. monosperma* flowers have been shown as anti-hyperglycemic, anti-diabetic and anti-fertility activity (Somani et al., 2006; Razdan et al., 1970). The Same extract has also possess hepatoprotective and anti-tumorigenic effect (Sehrawat and Sultana, 2006; Mathan et al., 2011). Whereas, the petroleum ether extract of *B. monosperma* flowers has been showed to exhibit anticonvulsive activity, due to the presence of triterpenes (Kasture et al., 2002). These constructive effects were attributed to several class of compounds identified in *B. monosperma* flowers extracts.

The preliminary reports of *B. monosperma* flower extract had showed the cytotoxic effect against human colon cancer cells. So far there are no reports of this extract against wnt/β-catenin signaling mediated CRC on SW480 cells. Based on these reports, this present study was aimed to assess the *n*-butanol fraction of *B. monosperma* floral extracts (NBF-BMFE) inhibitory effects on Wnt/β-catenin signaling proteins against CRC in SW480 cells.

2. Materials and methods

2.1. Preparation of plant extracts and fractions

The *B. monosperma* flowers were collected from the Bharathidasan University Campus, Tiruchirappalli. The voucher specimen was deposited in the Department of Plant Science, Bharathidasan University (Tiruchirappalli, Tamil Nadu, and India). The *B. monosperma* flowers were dried and ground into a fine powder and their preparation of extract has described previously (Wagner et al., 1986). In brief, the dried powder (500 g) of *B. monosperma* flower was extracted with methanol in a Soxhlet extractor for 20 h and solvent was removed under reduced pressure in a rotatory evaporator. From that, we obtained 130 g of an orange powder. Further, the 20 g of total methanol extract was partitioned between water and Ethyl acetate (3 times). Then after removal of the ethyl acetate fraction, the remaining water phase was treated with *n*-butanol (3 times). Both the ethyl acetate and the *n*-butanol fractions were dried using sodium sulfate.

2.2. Chemicals and antibodies

Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich, USA. The Fetal bovine serum (FBS), Trypsin-EDTA, Lipofectamine 3000 and penicillin-streptomycin antibiotic were obtained from Invitrogen, USA. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dichloro-dihydro-fluorescein diacetate (DCFH-DA) and Silibinin was purchased from Sigma Aldrich, USA. The polyvinylidine difluoride (PVDF) membrane was purchased from Pall life sciences, USA. Antibodies to β-catenin, APC, GSK-3β, cyclin D1, *c-myc* and β-actin were obtained from Santa Cruz Biotechnology, USA. The secondary antibodies, horse radish peroxidase (HRP) conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Santa Cruz Biotechnology, USA.

2.3. Cell culture

Human colon cancer cells SW480 was obtained from American Type Culture Collection (ATCC), Rockville, USA. HCT-116, HT-29 and SW-620 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator.

2.4. MTT assay

Cell growth was assessed by using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) as described previously (Reddy et al., 2006). At first, 5×10^4 cells per well was seeded in 96-well plate and after 24 h the cells were incubated with different concentrations of NBF-BMFE and Silibinin up to 1000 µM for 24 and 48 h. After incubation, the medium containing compounds were removed and 100 µL MTT (5 mg/ml in PBS) was added to each well. After 3 h incubation in dark, MTT was discarded and 150 µL DMSO was added to each well. Absorbance at 570 nm was measured with an ELIZA micro plate reader. The percent cell viability was then calculated.

2.5. Measurement of intracellular reactive oxygen species (ROS)

The measurement of intracellular ROS formation was based on the oxidative conversion 2′,7′-dichlorofluorescein- diacetate (DCFH-DA) to fluorescent compound dichloroflouorescin (DCF) (Jia et al., 2006; Lin et al., 2006). After treatment, SW480 cells were harvested and suspended in 0.5 ml PBS containing 10 µl DCFH-DA for 15 min at 37 °C in the dark. DCFH-DA was taken up by the cells and deacetylated by cellular esterase to form a non-fluorescent product DCFH, which was converted to a green fluorescent product DCF by intracellular ROS produced by treated SW480 cells. Fluorescent measurements were made with excitation and emission filters set at 488 and 530 nm, respectively. Fluorescence microscopic images were taken using blue filter (450–490 nm).

2.6. Cell cycle analysis

The SW480 cells were cultured in T25 flasks and incubated with the respective IC50 doses of NBF-BMFE for 24 and 48 h. After incubation, cells were harvested and fixed in 70% (v/v) ice-cold ethanol. Fixed cells were washed with PBS, incubated with 1 mg RNase/ml for 30 min before they were stained with propidium iodide. Finally, cell cycle distributions were analysed in a FACS caliber flow cytometer (BD BioSciences) and the percentage of cells in each phase was determined (Palanivel et al., 2013).

2.7. β-Catenin/Tcf transcription reporter assay

The SW480 cells were plated in 6-well plates, grown to 80–90% confluence and transiently transfected with the plasmids of TOPflash and FOPflash, respectively. It has 3 copies of the Tcf/Lef binding sites in the upstream of a thymidine kinase (TK) promoter and the firefly luciferase gene. Fop flash has mutated copies of Tcf/Lef sites and is used as control for measuring nonspecific activation of the reporter. All transfections were performed with Lipofectamine 3000 reagent and 1.8 µg of TOPflash or FOPflash plasmids. After transfection, cells were incubated in medium the IC50 value of NBF-BMFE for 24 h and 48 h and then lysed with reporter lysis buffer at harvest. A luciferase activity assay was conducted in a white 96-well plate and detected with a Victor × 2 multimode microplate reader (PerkinElmer, USA). Finally, steadylite plus reagent (PerkinElmer) was added to each well. Equal volumes of cell culture medium and steadylite plus reagent were mixed well and waited 10 to 15 min for complete cell lysis and to allow full