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Physiological Response of Human Epithelial Squamous Carcinoma (HEp-2) cells to a Di-Herbal Formulation

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ABSTRACT: *Senna alata* and *Senna podocarpa* are medicinal plants widely used in complementary and alternative medicine in the treatment of various diseases, in southwestern Nigeria. The *in vitro* cytotoxic and genotoxic potential of a formulation composed of methanolic leaf extract of *S. alata* (75%) and *S. podocarpa* (25%), on Human Epithelial Squamous Carcinoma (HEp-2) cells was investigated. Hep-2 cells were exposed to graded (0 - 933µg/ml) concentrations of the formulation for 48 hrs after which cytotoxicity and genotoxicity were assessed using the WST-1 assay, comet assay as well as DNA fragmentation assay. The formulation did not significantly ($p > 0.05$) alter cell viability at the doses tested. The current study further provided evidence that 186.5µg/ml and 559.5µg/ml of the formulation did not induce DNA Damage in HEp-2 cells given the absence of tail moments from the visible comet head. The results suggest that the formulation did not exhibit anti-proliferative activity, but rather, may contain some growth promoting principles.
Key words: *Senna alata*, *Senna podocarpa,* Hep-2 cells, Cytotoxicity, CAM.

Introduction

Cancer is a potentially fatal disease caused mainly by environmental factors that mutate genes encoding critical cell-regulatory proteins (Malcom, 2001; Patel *et al.,* 2009). World Health Organization (2009), reported 10 million new cancer cases worldwide in 1996 and 6 million deaths attributed to cancer; as at 2004, worldwide death group attributed 7.4 million deaths (around 13% of all deaths) to cancer and by 2020, statistical predictions show that there will be 20 million new cases and 12 million deaths. It has thus been reported as a dreadful disease and as one of the leading cause of death around the world, therefore any practical solution in combating this disease is of paramount importance to public health (Rao *et al.,* 2008; Díaz-García *et al.,* 2013).

Radiotherapy, chemotherapy and surgery have hitherto been found to be the key tools for cancer treatment (Fritz *et al.,* 2013) but these treatments have been found to cause severe systemic side effects and as such several therapeutic approaches have been developed to overcome the complexities of different cancers (Meiyanto *et al.,* 2012), in which case, searches and discoveries of new drugs against cancer, especially those derived from the natural products, is increasing throughout the world (Díaz-García *et al.,* 2013).

The head and neck squamous cell carcinoma (HNSCC) is the tenth most common human cancers of which the laryngeal carcinoma is reportedly the eleventh most common kind of cancer in men (Fan *et al.,* 2004; Marioni *et al.,* 2006). Human Epithelial Squamous Cell Carcinoma (HEp-2) is characteristically polyploid, derived from the larynx with characteristic plating efficiency and 44% Hela-like (Enlander *et al.,* 1974; Freshney, 2005). This carcinoma cells are found to be appropriate for investigating cancers (Webster *et al.,* 1991) and for experimental models associated with anti-

cancer therapy (Nakayama *et al.,* 2005). Yan *et al.* (2013) therefore suggests the desperate need for novel effective chemotherapeutic agents in the treatment of laryngeal cancer to improve survival and to enhance larynx preservation.

Herbalism or Botanical medicine, though as old as the origin of man, has been described as the practice of using medicinal plants in curing ailments (Evans *et al.,* 2002). This practice has remained unrecognized by the developed countries and has hitherto been described as indigenous medicine or traditional medicine. About two-third of the world population in the developing countries have relied solely on this folklore medicine as their primary means of health care (Sumner, 2000), due to lack of basic health care and personnel (Okpuzor *et al.,* 2008) as well as its potency in the management and treatment of diseases (Elujoba *et al.,* 2005).There has been an increased need to search for new compounds from natural source with selective cytotoxic activity in the treatment of cancer, and thus the search for novel drugs to combat diseases and infections has gingered the interest of scientists all over the world in medicinal plants remedies (Dahanukar *et al.,* 2000; Patel *et al.,* 2010).

Senna alata (Yoruba: Asunwon oyinbo) and *Senna podocarpa* (Yoruba: Asunwon egba) have been reported to be employed in traditional medicine for their potent effect against human diseases (Levin *et al.,*1979; Elujoba *et al.,* 1994; Akanmu, 1999; Evans *et al.,* 2002; El-mahmood *et al.,* 2008).

Senna alata is known to contain some secondary metabolites like resin, saponin, phenols, flavonoids, anthraquinone glycosides and alkaloids, carbohydrates, diterpenes (Levin *et al.,* 1979; Akinde *et al.,* 1999; Owoyale *et al.,* 2005; Makinde *et al.,* 2007; El-mahmood *et al.,* 2008). *Senna podocarpa* contains anthraquinone derivatives responsible for its laxative properties (Elujoba *et al.,* 1989, 1994; Akanmu, 1999). Constituents of the leaves and pods of *S. podocarpa* that have been identified include rhein, emodin, chrysophanol and other combined and free anthraquinones (Elujoba *et al.,* 1994).

The cytotoxicity and genotoxicity of some medicinal plants/herbs extracts, infusions, essential oils and fractions of many extracts have been widely evaluated (Sobita and Bhagirath, 2005). Adebesin *et al.* (2013) reported cell cytotoxicity due to exposure of K562 leukaemic cell line to graded concentrations of methanolic extract of *Senna alata* or *Senna podocarpa* leaf, thus this present study investigated the response of Human Epithelial Squamous Carcinoma (HEp-2 Cells) to a Di-Herbal formulation (a proportional mixture of the methanol fraction of *Senna alata* and methanol fraction *Senna podocarpa* leaves)

Materials and Methods

Plant material and formulation

Senna podocarpa and *Senna alata* plants were obtained from Iyin-Ekiti, Ekiti State, and Isara, latitude 54.8236°W and longitude 125.1522°W in Ogun State Nigeria, respectively. Both plant materials were authenticated with voucher specimen (LUH5511) and deposited at the Herbarium of the University of Lagos (UNILAG), Akoka. Crispy air dried leaves of the plants (*Senna alata* and *Senna podocarpa*) were homogenized to powdery form and 150 g of each was extracted separately with 350 ml of 80 % methanol. In accordance with the fractionation protocol of Yeşilada and Küpeli (2002), 7 g of each 80 % methanolic crude extract was fractionated twice with 50ml methanol repeatedly and dissolved in phosphate buffered saline to prepare a final concentrated volume of 18.8 mg ml^{-1} (18800 µg/ml) and 18.2 mg ml^{-1} (18200 µg/ml) respectively and 75 % of the sterile volume of *S. alata* was mixed with 25 % of the sterile volume of *S. podocarpa* to obtain 1865 µg/ml of the combined extract. For the purpose of this study, the mix was named Extract 'M' and stored at 4°C in the refrigerator until use.

Culture conditions

HEp-2 cell stock in cryovial frozen in Liquid Nitrogen was quickly thawed by swirling gently in a 37 °C water bath in such a way that the O-ring and cap of the cryovial are kept out of the water to reduce the risk of contamination. The cells were transferred from cryovial into 15 mL conical tube containing 10 mL of culture medium supplemented with 10 % fetal bovine serum and 1% penicillin-streptomycin (Sigma Aldrich). The suspension was centrifuged at 500 rpm for 5 minutes, the supernatant was discarded and the pellet was re-suspended in 10 mL of culture medium. A 10 µL aliquot of this cell suspension was transferred into Eppendorf tube for Trypan blue count while 990 µL aliquot of cell suspension was transferred into culture flask and incubated at 37 °C in a humidified atmosphere of 5 % CO_2.The medium was changed every 3-4 days and cells passaged into new culture flasks when observed to have reached 90% confluent monolayer.

Cell viability Assay (WST-1)

The di-herbal formulation was tested for *in vitro* cytotoxicity in accordance with the protocol of Ishiyama *et al.* (1993) using HEp-2 cells. Cytotoxicity was measured by the reduction of tetrazolim salt - WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] cell viability and proliferation assay kit (ScienCell, USA) - to colored formazan (water soluble) compounds by succinate-tetrazolium reductase which exists in viable cells. Briefly, HEp-2 cells (1.14×10^5 cells/mL) were seeded into 96-well plates, then exposed to medium containing graded (0-933 µg/mL) concentrations of the di-herbal formulation and incubated for 48 hours. After the incubation period, WST-1 reagent (10 µl of 3.26 mg/ml) was added into each well, the plates were carefully shaken and the cells incubated for another 3hrs after which the absorbance values of each well at $OD_{450} - OD_{630}$ were read using a microplate Reader (Molecular Device, Menlo Park, USA).

% Viability= [(Absorbance of test / Absorbance of control) x 100]

A dose-response curve was plotted to enable the calculation of the concentration that inhibited growth of 50 % (IC_{50}) of the HEp-2 cells.

Genotoxic Assay

Seeding of Plate for DNA Fragmentation and Comet Assay

Six well plates were seeded with HEp2 cell suspension (1.14×10^5 cells/ml) and incubated for 24 hrs to allow the cells attach and stabilize. Following this incubation period, the cells were exposed to 186.5 µg/mL and 559.5 µg/mL concentration of the extract and afterwards incubated for 72 hrs in the humidified 5 % CO_2 Incubator set at 37 °C. After the incubation period, the culture medium was discarded and the wells were washed with PBS, trypsinized, incubated to allow for detachment of cells and then suspended in 500µL of fresh medium to stop the action of trypsin.

The plate was swirled gently and the cell suspensions were aspirated into pre-labeled Eppendorf tubes. The tubes were centrifuged at 2000 rpm for 5mins, the supernatants were discarded, cell pellets were re-suspended in 500 µl ice-cold 1X PBS (Ca^{++} and Mg^{++} free) and centrifuged. The supernatants were removed and the cell pellets re-suspended in 50 µL of ice-cold 1X PBS (Ca^{++} and Mg^{++} free), 40 µl of this cell suspension was used for DNA fragmentation assay and 10 µl was used for Comet assay

DNA fragmentation Assay

DNA Fragmentation was carried out following the protocol of Kotamraju *et al.* (2000) with slight modification. The evaluation is based on the induction of apoptosis leading to internucleosomal cleavage of DNA into fragments visible as a ladder pattern when resolved on Agarose gel electrophoresis.

Single Cell Gel Electrophoresis (Comet assay)

Comet assay in accordance with the protocol of Ostling and Johanson (1984) was undertaken with slight modification. This assessment is based on the use of gel electrophoresis to show the potential of a test substance to induce DNA damage which is visible under florescent microscope as a comet with tail within the isolated cell.

Results

Figure 1 shows that Extract 'M' supported the growth of HEp-2 cells exposed to it.

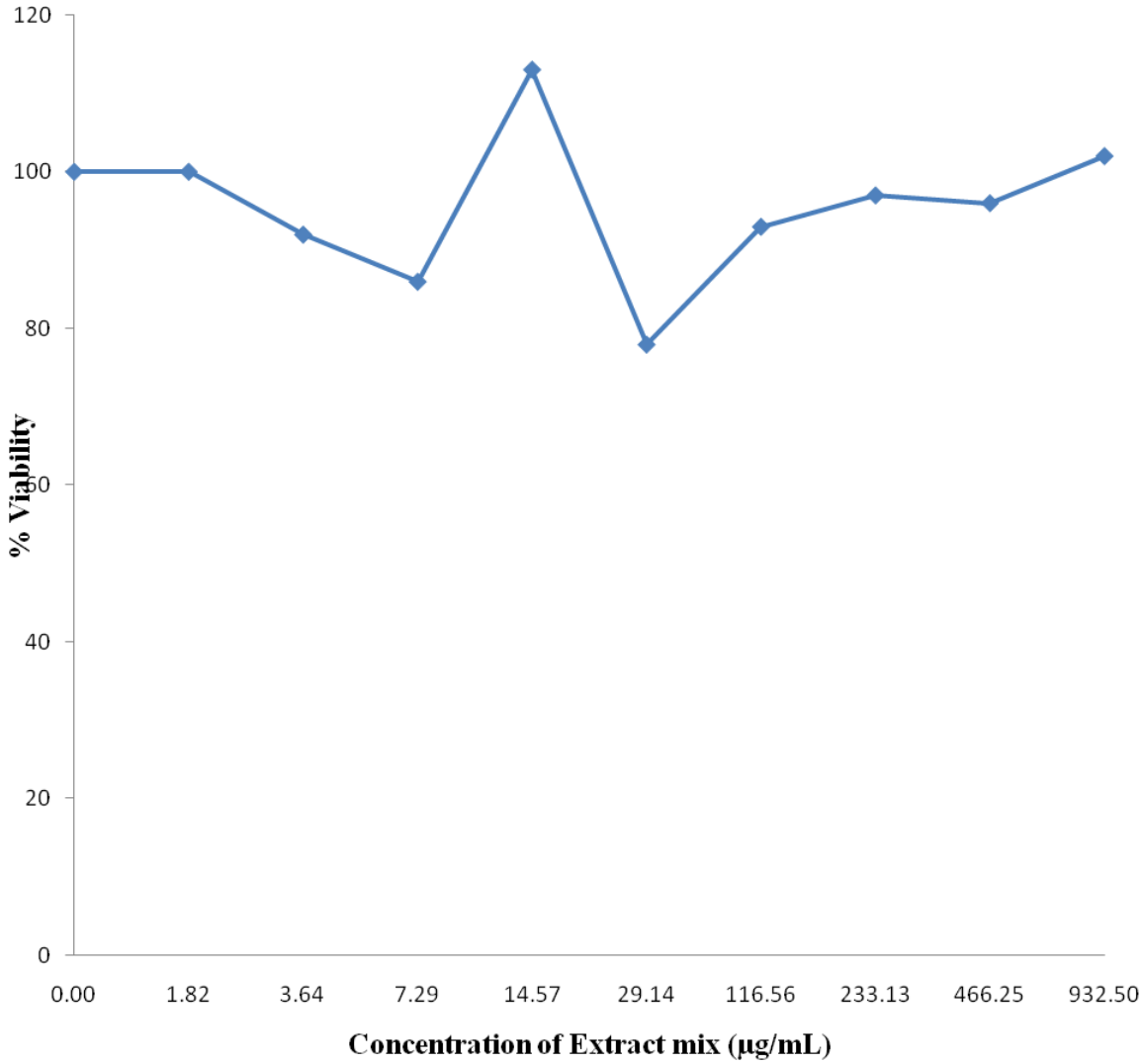

Figure 1: Cytotoxic effect of Extract 'M' on HEp-2 cells. A 100µL (1.14×10^5cells/mL) aliquot of HEp-2 cell suspension was seeded in 96-well plate. Upon incubation period, the cells were exposed to graded concentration of Extract 'M', incubated for 48hours and

Figure 2 shows that the tested extract concentration did not induce comet formation due to the absence of tail moments from the comet head; and in comparison with the laddering pattern in the control groups lanes 4, 5 and 6 (graded concentrations of 1 % Triton-X), the concentrations in lane 2 and 3 (186.5 µg/mL and 559.5 µg/mL respectively) did not induce any DNA laddering pattern where M denotes DNA Marker.

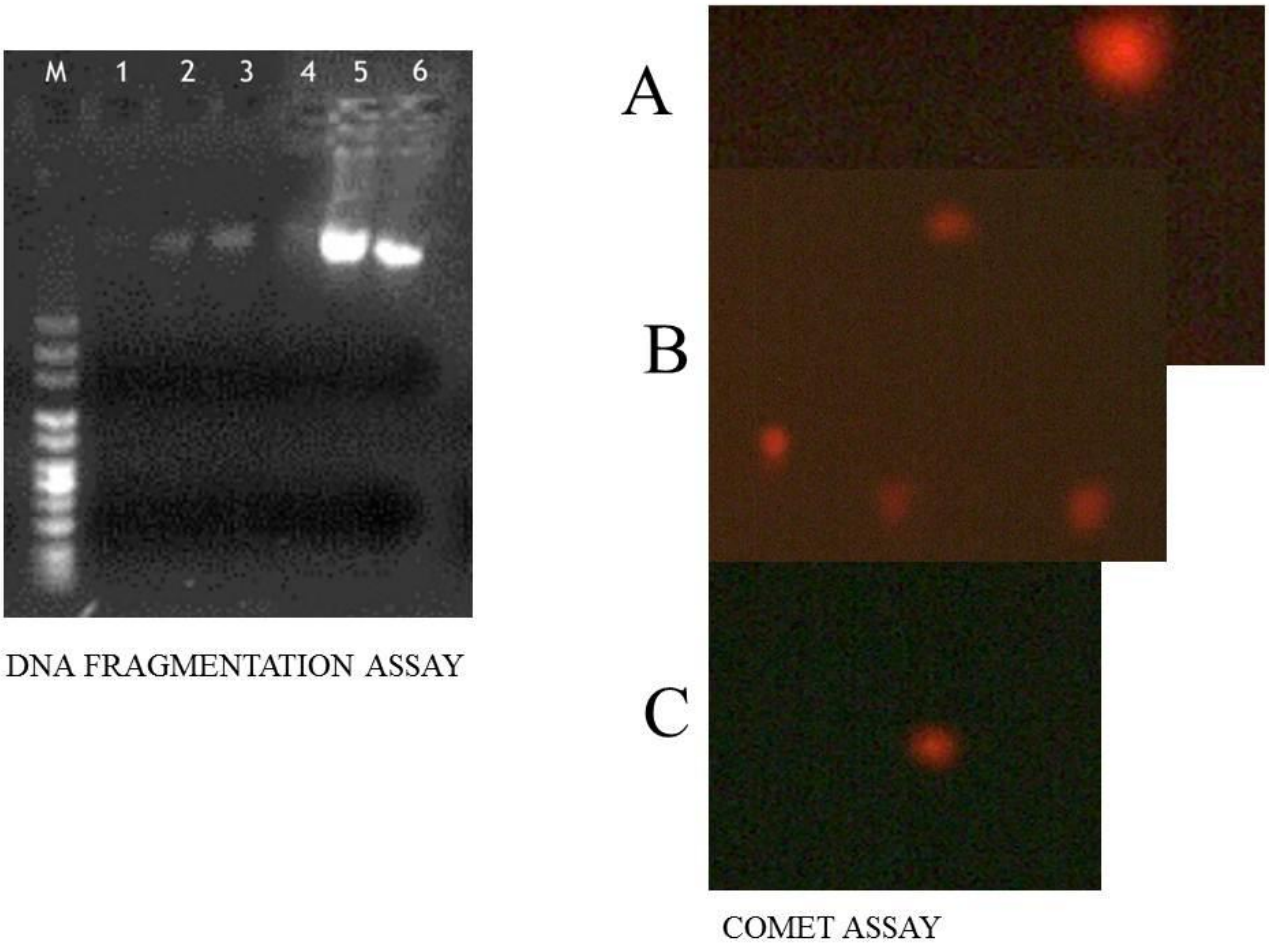

Figure 2: Genotoxic effect of Extract 'M' on HEp-2 cells. A 500 µL (1.14×10^5 cells/mL) aliquot of HEp-2 cell suspension was seeded in 6-well plates. Upon incubation period, the cells were exposed to 186.5 µg/mL and 559.5 µg/mL of Extract 'M', incubated for 72 hours and genotoxicity was evaluated using DNA Fragmentation and Comet assays

Discussion

The anticancer property of plant materials have been identified to serve as palliative measures with little or no side effects (McKinlay *et al.,* 1989). In a study conducted by Adebesin *et al.* (2013) where K562 leukaemic cell lines were exposed to the graded concentrations of either rmethanolic extract of *Senna alata* leaves or methanolic extract of *Senna podocarpa* leaves, the result show significant cytotoxic effects on the cells using WST-1 assay. The present work however shows that the formulation did not significantly ($p> 0.05$) alter cell viability at the doses tested. Farah *et al.* (2013) reported the exposure of human Leukemic cell lines (U937 and Molt4) to ethanolic tumeric extract and the toxicity was measured using Comet assay where the extract was found to show significant apoptogenic and DNA damage activity against the two cell lines. More so, α-Tocopherol and the ethylacetate fraction of *Acalypha wilkesiana* leaf extract has been reported by Su-wen *et al.* (2013) to have a complementary antiproliferative efficacy against U87MG (Human Brain cells) and A549 (Lung cancer cells) as evaluated by Comet assay. The current study provided evidence that 186.5µg/mL and 559.5µg/mL of the extract 'M' did not induce DNA Damage in the HEp-2 cells observed in the absence of tail moments from the comet head. Yedjou *et al.* (2008) exposed MCF-7 to *Vernomia amygdalina* leaf extract and the data generated from the comet assay analysis indicated a minimal dose-dependent increase in DNA damage in the MCF-7 cells. Maheswarappa *et al.* (2013) treated HEp-2 cells with graded concentrations of anticancer drug, prodigiosin and reported that the drug exhibited significant genotoxic effects on the cells as evaluated with Comet assay. The Di-Herbal formulation did not induce significant genotoxic damage in HEp-2 cells at the concentrations tested.

The results as obtained in this present study having being investigated using WST-1, Comet assay as well as DNA fragmentation assay have provided evidences that the Di-Herbal formulation has no significant antiproliferative effects on HEp-2 cells, rather have been found to support the viability of the cell population and as such may contain some growth promoting principles.

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