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Genotoxic and chemopreventive potentials of ethanol leaves extract of *Annona* muricata on *N*-Ethyl-*N*-Nitrosourea-induced pro-leukaemia carcinogen in mice model by bone marrow micronucleus assay

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ABSTRACT

Background. Studies have proven the effect of several agents, including natural products, to induce, prevent and treat genotoxicity through experimental models and clinical trials. In this study, the genotoxic preventive potential of *Annona muricata* ethanol extract on *N*-Ethyl-*N*-Nitrosourea (ENU)-induced pro-leukaemia in mice models using micronuclei formation in bone marrow was assessed.

Materials and methods. Forty-eight mice weighing 18-24g were randomly divided into six groups of eight mice. The mice were intravenously administered 20mg/kg of NEU 48 hourly 3 times, 80mg/kg of NEU 48 hourly 3 times. The negative control was fed with feed and water only. We introduced 0.2ml (0.1g/ml) ethanolic extract of Annona muricata for 3 weeks prior to NEU low dosage administration, 0.2ml (0.1g/ml) ethanolic extract of Annona muricata for 3 weeks prior to ENU high dosage and Annona muricata (ethanolic extract) administration, and gave commercial diet to the adverse/ toxicity group. The bone marrow was harvested, smeared and stained using MayGrumwald. The procedure enabled the determination of micronucleus polychromatic erythrocytes (MNPCEs) microscopically.

Results. Groups exposed to various dosages of the ENU yielded significantly increased MNPCEs, with group B producing higher MNPCEs. The groups treated with the extract displayed a significant reduction in the MNPCEs despite prior exposure to concentrations of NEU. The adverse group displayed no difference in MNPCEs compared with the negative control.
Conclusions. The ENU induced genotoxicity depending on its concentration. The extract displayed a profound capacity to prevent genotoxicity and alleviate leukaemia with good tolerance.

Introduction

Leukaemia, the eighth to twelfth most common cancer generally, with an increasing incidence trend, is a global public health concern as more than 420,000 new cases and over 300,000 cancer deaths from leukaemia occur worldwide [1–3]. Notable geographical disparities evidence this as a result of significant factors such as quality health system with adequate accessibility and some etiological factors like gene-environment interaction [4].

Genotoxicity is a series of irritation to a cell's genetic makeup, which can result in adverse outcomes such as cancer, inherited mutation, ageing/other developmental toxicity and several diseased states [5]. The major classes of genotoxic materials include mutagens, carcinogens and teratogens. Their genotoxic effects could be categorized as mutational, clastogenic and aneugenic [5, 6]. The most common chemical carcinogens identified in studying the progression of leukaemia are *N*ethyl- *N*-nitrosourea (ENU), *N*-methyl-*N*-nitrosourea (MNU), dimethyl benzanthracene (DMBA), benzo (a) pyrene (BaP), amongst others [7]. N-ethyl-*n*-nitrosourea (ENU) (C3H7N3O2), a potent mutagen, acts by transferring its alkyl (ethyl or methyl) group to the nucleobases in nucleic acids in the bone marrow, thereby generating leukaemogenesis [8, 9].

Furthermore, the internationally accepted genotoxicity assessment methods are some in-vitro and in-vivo assays, including the mouse lymphoma gene mutation assay (MLA), the micronucleus assay, the Ames bacterial mutagenicity test, the Comet assay, the chromosome aberration test and sister chromatid exchange assays. All the methods assess DNA damage by diverse mechanisms, like intragenic mutations, chromosomal rearrangements or deletions, loss or gain of whole chromosomes (aneuploidy) or chromosomal segments and other genotoxic effects [10–12].

Herbal supplements and formulations have been experimented on severally to have beneficial, prevention, and therapeutic effects on the physiological function and reversal of diseased states in the body system [12–14]. There are several reports depicting their beneficial effects. The recently discussed ones include boosting the immune system, regulating oxidation, enhancing memory, acting as an antidepressant and an antidiabetic means, and chemotherapeutic agents in cancer [15–18]. We have identified the active ingredients in the aqueous or ethanolic forms of plants and herbs and their potencies. The toxicity tendencies of these herbs have also been examined. Studies are still emerging to depict the efficacy of more herbal products in treating carcinogen-induced genotoxicity and their systemic effects.

Annona muricata, also known as soursop, guanabana and commonly graviola, is an efficacious medicinal tree. Indigenous communities of Africa and South America use different parts of the plant extensively to perform numerous ethnomedicinal activities. Phytochemical analysis has identified annonaceous acetogenins as the major constituents of the leaves, bark, roots and fruits [19]. In addition, several studies have distinguished the anti-arthritic, hepatoprotective, antimalarial, antidiabetic, anticancer, and anticonvulsant effects [20, 21].

Despite reported evidence of the efficacy of A. murricata in some cancers, there is a need to determine the effects on ENU-induced pro-leukaemia in experimental models as an in-vivo study. Furthermore, the study should use a micronucleus assay to determine genotoxicity and any other stage of transformation for possible future application in cancer prevention, therapy, control, and eradication. Hence there is a reason for this study.

Materials and Methods

Procurement, Identification and Extraction of *Annona muricata* leaves.

The leaf samples of *Annona muricata,* locally called Apekan, Tuwon biri and Sawansop in Yoruba, Hausa and Igbo languages of Nigeria, respectively, were gotten from a local farm at Osogbo city, Osun state, Nigeria. It was further identified and authenticated at the Department of Pharmacology, Obafemi Awolowo University Ile-Ife, Nigeria.

Ethanol Extraction of the leaves

Fresh matured leaves of *Annona muricata* were separated from the stalk, air-dried at room temperature (24^0C), pulverized and crushed into a fine powder using a grinding machine and weighed. Ethanolic (Absolute) extracts of the plant were prepared by soaking 500 g of the dry powdered plant material in two (2) litres of absolute ethanol and then kept at room temperature for 48 hours (for thorough extraction). At the end of the 48 hours, the extracts were filtered first using a Whatman filter paper No.42 (125 mm) and cotton wool. The harvested ethanol extract of *Annona muricata* leaves was then dried off in an oven at 37^0C and stored at 4^0C. Subsequently, on each day of the experiment, an aliquot portion (1 g) of the crude plant extract residue was weighed and dissolved in distilled water (10 ml) for use.

Experimental Models

The experimental models included forty-eight (48) young Swiss Albino mice weighing between 18 g and 24 g and purchased from the animal house of Ladoke Akintola University of Technology (LAUTECH), Mercyland, Osogbo, Nigeria. The mice were divided into six (6) groups of eight (8) mice each, made to acclimatize to the new animal house conditions for one week at the department of Medical Laboratory Science, Ladoke Akintola University of Technology (LAUTECH), Mercyland, Osogbo, Nigeria before the commencement of the experiment. They were fed with a standard commercial pellet with clean water *ad libitum*. The animal house was ascertained to be pathogen-free, room temperature maintained at 28 ± 2^0C, and adequate ventilation ensured with 12 hours light/dark cycle.

Experimental procedures involving the experimental animals and their care were conducted in compliance with the Guidelines for Care and Use of Laboratory Animals in Biomedical Research promulgated by the Canadian Council on Animal Care (2003) [23]. Accordingly, the Animal Care and Use Ethical Committee of the College of Medicine and Health Science, Ladoke Akintola University of Technology (LAUTECH), Osogbo, Nigeria, granted the Ethical Approval.

Preparation and Administration of ENU for Leukaemia Induction

The *n*-ethyl-*n*-nitrosourea (ENU) was procured from Sigma Aldrich (Germany) and stored at −20°C as indicated by the manufacturer. It was reconstituted daily with normal saline by measurement of the average body weight (kg) of each mouse with corresponding carcinogen weight (mg) across all groups except the negative control and adverse reaction group (**Table 1**). The mice were administered twenty (20) mg/kg and eighty (80) mg/kg body weights of the solutions intraperitoneally (IP) with a sterile 26-gauge – needle 48 hourly three times (**Table 1**). The mice were then monitored closely to determine the induction of a pro-leukaemia state and other reactions on the administration of extracts.

Administration of Ethanolic Extract and Acute toxicity

One gram of *A. muricata* (ethanolic crude extract) was dissolved in 10 ml of distilled water to make

Table 1. Experimental Protocol

Groups (6 mice/group, 18–24kg)	Treatment	Inference
A	20mg/kg NEU 48 hourly thrice	Leukaemia induction by low dosage
B	80mg/kg of NEU 48 hourly thrice	Leukaemia Positive by high dosage
C	Feed and Water	Negative Control
D	0.2ml (0.1g/ml) extract +20mg/kg NEU 48 hourly thrice	Preventive group on low dosage of NEU
E	0.2ml (0.1g/ml) extract + 80mg/kg of NEU 48 hourly thrice	Preventive group on high dosage of NEU
F	0.2ml (0.1g/ml) extract + Feed and Water	Adverse Group

a concentration of 0.1 g/ml of the prepared extract. The mice in the preventive groups D and E (**Table 1**) were administered the *A. muricata* with 0.2 ml volume orally by gavage once daily with the aid of oral cannula and sterile syringes for 3 weeks. The adverse group F mice were administered the same concentration and volume of extracts for 3 weeks along with feed and water. They were then observed toxicity, behavioural changes and mortality the administration period. The ethanol leaf extract lethal dose (LD50) had been determined to be above 5000 mg/kg [23].

Sample Collection and Animal Sacrifice

Sample collection commenced precisely three weeks after the last exposure to ENU in each induction group A and B, the negative control group C, and the adverse/toxicity group F.. Also, the sample collection commenced in the preventive at the fifth week after three weeks of the last exposure to ENU administration in groups D and F. At the end of the ninth week of the experiment, the animals from each group were terminated ethically by cervical dislocation, and both femurs were surgically removed and placed in a petri dish containing normal saline. The bone marrow was flushed with 1 ml of fetal calf serum (FCS) into 1.5 ml Eppendorf Tubes, centrifuged at 2000 rpm for 5 minutes and the supernatant removed. The pellets were then suspended in another 1 ml of FCS in Eppendorf Tubes, well-mixed, and centrifuged at the same rate. Finally, the supernatant was removed and discarded; 0.05 ml of FCS was added to the pellet and mixed properly to form a viscous suspension. The smear was made from the suspension on a clean, grease-free slide with drops of the viscous suspension and air-dried overnight.

Staining for Micronucleus (MN) test

Staining was performed as described by Schmidt (1976), Alabi and Bakare (2011), and Oyeyemi *et al.* (2015) [24–26]. First, the smear was fixed in 70% methanol for 3 min and air-dried for 24 hours. It was then stained with 0.4% May-Grumwald stain for 3 min. It was immediately transferred into another Coplin Jar containing an equal volume of May-Grumwald and distilled water to allow further staining for 3 min. Next, the stained slides were rinsed in distilled water and air-dried for 24 hours. After 24 hours, the air-dried slides were stained in 5% Giemsa stain for 5 minutes, rinsed in distilled water and air-dried for 24 hours, after which it was dipped in Xylene and mounted in DPX at 45^0C.

Enumeration of micronuclei cells in MN test

The slides were scored under 100X (oil immersion) objective using a light microscope to score the number of micronucleus polychromatic erythrocyte (MNPCEs) out of 1000 polychromatic erythrocytes per mice. The differential staining of the bluish–purple polychromatic erythrocytes (PCEs) and pinkish normochromatic erythrocytes (NCEs), as well as the relative size of the erythrocytes, were indices for differentiating them (Schmidt 1976; Alabi and Bakare 2011; Oyeyemi *et al.*, 2011).

Statistical Analysis

Data obtained were analyzed using IBM SPSS for Windows, version 25.0 (SPSS Inc., Chicago, IL), and the results were expressed as mean ± SEM (standard error of the mean). PCE, NCE, and MNPCE values were calculated to derive percentages, and significant differences were tested using analysis of variance (ANOVA). Values considered significant at $p \leq 0.05$

Results

Pro-Leukaemia Induction by Micronucleus Assay

The number of micronucleus polychromatic erythrocytes per polychromatic erythrocytes was counted, as seen in **Figure 1**, and displayed the resultant pro-leukaemia induction effect of the ENU in the animal. It was visible as a simultaneously increased trend in the low and high ENU dosage groups A and B (**Table 2** and **Figure 2**) between weeks 2 and 5, which are distinctly significant and farther from the other groups in the diagrammatic representation. Also, groups D and E, where ENU was administered after the extract administration, displayed an increase in their mean MNPCE values in the line graph according to the administered ENU dosage (**Figure 2**).

In **Table 2**, significant differences were observed when group A was compared with the other 5 groups ($p < 0.05$) with a more significant increase in group B compared with the other 5 groups. Despite their preventive design, this observation is similar in groups D and E.

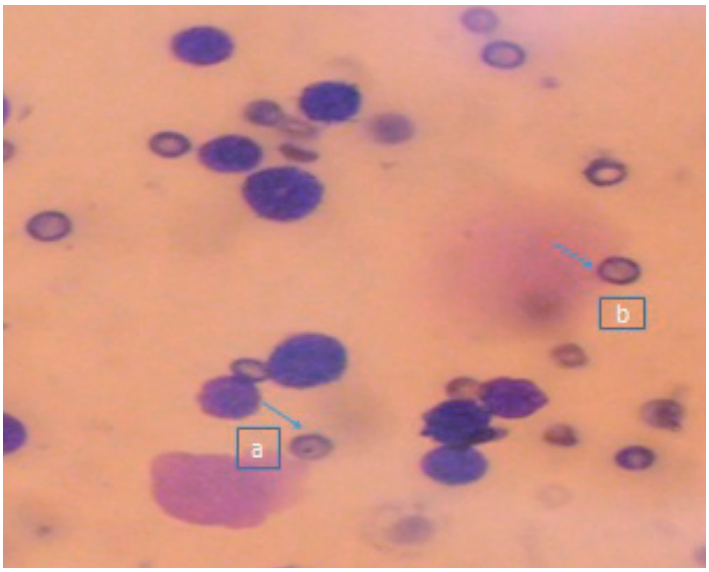

Figure 1. Micronuclei in mice administered A. murricata after exposure to *n*-ethyl-*n*-nitrosourea. A: Micronuclei polychromatic erythrocyte; B: Normal polychromatic erythrocyte

Table 2. Mean ± Standard deviation of micronucleus polychromatic erythrocyte cells (MNPCE) population among the groups across the weeks of exposure

Week Number	Groups (Mean ± Standard deviation of MNPCE)					
	A	B	C	D	E	F
1	-	-	-	-	-	-
2	14.13 ± 0.56	50.45 ± 2.31	4.52 ± 0.34	-	-	4.53 ± 0.45
3	15.01 ± 0.18	53.77 ± 1.95	4.61 ± 0.67	-	-	4.62 ± 0.23
4	16.11 ± 0.22	56.95 ± 2.01	4.91 ± 0.88	-	-	5.63 ± 0.45
5	19.64 ± 1.68	68.23 ± 1.56	4.82 ± 0.75	6.15 ± 0.49	17.11 ± 1.11	5.92 ± 0.67
6	-	-	-	7.85 ± 0.98	20.34 ± 2.34	-
7	-	-	-	8.01 ± 1.01	25.11 ± 1.67	-
8	-	-	-	10.07 ± 0.67	30.54 ± 2.34	-

Group A – Leukaemia induction by a low dosage; Group B – Leukaemia induction by a high dosage; Group C – Negative Control; Group D – Preventive group on a low dosage of N-Nitroso-N-ethylurea; Group E – Preventive group on a high dosage of N-Nitroso-N-ethylurea; Group F – Adverse/Toxicity group.

Acute Toxicity of *A. murricata*

The oral administration of 0.2 ml of 0.1 g/ml concentration of the ethanolic extract of *A. murricata* for 3 weeks continuously in group F did not yield behavioural changes, toxicity signs or mortality. Furthermore, the mean±SD MNPCE values for group F were not significantly different from the negative group C ($p > 0.05$), which proved good extract tolerance.

A. murricata preventive effect on genotoxicity

The chemopreventive potential of A. murricata ethanolic extract was displayed based on the ENU concentration administered in groups D and E. An increase in MNPCE level in both groups was lower than in groups A and B because of the extract administration (**Figure 2**). Also, there was a significant decrease when group D was compared with A and B ($P < 0.05$) and an increase when compared with other groups ($P > 0.05$), and the same observation was recorded when group E was compared across other groups.

Discussion

Several studies have proven the use of herbal or plant extracts in the control and management of diseases efficient. Coupled with the cheap and affordable nature of such products, especially in

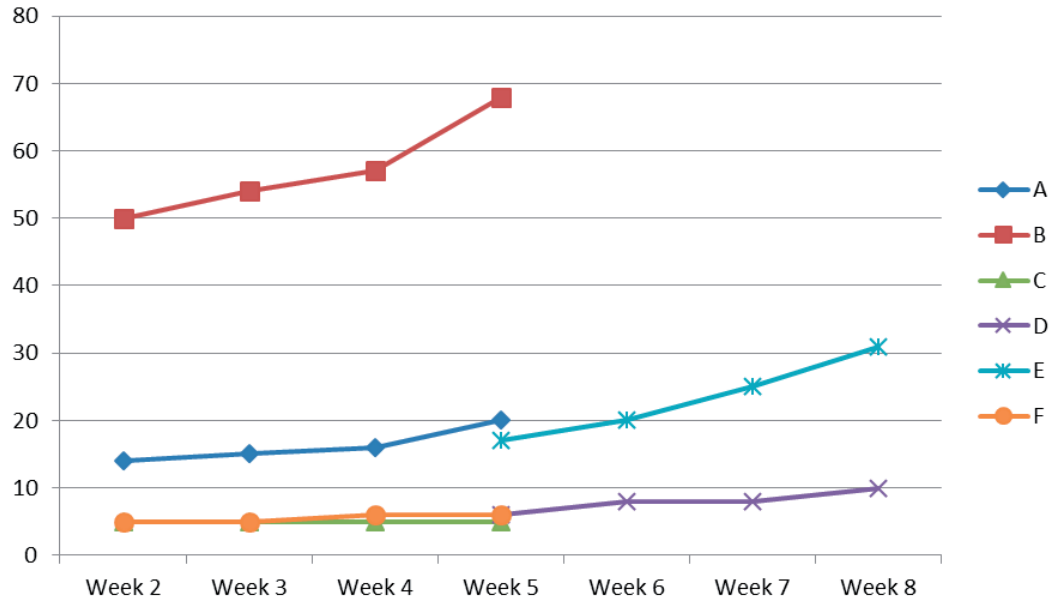

Figure 2. Mean micronucleus polychromatic erythrocyte cell (MNPCE) population among the groups across the weeks of exposure

Table 3. Comparison of Mean ± Standard deviation of MNPCE across the various groups

Week number	Groups (Mean±Standard deviation of MNPCE)					
	A	A vs. B	A vs. C	A vs. D	A vs. E	A vs. F
1	-	-	-	-	-	-
2	14.13 ± 0.56	50.45 ± 2.31*	4.52 ± 0.34*	-	-	4.53 ± 0.45*
3	15.01 ± 0.18	53.77 ± 1.95*	4.61 ± 0.67*	-	-	4.62 ± 0.23*
4	16.11 ± 0.22	56.95 ± 2.01*	4.91 ± 0.88*	-	-	5.63 ± 0.45*
5	19.64 ± 1.68	68.23 ± 1.56*	4.82 ± 0.75*	6.15 ± 0.49*	17.11 ± 1.11*	5.92 ± 0.67*
6	-	-	-	7.85 ± 0.98*	20.34 ± 2.34*	-
7	-	-	-	8.01 ± 1.01*	25.11 ± 1.67*	-
8	-	-	-	10.07 ± 0.67*	30.54 ± 2.34*	-
Week number	Groups (Mean±Standard deviation of MNPCE)					
	B	B vs. C	B vs. D	B vs. E	B vs. F	E vs. F
1	-	-	-	-	-	-
2	50.45 ± 2.31	4.52 ± 0.34*	-	-	4.53 ± 0.45*	-
3	53.77 ± 1.95	4.61 ± 0.67*	-	-	4.62 ± 0.23*	-
4	56.95 ± 2.01	4.91 ± 0.88*	-	-	5.63 ± 0.45*	-
5	68.23 ± 1.56	4.82 ± 0.75*	6.15 ± 0.49*	17.11 ± 1.11*	5.92 ± 0.67*	17.11 ± 1.11
6	-	-	7.85 ± 0.98*	20.34 ± 2.34*		20.34 ± 2.34
7	-	-	8.01 ± 1.01*	25.11 ± 1.67*		25.11 ± 1.67
8	-	-	10.07 ± 0.67*	30.54 ± 2.34*		30.54 ± 2.34
Week number	Groups (Mean±Standard deviation of MNPCE)					
	C	C vs. D	C vs. E	C vs. F	D vs. E	D vs. F
1	-	-	-	-	-	
2	4.52 ± 0.34	-	-	4.53 ± 0.45	-	4.53 ± 0.45
3	4.61 ± 0.67	-	-	4.62 ± 0.23	-	4.62 ± 0.23*
4	4.91 ± 0.88	-	-	5.63 ± 0.45	-	5.63 ± 0.45*
5	4.82 ± 0.75	6.15 ± 0.49*	17.11 ± 1.11*	5.92 ± 0.67	17.11 ± 1.11*	5.92 ± 0.67*
6	-	7.85 ± 0.98*	20.34 ± 2.34*	-	20.34 ± 2.34*	
7	-	8.01 ± 1.01*	25.11 ± 1.67*	-	25.11 ± 1.67*	
8	-	10.07 ± 0.67*	30.54 ± 2.34*	-	30.54 ± 2.34*	

Group A – Leukaemia induction by a low dosage; Group B – Leukaemia induction by a high dosage; Group C – Negative Control; Group D – Preventive group on a low dosage of N-Nitroso-N-ethylurea; Group E – Preventive group on a high dosage of N-Nitroso-N-ethylurea; Group F – Adverse/Toxicity group. *- p significant at 0.05 ($p \leq 0.05$)

low-resource and income countries, A. muricata is not an exception to those qualities [12–16, 26]. Therefore, the active ingredients of the A. muricata ethanolic leaf extracts have been analyzed for the content of acetogenins, alkaloids, flavonoids (phenolic compounds), such as Sparteine, Anthocyanin, Sapogenin, Morphine, Phenol, Quinine, Ribalinidine, Ephedrine, Resveratrol, Catechin, Saponin, Oxalate and Quercertin [27], steroids, and saponins [28]. All the substances have specific and multiple effects on cell constituents depending on their concentration and target tissues. These abilities include, for example, antidiabetic, anticonvulsant, antioxidant, cardioprotective, antimicrobial, and anti-carcinogenic activities [23, 27].

In this study, different ENU concentrations were observed to induce pro-leukaemia with resulting leukaemia states in the models, evidenced by the increasing trend of the values of MNPCE counted per group. The ENU has been studied to possess leukaemogenetic property based on its ability to transfer alkyl to the nucleobases of cellular nucleic acid by an enzymatic reaction [9]. Therefore, this study emphasises the agent's ability and efficacy in inducing leukaemia for study and other research purposes. Furthermore, the study has proven that a required form of induction, especially the gravity of the leukaemia state, determines ENU ability to perform such a task successfully.

A.muricata has been shownto possess anticancer abilities by suppressing tumor growth and induced apoptosis of various cancer cell lines [19, 29–31]. The study further depicts its antigenotoxic and chemopreventive attributes manifested by the drastic reduction in the genotoxic effect of ENU and evidenced by the consistent reduction in the MNPCE value in the preventive groups. The protective ability of A. muricata leaf extract to withstand carcinogenic agents in inducing pro-leukaemic conditions and eventually full-blown leukaemia was displayed. It can be deduced from the high concentration of various acetogenins, phenols and alkaloids. The substances have been studied to possess anticancer properties. The acetogenins inhibit the mitochondrial complex I due to their bis-THF structure in vitro, thereby preventing replication of the cancerous cell lines [31]. It is characterized by its unbranched C32 or C4 fatty acid with γ-lactone at the end of the cytoskeleton. This structure is highly reactive against cancer as it deprives the cells of ATP supply to the mitochondria, resulting in apoptosis [32–34]. On the other hand, the efficacy of alkaloids of plant derivatives has been proven in oncogenesis suppression by modulating key signalling pathways involved in proliferation, cell cycle, and metastasis [32, 33].

Furthermore, a previous study on gas chromatography-mass spectrometry (GC-MS) analysis of the essential leaf oil of A. murricata has further indicated terpenes, terpenoids and δ-cadinene as the significant essential oil and compounds of the leaves with 50.26% 34.24% and 22.58%, respectively. Other constituents include, amongst other sesquiterpenes, alkanes,*r*- Cadinol, esters, β-Caryophyllene, α-Copaene, Ledene oxide II, and Octadecane [37]. The phytochemicals acetanogennins, flavonoids, alkaloids with the terpenes and δ-cadinene essential oil forms are the constituents suspected to be the main antigenotoxic contributors in the studied extract. The presence of these constituents severally inhibits the proliferation of MCF-7, MDA-MB-231 and 4T1 breast cell lines, initiate cytotoxicity on histiocytic lymphoma cell lines, pancreatic cancer cells, immortalised human keratinocytes, HaCat, normal human liver cells, WRL-68, and human skin malignant melanoma, A375 [38–42].

Some essential minerals such as calcium, sodium, potassium, iron, magnesium, and zinc are also abundantly present in A. muricata [43]. These elements generally contribute to the normal physiologic function of the cell primarily by the enzymatic catalytic system and oxidation-reduction reaction aiding cellular functions. Therefore, their presence in the leaf extracts has yielded similar outcomes in the A. muricata administered models hence down-regulating the induced genotoxicity.

Furthermore, this study established the ability of the mice models to tolerate *A. muricata* well enough. Hence the normal state observed with MNPCE values as seen in the negative control. Although the same leaf extract concentration was administered, the rate at which the MNPCE differed in the positive groups with the low and the high ENU dosage was also replicated in the chemopreventive groups. The fact points to consistency in the extract's ability to eradicate the leukaemic condition irrespective of the dos-

age of the ENU. This observation still affirms the anti-genotoxic ability of A. *muricata* irrespective of the extent of genotoxicity or leukaemia development.

In conclusion, the ENU-induced genotoxicity manifested by the pro-leukaemia state and detected with the bone marrow micronucleus assay in the animal model proved its capacity to induce malignancy and pose a risk factor on exposure. The studied A. muricata ethanolic leaf extract prevented further induction of genotoxicity and pro-leukaemia in the ENU-induced model, which was recognised by a reduced MNPCE count in the preventive groups based on the ENU dosages. The proven active antigenotoxic phytochemicals and essential oils of A. muricata include acetanogennins, flavonoids, alkaloids, terpenes, and δ-cadinene. The anti-genotoxic activity of A. muricata was also seen to be potent in the case of a high ENU carcinogenic agent dosage, with an observable tolerance of the extract when administered within the required dosage to the non-induced mice models.

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Conflict of interest statement
The authors declare no conflict of interest.

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