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Genotoxic and Cytotoxic Activity of Aqueous Extracts of *Croton membranaceus* in Rodent Bone Marrow and Human Benign Prostate Hyperplasic Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Authors GAA and SA designed the study, and wrote the protocol. Author BA performed the statistical analysis and author ESY wrote the first draft of the manuscript. Authors GAA, ESY and DKA managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Croton membranaceus* is a plant cherished by traditional healers in Ghana for its anti-prostate cancer properties. Its cytotoxic effect as well as safety has been proved. However, to the best of our knowledge, no study has been conducted to assess its genotoxic and cytotoxic potential using the rodent bone marrow and colony formation assays respectively.
Aims: This study is aimed at investigating the cytotoxic and genotoxic effects of the aqueous root

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extract of *Croton membranaceus* (CMARE) using the colony formation and rodent bone marrow assays respectively.
Study Design: This was an experimental study.
Methodology: To determine the cytotoxic effect, BPH-1 cells were seeded in 6-well plates at a density of 1.0 x 10^5 cells/well in 2 mL culture medium (plus 10% FBS) and incubated for 24 h. After treatment with 0, 1, 3 and 5 mg/ml of CMARE for 48 h, the cells were collected and further treated with fresh medium in the absence of CMARE, and reseeded into new 6-well plates at a density of 1.0 x 10^3 cells / well. After 10 days incubation, colonies were fixed in 10% formalin, crystal violet-stained and counted. Genotoxicity was determined by the bone marrow assay using 30 male Sprague-Dawley rats divided equally into three groups. Animals in the treatment, positive and negative control groups were administered 3000 mg/kg CMARE, N-nitroso-N-methylurea and saline respectively.
Results: Cytotoxicity significantly occurred at 3 and 5 mg/ml, compared to the control. The bone marrow assay showed a significant difference between CMARE-treated animals and negative controls when the polychromatic erythrocyte-normochromatic erythrocyte (PCE-NCE) ratios were compared. Furthermore, there was a positive correlation between CMARE-treated animals and positive controls when their micronucleated polychromatic erythrocytes (MNPCE) were compared, indicating similar genotoxic potential between very high CMARE doses and the positive control.
Conclusion: These results show that CMARE has both cytotoxic and genotoxic potential.

Keywords: Genotoxicity; cytotoxicity; Croton membranaceus; micronucleated cell.

ABBREVIATIONS

CSRPM = Center for Scientific Research into Plant Medicine
FBS = Fetal bovine serum
GAFCO = Ghana Agro food Company
MNPCE = Micronucleated polychromatic erythrocytes
MTD = Maximum tolerated dose
PCE-NCE = Polychromatic erythrocyte-normochromatic erythrocyte
SEM = Standard error of the mean

1. INTRODUCTION

Croton membranaceus Müll.Arg., a monoecious herb belonging to the Euphorbiaceae family, is a medicinal plant cherished in several countries around the world for its numerous pharmacological and socio-cultural effects. Anecdotal evidence suggests that its leaves are used to aromatize tobacco in the Bahamas, whilst in the West African country of Nigeria they are used as a tonic and aromatic bitters which enhance digestion. Similarly, essential oil extracted from the back is utilized in aromatherapy for the treatment of cough, fever, flatulence, diarrhea and nausea [1].

Concoctions of the herb are used by herbalists and traditional healers in Ghana for the management of various medical conditions, especially cancer of the prostate. Incidentally, its cytotoxic activity against various human cancer cell lines has been demonstrated. This is said to be as a result of the presence of six compounds including a novel furano-clerodanediterpenoid [12-oxo-15,16-epoxy- 3,13(16), 14-clerodatrien-17,18-dioic acid dimethyl ester] for which was suggested the trivial name "crotomembranafuran", the glutarimide alkaloid, julocrotine, p- sitosterol, p-sitosterol-3-D-glucoside, the labdanediterpenoid [labda- 8(17), 13E-dien-6a, 15-di-O-glucopyranoside] commonly called gomojoside H and DL-butane-1,2,3,4-tetraol (DL-threitol) [2,3]. Furthermore, its usefulness in the treatment of secondary bacterial infections has been shown to be due to its potent antimicrobial activity [4].

Recently, the root extract of *C. membranaceous* has been shown to be safe in acute toxicity studies. In that study, the 3000 mg of the extract administered per kg body weight of rats did not show signs of general toxicity or mortality [1]. To the best of our knowledge, no study has yet been conducted on its genotoxic effects in rodent bone marrow and cytotoxicity against BPH-1 cells.

In this study, the rodent bone marrow and the colony formation assays were used to assess the

genotoxic and cytoxic potential of the root extract of the plant.

2. MATERIALS AND METHODS

2.1 Plant Material

Whole plants of *C. membranaceus* were collected from the Gyekiti Forest Reserve area in the Eastern region of Ghana by Dr. Archibald Sittie of the Center for Scientific Research into Plant Medicine (CSRPM). The plants were identified by its vernacular names by the farmers and authenticated by taxonomists from the CSRPM herbaria, where voucher specimens (CSRPM 2110) have been kept for reference purposes. The roots were carefully cut off and washed with water, sun dried for two weeks, pulverised, packaged in sample bottles, labelled appropriately and stored at room temperature (25 -27ºC).

2.1.1 Preparation of aqueous extract (CMARE)

The method used by Asare et al. [1] was used to prepare the aqueous extract of *C. membranaceus*. One thousand grams (1000 g) of dry powdered *C. membranaceus* root was macerated for 24 hours with 4000 ml of distilled water and boiled for one hour. The extract was filtered through medical gauze (2 mm x 2 mm pore size) to separate it from the residue. Another 3000 ml of distilled water was added to the residue, macerated further for another 24 hours, and the above procedure repeated to obtain a second extract. The extracts were pooled and freeze-dried using Freeze Dryer Gamma 1-16/2-16 LSC (2004 version). The dry yield of the extracts was weighed (22.5 g) and stored in a sealed container in a refrigerator between 2ºC and 8ºC until use.

2.2 Chemicals

All chemicals used [N-nitroso-N-methylurea, Sodium Chloride, fetal bovine serum (FBS), May-Grunweld and Giemsa stains] were of analytical grade, and purchased from Sigma chemical company, St. Louis, Missouri, USA. Rosewell Park Memorial Institute Medium 1640 (RPMI 1640), and crystal violet stain were obtained from Sigma Chemicals (St. Louis, USA). Human BPH-1 cells were kindly provided by Professor Zhengfeng Hong (Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, China). All other chemicals, unless stated, were obtained from Sigma Chemicals (St. Louis, USA).

2.3 Animals

Thirty (30) young healthy male Sprague-Dawley rats aged six weeks with an average weight of 240 g, were used in this study. Rats were obtained from the Noguchi Memorial Institute for Medical Research and allowed to acclimatize for a period of five days before initiation of the study. The rats were kept under standard laboratory conditions and fed with commercial laboratory animal feed from the Ghana Agro food Company (GAFCO) ad libitum. Drinking water was also made available throughout the study period. Ethical approval for the study (STC-2009-02-3) was obtained from the scientific and technical committee of Noguchi memorial institute for medical research.

2.4 Cytotoxicity (Colony Formation Assay)

2.4.1 Cell culture

The human BPH-1 cells were cultured in RPMI Media 1640 Basic (L-Glutamine) containing FBS 10% (v/v), 100 Units/ml penicillin and 100 µg/ml streptomycin, and maintained at 5% CO_2 in a humidified incubator at 37ºC.

2.4.2 Preparation of phosphate buffered solution of CMARE

To enhance complete dissolution of CMARE powder, PBS was used as solvent to prepare a stock solution of 200 mg/ml. Serial dilutions of CMARE was further made with RPMI-Media 1640 Basic (L-Glutamine) in 10% FBS to obtain the required working concentrations.

2.4.3 Colony formation assay

The methods used by Rafehi et al. [5] and Shen et al. [6] were adopted for the colony formation assay. Briefly, BPH-1 cells were seeded in 6-well plates at a density of 1.0 x 10^5 cells/well in 2 mL medium (in 10% FBS) and incubated for 24 h. After treatment with various concentrations of CMARE (0, 1, 3 and 5 mg/ml) for 48 h, the cells were collected and treated with fresh medium in the absence of CMARE, and reseeded into new 6-well plates at a density of 1.0 x 10^3 cells/well. After incubation for 10 days in a 37ºC humidified incubator with 50% CO_2, the colonies formed

were fixed in 10% formaldehyde, stained with 0.01% crystal violet and counted using a stereomicroscope and an automatic "colony counter pen". Survival of cells was determined by normalizing the survival of control cells as 100%.

2.5 Genotoxicity Assay

2.5.1 Experimental procedure

Rats were randomly divided into three groups with ten (10) rats each. Animals in group one (GP1) were treated with the maximum tolerated dose (MTD) of the CMARE (3000 mg/kg body weight). Group two (GP2) rats served as the negative control, receiving normal saline (0.9%) orally. Rats in group three (GP3) served as the positive control, and were administered the mutagen N-nitroso-N-methylurea (10 mg/ml) intramuscularly.

The animals were observed for clinical signs of toxicity at various intervals after treatment. Half of the animals in each group were euthanized 24 hours after treatment, whilst the rest of the animals were euthanized 48 hours after treatment. Sacrifice consisted of euthanasia followed by excision of the left femur after the skin and muscle tissues were trimmed. Both ends of the bone tips were severed with bone snips, and the marrow flashed gently from the channel into a tube with fetal bovine serum (FBS, 2 ml per femur) into centrifuge tubes [7].

The preparation and staining of bone marrow cells were carried out according to the protocol of Schmid[5]. The tubes were centrifuged at 1000 rpm for 5 minutes, the supernatant removed and the cells re-suspended in a few drops of FBS. A thin film of bone marrow cells was prepared (2 slides per animal) and air dried overnight. The air-dried slides were fixed in methanol, stained with May-Grunweld and Giemsa as described by Schmid [8].

2.5.2 Scoring erythrocyte cells

The criteria for scoring followed that of Mac Gregor et al. [9] and OECD Guideline 474 [10]. At high magnification, two thousand erythrocytes were scored per animal. The erythrocytes were screened for the presence of micronucleited polychromatic erythrocytes (MNPCE) and micronucleated normochromatic erythrocytes (MNNCE). The ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) was then determined.

2.6 Statistical Analysis

Results were expressed as mean±SEM. The Student's t-test was used to compare MNPCEs and MNNCEs values and PCE to NCE ratios between treated and control animals for statistical significance. ANOVA was used to determine significant differences of colony count of the various concentrations. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1 Cytotoxicity (Colony Formation Assay)

Fig. 1 shows an increasing antiproliferative activity in BPH cells as the concentration of CMARE increased from 0 – 5 mg/ml. The quantitative analysis of the effect of various concentrations produced a dose dependent inhibition of growth of BPH-1 cells, with the highest dose tested (5 mg/ml) producing almost a 100% inhibition. Statistically significant differences were noticed at 3 mg/ml ($p<0.001$) and 5 mg/ml ($p<0.001$) (Fig. 2).

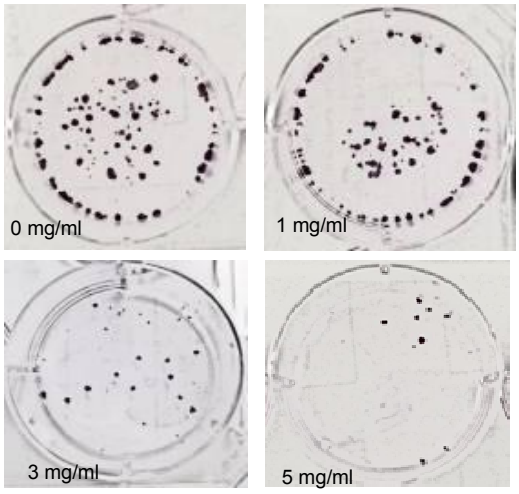

Fig. 1. Effect of CMARE on BPH-1 cell survival. Cells treated with CMARE in a dose-dependent manner are seen in the photomicrographs

BPH-1 cell survival was determined by anaysis of colony formation and the Images are representative of three independent experiments

3.2 Bone Marrow Assay

The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) in 2000

erythrocytes of rats euthanized 24 hours post treatment are shown on Table 1. The PCE: NCE ratio of the maximum tolerated dose group (0.22±0.02) was found to be similar to that observed for the negative control group (0.26±0.01). Both values were however found to be significantly higher than the value observed for the positive control group (0.06±0.00). The PCE: NCE ratio obtained for the maximum tolerated dose group 48 hours post treatment was however 34.6% lower than that realized for the negative control group (Table 2).

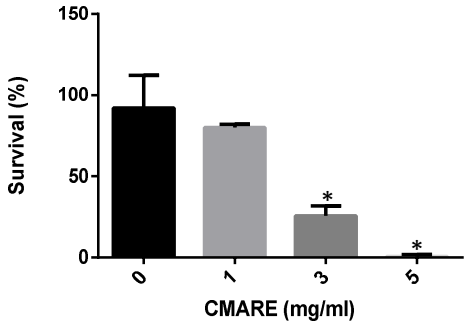

Fig. 2. Quantitification analysis of the effect of various concentrations of CMARE on BPH-1 cell survival after 48 hr of treatment

*The data were normalised to the survival of the control cells and presented as averages with SEM. *P < 0.001 compared to untreated BPH-1 cells. Data is representative of three independent experiments*

Table 1. Frequencies of micronucleated polychromatic erythrocytes (MNPCE) and polychromatic erythrocytes (PCE) in 2000 normochomatic erythrocytes (NCE) of rats euthanized 24 hours after treatment

Treatment	PCE/NCE ratio	MNPCE/PCE ratio
MTD	0.22±0.02	0.47±0.02
Positive control	0.06±0.00	0.49±0.02
Negative control	0.26±0.01	0.24±0.05 [a]

MTD, Maximum tolerated dose; [a]Value statistically significant (P<0.001) from MTD

On the other hand, a slight but insignificant difference in the MNPCE:PCE ratio was observed for the MTD and positive control groups of rats euthanized 24 hours after treatment (Table 1).

The value for the negative control group was, however, found to be 48.9% and 51.0% less than that obtained for the MTD and positive control groups, respectively. This was statistically significant (p<0.001), and an indication that the MTD and the positive control chemical could have similar effects on gene integrity. A similar outcome was observed in the 48 hour euthanasia group (Table 2).

Table 2. Frequencies of micronucleated polychromatic erythrocytes (MNPCE) and polychromatic erythrocytes (PCE) in 2000 normochromatic erythrocytes (NCE) of rats euthanized 48 hours after treatment

Treatment	PCE/NCE ratio	MNPCE/PCE ratio
MTD	0.17±0.01	0.47±0.05
Positive control	0.06±0.00	0.49±0.02
Negative control	0.26±0.01 [b]	0.24±0.05 [c]

MTD-Maximum tolerated dose; [b]Value significantly different from MTD (P < 0.05) and positive control (P<0.001), [c]Value significantly different from MTD and positive controls

4. DISCUSSION AND CONCLUSION

Colony formation assay has been used widely for assessing the cytotoxic potential of drugs and chemicals [11]. Administration of CMARE to BPH-1 cells resulted in the dose-dependent inhibition of colony formation, with significant antiproliferative effects observed with cells treated with 3 and 5 mg/ml. Findings were consistent with the antiproliferative effects of CMARE on prostate cells observed in our previous in vivo studies [12,13]. An unpublished data by Bayor [14] revealed that the cytotoxic potential was as a result of the presence of crotomembranofuran, β-sitosterol-3-D-glucoside and DL-threitol in the methanolic extract of *C. membranaceus.* The findings from this study could imply that the same chemicals identified by Bayor [14] could be eliciting the same observed effects in the current study

The micronucleus assay has been used widely to measure genotoxicity, both in vitro and in vivo, as part of a battery of tests recommended by regulatory agencies throughout the world for product safety assessment. The In vivo test is particularly relevant for assessing genotoxicity in that it allows consideration of factors of In vivo metabolism and other pharmacokinetic events. It also allows DNA-repair processes to occur. These properties make it a useful method for detection of the mutagenic effect of chemical substances [15].

Regulatory guidelines recommend that the high dose selected for the rodent micronucleus assay

should be the highest dose that can be administered without inducing lethality or excessive toxicity (maximum tolerated dose) or be administered at 2000 mg/kg [16,17]. Our previous acute toxicity study in CMARE using S-D rats employed the MTD of 3000 mg/kg b. wt. [1]. In this current study, the maximum tolerated dose, as given by Asare et al. [1] was assessed for genotoxicity.

When the normal proliferation of bone marrow cells is affected by a toxic agent, there is a decrease in the number of immature erythrocytes (PCE) in relation to the number of mature erythrocytes (NCE) [18]. It is considered that, a decrease of the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) in the micronucleus test is an indicator of bone marrow toxicity induced by mutagens [19]. In this study, treatment with the maximum tolerated dose of CMARE did not appear to have any significant effect on the PCE/NCE ratio 24 hours post treatment. However, by 48 hours post treatment the PCE/NCE ratio of the maximum tolerated dose group was clearly lower than that observed in the negative control group. This suggests that treatment with high doses of C. membranaceus has the potential of adversely affecting the normal proliferation of bone marrow cells, though not to the extent of the mutagen, N-nitroso-N-methylurea.

According to Muller and Streffer [19], the presence of micronuclei in cells is due to chromosomal damage during the last mitosis and they are the reliable indicators of genotoxicity of exogenous agents. Although there are several routes by which micronuclei may be produced, chromosomal damage is the predominant route by which they are formed. The frequency of micronuclei formation in polychromatic erythrocytes rodent bone marrow PCE is a very sensitive index of damage produced by chemical mutagens [20].

In this study, a significant increase in MNPCE/PCE ratio was observed in CMARE treated rats at 24 and 48 hours post administration compared to negative controls. Results from this aspect of the study obviously show that, treatment with the CMARE resulted in increasing the frequency of micronuclei formation, an indication that the maximum tolerated dose can induce genotoxicity. It is not however clear whether genotoxic effect of the extract is in any way related to the compounds responsible for its cytotoxic effect. Further studies will have to be conducted in that respect.

In conclusion, the cytotoxic potential of the extract on human BPH-1 cells has been demonstrated through the colony formation assay. Bone marrow assay has also proved for the first time that high doses of *C. membranaceus* aqueous root extract could also be genotoxic.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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