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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 14 (10): 578-583, 2011
ISSN 1028-8880 / DOI: 10.3923/pjbs.2011.578.583

Dichloromethane-methanol Extract from *Borassus aethiopum* Mart. (Arecaceae) Induces Apoptosis of Human Colon Cancer Ht-29 Cells

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Abstract: *Borassus aethiopum* MART (Arecaceae) is a plant used in traditional herbal medicine for the treatment of various diseases (bronchitis, laryngitis, antiseptic). In particular, their male inflorescences were reported to exhibit cicatrizing, antiseptic and fungicidal properties. In the present study, the biological activity of E2F2, an apolar extract from *Borassus aethiopum* male inflorescence was investigated on colon cancer HT29 cells. Phytochemical screening was carried according to methodology for chemical analysis for vegetable drugs. Cells proliferation was determined by the MTT assay and cells cycle distribution was analysed by using laser flow cytometer (Beckman coulter). The cytoskeleton organisation was examined under a laser scanning confocal microscope (Zess). Preliminary phytochemical analysis of E2F2 extract revealed the presence of sterols, triterpenes and saponosids. E2F2 extract (1 μg and 100 μg mL^{-1}) significantly inhibited cell proliferation by blocking cell population in G0/G1 phase. Flow Cytometric analysis of E2F2-treated HT29 cells showed that hypoploïd cell population (sub G1 phase) increased with processing time exposures. Immunofluorescence confocal analysis revealed a disrupt actin microfilaments network in E2F2 treated-cells with a significant reduction in actin stress fibres and appearance of a random, non-oriented distribution of focal adhesion sites. These data indicate that E2F2 extract has anti-proliferative and pro-apoptotic activities. Further studies are required to unravel the mechanisms of action of E2F2 extract.

Key words: Apoptosis, epithelial intestinal cancer, antiproliferative, medical flower, *Borassus aethiopum*

INTRODUCTION

Borassus aethiopum Mart (Arecaceae), also called Ronier palm, is a tall dioecious palm widely spread in Tropical and southern African savannah and open forest. It is a plant at once used in alimentation, in technology and in traditional medicine. In the field of alimentation, a study showed that the young shoots of *Borassius aethiopum* contains key nutriments which can help in nutritional supplementation in western Africa (Glew *et al*., 2005). In technology, the activated charcoal stemming from inflorescences of *Borassus aethiopum* is used as adsorbent (Nethaji *et al*., 2010). In Burkina faso, almost all parts of the palm are used medicinally for the treatment of abdominal pain, respiratory and inflammatory diseases. Combination of powdered *Borassus aethiopum* male inflorescences with shea butter is well known as cutaneous lesion remedy (Cassou *et al*., 1997).

In previous studies, using a bioassay-guided fractionation procedure, five fractions (E1F1, E2F2, E3F3, E4F4 and E5F5) from powdered *Borassus aethiopum* male inflorescences were extracted. Among these, the dichlomethane methanol extract E2F2 was found to exert significant antibacterial, antifungal, anti-inflammatory and antipyreti activities (Sakande *et al*., 2003, 2004a, b). Other studies on *Borassus flabellifer* flowers a plant belonging to the same family reported the isolation of an immunosuppressive 17 alpha substituted dammarane (Revesz *et al*., 1999) and a spirostane-type steroid saponins with antimetabolic activity (Yoshikawa *et al*.,

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2007). According to such important results on the flowers of *Borassus*, there is growing interest in the use of phytoextracts of this drug for the treatment of human diseases including cancer. Indeed, despite modern advancement in diagnosis, prevention and therapy, cancer is still the single largest cause of death for human worldwide even poor countries as Burkina Faso. Therefore, it is necessary for pharmaceutical and alternative medicinal industries to study and develop new and safe drugs (Yi *et al*., 2003; Kraft, 2009; Kim *et al*., 2010).

This study is also based on the fact that apoptosis mechanism is now well established as a novel strategy in cancer treatment (Ward *et al*., 2008; Tan *et al*., 2009; Yang *et al*., 2009).

In The present study, the cytotoxic and apoptogenic effect of dichloromethane-methanol E2F2 extract on human colon cancer HT29 cells lines was investigated.

MATERIALS AND METHODS

Materiel

Chemicals drugs and test agents:

- **Extraction solvents:** Dichloromethane and methanol (Prolabo, France)
- Phytochemical screening reagents from Prolabo, France: Potassium isobismuthite, KOH 2%, Ferric chloride d = 1,45, Sodium diphenylborate
- Cells and test agents from Laboratories Eurobio and AbCys, France:
  - Human colon cancer cells HT29
  - Dulbecco's minimal medium
  - Trypsin
  - Dimethyl Sulfoxyde (DMSO)
  - Trypan blue
  - Triton X-100
  - TRITC (tetramethylrhodamine isothiocyanate)-conjugated phalloidin
  - Propidium iodide
  - RNase A

Equipment

Material of extraction:

- Column of chromatography (Kieselgel 63-160 μ, Prolabo, France)
- Lyophilisator Alpha chris 1-2

Material of cells study:

- Hematocytometer (Beckman Coulter, USA)
- Multiscan MCC 340 microplate reader (Titertek, USA)
- Confocal microscope (Zeiss, LSM510)
- Flow cytometer (Beckman Coulter, USA)

Methods

Plant material and extraction: *Borassus aethiopum* male inflorescences were collected and identified by Professor Sita Guinko, Institute of natural products research of Ouagadougou (Vegetal Biology and Ecology Laboratory of UFR SVT). A voucher specimen AA1522 was deposited at the Herbarium.

The inflorescences were air-dried in the shade and powdered. The powder was exhaustively extracted by percolation with 3 L of dichloromethane-methanol (50:50). The fraction obtained (E2F2 extract) was evaporated under reduced pressure to obtain 10 g of residue (scheme 1). Phytochemical screening was carried out according to the methodology for chemical analysis for vegetable drugs (Ciulei, 1982).

Study on human colon cancer cells HT29: The study of E2F2 extract effect on human colon cancer cells HT29 was carried from February 2008 to may 2009. To study the ability of chemotherapeutic to induce apoptosis it was essential to accurately define this mode of cell death (Oh *et al*., 2001). Generally, the following traditional criteria are requested as morphological changes including chromatin condensation, cell nuclear fragmentation and DNA ladder. For this study following methods were used.

Cell culture: The human colon cancer HT29 cells were seeded at 3.10^5 cells/25 cm^2 flasks and cultured in Dulbecco's minimal medium supplemented with 20% fetal calf serum under a 5% CO_2 humidified atmosphere at 37°C and left to grow exponentially (Rouet-Benzineb *et al*., 2004).

Cell proliferation and viability: The cells were seeded in 96-well microplates and incubated overnight. The cells were treated with 1 and 100 μg mL^{-1} of the E2F2 extract or its vehicle, methanol (0.1%) for 24 h. The cells were harvested by trypsin digestion and cell viability was determined by trypan blue exclusion with a hematocytometer. The level of cell viability was measured at 0.5, 1, 2, 4 and 24 h.

Cell proliferation was determined by the MTT assay as described previously by Hu *et al*. (2002) and Kim *et al*. (2009).

Confocal visualization of actin cytoskeleton organization: Cells were grown on glass coverslips and washed twice with serum-free medium. The cells were then treated with different concentration of E2F2 for 4 h and 24 h. The treated cells were fixed with 3.7% paraformaldehyde in Phosphate Buffer Saline (PBS) at

room temperature and permeabilized with 0.1% Triton X-100 as described by Rao and Li (2004). The cells were stained for actin with TRITC (tetramethylrhodamine isothiocyanate)-conjugated phalloidin and examined under a laser scanning confocal microscope (Zeiss, LSM510).

Cell cycle analysis: Cell cycle analysis was performed to determine the proportion of apoptotic sub G1 hypoploid cells (Nicoletti *et al*., 1991).

Statistical analysis: Data were collected and analyzed by using Expo32 software. The results were expressed as Mean±SD from at least 3 independent experiments in tables and figures. Statistical analysis was performed using Student's t-test with $p<0.05$ as a criterion of significance.

RESULTS

Phytochemical screening: The phytochemical analyses of the dichloromethane methanol E2F2 extract from *Borassus aethiopum* male inflorescences revealed the presence of sterols, triterpens and saponoids.

E2F2 extract activity on HT29 cell viability and proliferation: Incubation of HT29 cells with E2F2 extract (Fig. 1) showed a significant inhibition of proliferation from the first hour with a 100 μg mL^{-1} concentration compare to control group ($p<0.01$). This inhibition was observed from 2 h of incubation with 1 μg mL^{-1} concentration ($p<0.05$). This inhibition was dose dependent. The cytotoxic effect on HT29 cells was accompanied with cell detachment.

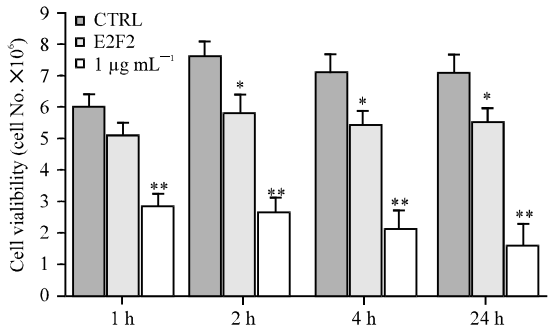

Fig. 1: Dose-response effect of E2F2 on HT29 proliferation. HT29 cells (10^5) were sealed in 12 well placed in triplicate samples. The cells were serum stored for 24 h and medium were supplemented with E2F2 (100 μ mL^{-1}) for different time period. Values are expressed as number of viable cells and each column represents Meean±SD of four experiments *$p<0.05$, **$p<0.01$ vs. control

Cell cycle analysis: Cell cycle distribution analyzed by using laser flow cytometer showed that E2F2 induces hypoploidic cells (Table 1, Fig. 2). The percentage of

Table 1: Pro-apoptotic effects of E2F2 on colon cancer HT29 cells

Time (h)	Treatment	Hypoploid cells (min-max) (%)	Diploid cells (min-max) (%)
0.5	Control	0.68 (0,48-0.89)	99.31 (99.10-99.51)
	[E2F2] 1 μg mL^{-1}	1.86 (1.46-2.26)	98.12 (97.72-98.51)
	[E2F2] 100 μg mL^{-1}	2.48 (1.98-2.99)	97.43 (96.93-97.94)
1	Control	0.85 (0.75-0.96)	99.15 (99.06-99.25)
	[E2F2] 1 μg mL^{-1}	1.35 (1.00-1.70)	98.75 (98.40-99.1)
	[E2F2] 100 μg mL^{-1}	5.99 (5.29-6.69)	93.83 (93.12-94.53)
2	Control	0.97 (0.92- 1.02)	99.09 (99.04-99.14)
	[E2F2] 1 μg mL^{-1}	5.92 (5.02- 6.83)	93.35 (92.44-94.25)
	[E2F2] 100 μg mL^{-1}	12.33 (11.03-13.63)	87.34 (86.04-88.64)
4	Control	1.12 (0.82-1.42)	98.83 (98.53-99.13)
	[E2F2] 1 μg mL^{-1}	7.96 (6.46-9.45)	93.44 (91.94-94.94)
	[E2F2] 100 μg mL^{-1}	10.96 (9.95-11.96)	88.14 (87.16-98.14)
24	Control	2.2 (1.70-2.70)	97.85 (97.34-98.36)
	[E2F2] 1 μg mL^{-1}	11.59 (9.90-13.30)	88.52 (86.83-90.22)
	[E2F2] 100 μg mL^{-1}	25.86 (20.85-30.86)	74.59 (69.60-79.59)

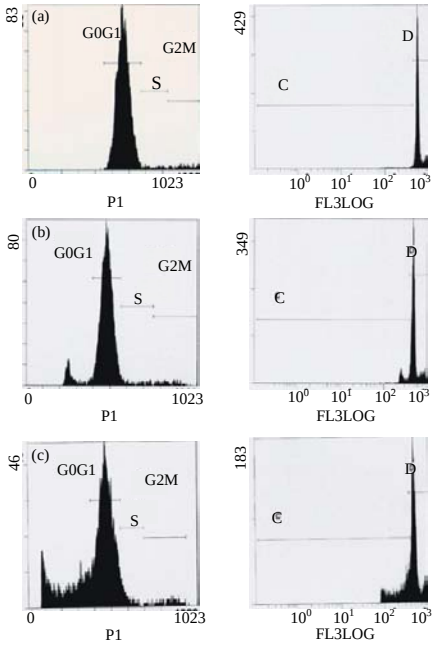

Fig. 2(a-c): E2F2 extract induces hypoploid peak in HT29 cells. (a) Control 24 h, (b) E2F2 (1 μg mL^{-1}) and (c) E2F2 (100 μg mL^{-1}). Panels represent cytometric flow analysis. Shown a typical cell cycle profile of control-or E2F2-treated cells. Cell were seeded at low density, grown at 70-80% confluence and were incubated with or without 1 μg mL^{-1} E2F2. Cells were subjected to Propidium Iodide (PI) staining as described in experimental procedure and 10,000 events were counted (X axis: FL3 log, Y axis: Cells count)

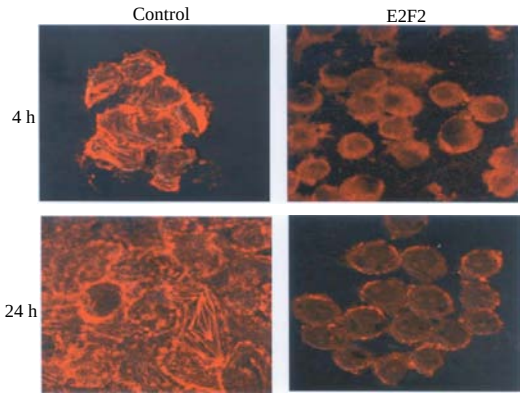

Fig. 3: E2F2 induces reorganization of actin cytoskeleton. HT29 cells were grown at 70-80% confluence and were incubate with or without 1 μg mL^{-1} E2F2. Cells were fixed and stained with TRITC-conjugated phalloidin for detection of F-action cyoskeletal organization using immunoflourescence micros copy. Confocalvies of colon cancer HT29 cells show that stress fibers are well defined in control cell whereas actin network is precisely disorganized in E2F2-treated cells

hypoploidic cells increased from 1.86% after 1 h exposure with 1 μg mL^{-1} concentration to 11, 59% after 24 h. With 100 μg mL^{-1} of E2F2 extract exposure the percentage of hypoploidic cells was 25, 86% after 24 h. In HT29 cells treated with E2F2 extract there was an appearance of hypoploidic peaks (Fig. 2). This apoptotic effect of E2F2 on human colon cancer HT29 was dose dependent. Moreover, SubG1 cell population appeared and increased as function of time. suggesting a genotoxic role of E2F2 extract on cancer HT29 cells.

This cytometric analysis revealed that E2F2 fraction induced cell death by producing hypoploidic cells.

Confocal visualization of actin cytoskeleton organization: Exposure of human colon cancer HT29 cells to 100 μg mL^{-1} of E2F2 extract, led to a reorganization of actin filaments, a characteristic feature of apoptosis (Fig. 3). Confocal analysis of phalloidin-staining of F-actin showed that attached cells become more flattered and elongated in E2F2-treated than control cells.

DISCUSSION

The chemical analysis of the extract E2F2 of *Borassus aethiopum* revealed the presence of substances such as sterols, triterpens and saponoids whom known pharmacological activities can justify the use in traditional medicine. The presence of these same constituents was already reported by Lanza *et al.* (1962). Other authors also isolated different steroids saponins from the flowers of Palmyrah palm (Borassus flabellifer) a plant belonging to the same family as *Borassus aethiopum* (Yoshikawa *et al.*, 2007; Keerthi *et al.*, 2009) .

The study of the activity of the extracts of *Borassus aethiopum* on cancer cells HT29 showed derangements of cytoskeleton witch may be a cell death signal as described by Khan (2010). It is well known that disruption of cytoskeleton actin filaments can affect multiple cell functions including motility, signal transduction, cell division, with cell death as an ultimate action (Suria *et al.*, 1999; Yamazaki *et al.*, 2000; White *et al.*, 2001). Apoptosis plays an essential role in controlling cell numbers in many developmental and physiological settings and in chemotherapy-induced tumour cell killing. The hindrance of this process leads to the development of many severe diseases and disorders, including tumours. Selective induction of the apoptosis process may become the fundamental strategy in developing anti-cancer drugs (Kim *et al.*, 2010). Most available chemotherapy drugs break down tumour cells by inducing apoptosis as reported by Bremer *et al.* (2006), Yang *et al.* (2009) and Sun and Peng (2009). But it is important to discriminate between cells that were experiencing apoptosis and cells undergoing necrosis as a result of the lethal effects of plant extract. Indeed, the hepatotoxic syndrome of *Borassus flabellifer* extract was previously reported due to water soluble saponins like neurotoxins (Keerthi *et al.*, 2009).

The chemical groups brought to light in the extract E2F2 could explain the observed apoptosis effect. It is for example the case of phytosterols. Indeed the β-sitosterol is capable of leading the apoptosis of cancer cells HT116. The apoptosis activity of the β-sitosterol would be bound to the activation of the caspase 3 and 9 on one hand and on the other hand it decreases the expression of anti-apoptosis proteins Bcl-2 and Bax (Choi *et al.*, 2003). Also glycosides steroids (gitoxigenin) isolated from *Digitalis purpurea* are capable of leading the apoptosis of TK-10 which are cancer cells of renal adenocarcinoma (Lopez-Lazaro *et al.*, 2003).

The extract E2F2 also contains triterpenes which are also quoted among substances stemming from plants and endowed to be able to apoptosis. It is the case of colostral that is an isolated triterpen of plants of the family of Celastraceae endowed with an anticancer and anti-inflammatory activity. The colostral leads the apoptosis on the cancer cells of leukaemia (HL-60) by fragmentation of the DNA and the inhibition of the topoisomerase II (Nagase *et al.*, 2003). Other parts the

effect of *Spirulina* species extracts containing various terpenes as carotenoid compounds and tocopherols on the viability of Ehrlich Ascites Carcinoma Cells (EACC) were evaluated. All algae extracts at different concentration of 200 and 400 ppm significant reduced the cell viability ranged from 89.11 to 5.25%. These extracts did not induce any significant changes in DNA fragmentation of treated EACC compared with untreated cells. These finding suggest that algae extracts may be reduce cell viability by other mechanism such as membrane lyases instead of apoptosis (Abd El-Baky *et al*., 2003; Bin-Meferij, 2009; Sixabela *et al*., 2011).

Finally saponins contained in the extract E2F2 could justify also its apoptosis activity. Indeed the saikosaponines of *Bupleurum* spp. is endowed with apoptosis activity on lineages of liver carcinoma cells Hep-2. The apoptosis mechanism of these saponins is based on the activation of caspases 3 and 7, the cleavage of the Poly-ADP-Ribose-Polymerase (PARP) and the fragmentation of the DNA (Chiang *et al*., 2003).

The powerful apoptosis activity of E2F2 should be bound to the synergy of these various molecules which it contains (phytosterols, triterpens and saponins).

CONCLUSION

This study clearly demonstrates that E2F2 extract from *Borassus aethiopum* strongly inhibits cell proliferation and induces apoptosis in HT29 cells. In summary, this study demonstrate for the first time that E2F2 extract from *Borassus aethiopum* may possess anticancer properties. Further investigations would be necessary to understand this apoptosis mechanism and to evaluate the potential clinical efficacy of E2F2 in apoptosis process for cancer therapy.

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