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Genotoxicity and anti-genotoxicity of aqueous extracts of herbal recipes containing *Luffa cylindrica* (L), *Nymphaea lotus* (L) and *Spondias mombin* (L) using the *Allium cepa* (L) assay

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Management of diseases with medicinal plant is an ancient practice that has improved in recent years. Extracts of *Luffa cylindrica* (Linn), *Nymphaea lotus* (Linn) and *Spondias mombin* (Linn) are used for traditional management of cancer in Nigeria. Four recipes prepared from the combinations of two or three of these plants; *L. cylindrica, N. lotus* and *S. mombin* (LNS), *N. lotus* and *S. mombin* (NS), *N. lotus* and *L. cylindrica* (NL), and *S. mombin* and *L. cylindrica* (SL), were evaluated for genotoxicity and anti-genotoxicity using the *Allium cepa* chromosome aberration and root growth inhibition assay. Five concentrations (1, 2.5, 5, 10 and 20%) of each recipe were considered. There was a concentration-dependent inhibition of root growth and reduction of mitotic index in each of the recipe compared with the negative control. All the recipes induced chromosomal aberrations but not significant ($p<0.05$) at tested concentrations. The extract of LNS reduced the frequency of chromosomal anomalies induced by lead nitrate. These show the potential of tested extracts to induce and ameliorate cytogenetic damage in *Allium cepa*.

Keywords: Medicinal plant, recipe, mitotic index, chromosomal aberration, *Allium cepa*.

INTRODUCTION

The increasing use of medicinal herbs is a clear evidence of public interest in having alternatives to conventional medicine. However, despite the profound therapeutic advantages possessed by medicinal plants, some of their constituents have been found to be potentially toxic, mutagenic, carcinogenic and teratogenic (Ping et al., 2012). Long-term use of herbs to treat or manage diseases can induce cellular damages (Oyedare et al., 2009) and thus increase the side effects and potential toxicity of the medicinal plants, hence, the need to assess their potential toxicity. *Luffa cylindrica, Nymphaea lotus* and *Spondias mombin* are medicinal plants commonly used in Nigeria in the traditional management of cancer. They have been reported to have great medicinal value,

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with treatment of intestinal disorders, antioxidant, anti-inflammatory, antiviral, antiparasitic, antibacterial, antimicrobial, antihepatotoxic, and antihelminthic activities (Ajao and Shonukan, 1985; Corthout et al., 1994; Ademola et al., 2005; Muthumani et al., 2010; Igwe et al., 2011; Velmurugan et al., 2011). Herbal preparations in Nigeria are mostly made as recipe. It is generally believed that herbs, when used in combination are more active than when used individually. In a previous study (Oyeyemi and Bakare, 2013), we evaluated the genotoxic and antigenotoxic effects of aqueous extracts of *L. cylindrica, N. lotus* and *S. mombin* in *Allium cepa.* Herein, we investigated the cytogenotoxicity of aqueous extracts of four recipes containing two or three of these plants using the *Allium cepa* assay. In addition, we also evaluated the anti-genotoxic effects of the recipe having a combination of the three plants.

MATERIALS AND METHODS

Collection and Identification of plants

The leaves of *S. mombin,* whole plants of *N. lotus* and fruits of *L. cylindrica* were collected within the premises of the University of Ibadan, Nigeria and then taken to the University of Ibadan herbarium for authentication where voucher specimens (*S. mombin* UIH-22350, *N. lotus* UIH-22349, *L. cylindrica* UIH-22348) were deposited. The leaves and whole plants were washed with tap water, shade dried, ground and stored in the dark while the fruits were washed with tap water and used fresh.

Preparation of extracts

Four different recipes were prepared from these plants using the following combinations:

1. *L. cylindrica, N. lotus* and *S. mombin* (LNS).
2. *N. lotus* and *S. mombin* (NS).
3. *S. mombin* and *L. cylindrica* (SL).
4. *N. lotus* and *L. cylindrica* (NL).

For LNS, 15 g each of ground *S. mombin* and *N. lotus* and 20 g of *L. cylindrica* were boiled in 1 L of tap water. For NS, NL and SL, 25 g each of the combined plant materials were boiled in 1 L of tap water. The resultant mixture from each combination was filtered using Whatman® no.1 (11 µm) filter paper and the filtrate of each preparation taken as the stock solution was kept at 4°C until use.

Allium cepa assay

Onions (*Allium cepa*, L., 2n = 16, Family *Amaryllidaceae*) obtained commercially in Ibadan, Nigeria, were sun-dried for 2 weeks and used in the modified *A. cepa* assay (Fiskesjo, 1997; Bakare et al., 2009) to evaluate the potential genotoxic effects of the recipes and anti-genotoxic effects of LNS only. Twelve onion bulbs were used per concentration of each of the test samples. Five concentrations (v/v): 1, 2.5, 5, 10 and 20% of each recipe were used. Tap water and lead nitrate (10 ppm) solution were utilized as the negative and positive control, respectively.

For the genotoxicity study, a series of 12 bulbs were placed on top of 100 ml beakers filled with the different concentrations of each of the recipe (85 to 100 ml of each of the recipe depending on the size and placement of the onion on the beaker) and incubated in the dark at room temperature for 72 h with the test samples being changed at 24 h interval. The same number of bulbs and treatment were used for the controls. At 48 h, the meristematic region of the roots from 2 bulbs was cut and processed for slide preparation. In the antigenotoxicity study, a series of 12 bulbs were placed on top of beakers filled with lead nitrate (10 ppm) solution for 24 h. After the lead nitrate treatment, the bulbs were treated with five different concentrations of LNS for 48 h and incubated in the dark at room temperature. At 24 h of treatment with LNS, the meristematic region of the roots from 2 bulbs was cut and processed for slide preparation.

Root growth and cytogenetic analysis

In the genotoxicity and anti-genotoxicity studies, the length of the roots of the remaining 10 onion bulbs at each concentration were measured (in cm) at 72 h and used as an index of general toxicity (root growth inhibition). From the weighted averages for each concentration, the percentage root growth inhibition in relation to the negative control and the EC_{50} for each extract was determined (Fiskesjo, 1985). The American Society for Testing and Materials (ASTM, 1994) minimal statistical guidelines for conducting early seedling growth tests were used in the analysis of measured root length.

For the slide preparation, the cut root tips were fixed in ethanol: glacial acetic acid (3:1, v/v). After which the roots were hydrolyzed in 1N HCl at 60°C for 5 min and then washed thrice in distilled water. Root tips (2 to 3) were squashed on each slide and stained with acetocarmine for 10 min. Six slides were prepared for each concentration out of which four were used for microscopic observation at 1000× magnification (4000 cells were observed per concentration). Chromosomal aberrations were characterized and classified. The mitotic index was calculated as the number of dividing cells per total cells scored at each concentration. The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored at each concentration of the extract (Bakare et al., 2009).

Statistical analysis

The SPSS 17.0® software package was utilized. Data on root length, mitotic index and chromosomal aberrations were compared using analysis of variance (ANOVA) followed by Dunnett test ($p<0.05$). The EC_{50} was determined from the root length data using Probit regression analysis. Correlation between root length and mitotic index was determined using Pearson correlation coefficient.

RESULTS

The result of the root length parameters is presented in Table 1. There was a marked inhibition of root growth at all tested concentrations for all the recipes. The inhibition of root growth was concentration dependent for all the recipes except for NL. The EC_{50} values obtained are 12.4, 12.2, 24.8 21.8 and 13.9% for LNS, NS, SL NL and LNS+ lead nitrate respectively. The results of microscopic effects are summarized in Tables 2 to 6. A decrease in the mitotic index (MI) value was observed at the tested concentrations of each of the recipe. The MI was positively correlated (LNS, r = 0.43; NS, r = 0.15; SL, r =

Table 1. Effect of different combinations of aqueous extracts of *Spondias mombin, Nymphea* lotus and *Luffa cylindrica* with or without Lead nitrate on root growth of *Allium cepa.*

Conc.(%)	LNS		NS		SL		NL		LNS+$PbN0_3$	
	Mean±SD	Growth in % of control	Mean±SD	Growth in % of control	Mean±SD	Growth in % of control	Mean±SD	Growth in % of control	Mean±SD	Growth in % of control
NC	4.32±0.44	100.0	4.32±0.44	100.0	4.32±0.44	100	4.32±0.44	100	4.37±0.38	100.0
1.0	4.03±0.81	93.3	3.65±0.47*	84.5	4.69±0.36	108.6	3.46±0.47*	80.1	4.36±0.58	99.8
2.5	4.11±0.65	95.1	3.24±0.53*	75.0	4.25±0.60	98.4	3.97±0.77	91.9	3.70±0.51	84.7
5.0	2.75±0.28*	63.7	2.53±0.48*	58.6	4.23±0.76	97.9	2.87±0.55*	66.4	2.65±0.54*	60.6
10.0	1.67±0.24*	38.7	1.86±0.41*	43.1	3.02±0.40*	69.9	3.00±0.43*	69.4	2.13±0.51*	48.7
20.0	1.42±0.13*	32.9	1.57±0.19*	36.3	3.17±0.58*	73.4	2.57±0.55*	59.5	1.77±0.36*	40.5
PC	4.65±0.70	107.6	4.65±0.70	107.6	4.65±0.70	107.6	4.65±0.70	107.6	4.52±0.90	103.4
EC50 (%)		12.4		12.2		24.8		21.8		13.9

*Values are significant compared to the negative control (tap water) at p<0.05. LNS = combination of extracts of *L. cylindrica, N. lotus* and *S. mombin.* NS = combination of extracts of *N. lotus* and *S. mombin.*SL = combination of extracts of *S. mombin* and *L. cylindrica.* NL = combination of extracts of *N. lotus* and *L. cylindrica.* NC = Negative control (tap water). PC = Positive control (10 ppm Lead nitrate).

Table 2. Cytological effects of the combination of aqueous extracts of *Spondias mombin, Nymphea* lotus and *Luffa cylindrica* (LNS). on *Allium cepa* cells

Conc.(%)	No of dividing cells	MI	Disturbed spindle	Sticky chromosome	Distributed metaphase	Chromosome lag	C-mitosis	Bi-metaphase cells	Anaphase bridge	Total aberration	Frequency of aberration (%)/total cells scored
NC	263	66	8	8	6	5	-	-	-	27	0.68±0.41
1.0	234	59	88	3	22	25	-	-	-	137	3.45±0.26*
2.5	145	36*	42	2	20	23	4	2	2	95	2.38±1.37*
5.0	197	49*	57	20	9	27	4	2	3	122	3.05±0.51*
10.0	191	48*	62	11	16	25	5	2	3	124	3.10±1.70*
20	144	36*	34	5	9	9	9	-	2	68	1.70±0.51
PC	172	43*	31	20	3	1	1	2	-	56	1.40±0.41*

*Values are significant compared to the negative control (tap water) at p<0.05. MI = mitotic index NC = Negative control (tap water) PC = Positive control (10 ppm Lead nitrate)

Table 3. Cytological effects of the combination of aqueous extracts of *Spondias mombin* and *Nymphea* lotus (NS) on *Allium cepa* cell.

Conc.(%)	No of dividing cells	MI	Disturbed spindle	Sticky chromosome	Distributed metaphase	Chromosome lag	C-mitosis	Bi-metaphase cells	Anaphase bridge	Total aberration	Frequency of aberration (%)/total cells scored
NC	263	66	8	8	6	5	-	-	-	27	0.68±0.41
1.0	283	71	50	4	8	27	-	1	3	93	2.33±0.45*
2.5	268	67	53	6	10	33	1	-	3	106	2.65±0.51*
5.0	228	57	54	3	7	24	-	1	2	91	2.28±0.57*
10.0	140	35*	33	16	15	29	-	1	-	94	2.35±0.65*
20.0	91	23*	27	10	4	14	-	1	-	96	1.40±0.50
PC	172	43*	31	20	3	1	1	2	-	56	1.40±0.41*

*Values are significant compared to the negative control (tap water) at p<0.05. MI: Mitotic index NC = Negative control (tap water). PC = Positive control (10 ppm Lead nitrate)

Table 4. Cytological effects of the combination of aqueous extracts of *Spondias mombin* and *Luffa cylindrica* (SL) on *Allium cepa* cells.

Conc.(%)	No of dividing cells	MI	Disturbed spindle	Sticky chromosome	Distributed metaphase	Chromosome lag	C-mitosis	Binucleated cell	Total aberration	Frequency of aberration (%) total cells scored
NC	263	66	8	8	6	5	-	-	27	0.68±0.41
1	132	33*	13	14	3	-	-	3	33	0.83±0.83
2.5	240	60	30	29	7	-	-	-	65	1.63±0.71*
5	188	47*	26	20	5	-	-	-	51	1.28±0.43*
10	162	41*	17	17	-	-	-	-	34	0.85±0.21
20	213	53*	17	7	3	1	1	1	32	0.80±0.22
PC	172	43*	31	20	3	1	1	2	56	1.40±0.41*

*Values are significant compared to the negative control (tap water) at p<0.05. MI: Mitotic index NC = Negative control (tap water). PC = Positive control (10 ppm Lead nitrate)

Table 5. Cytological effects of the combination of aqueous extract of *Nymphea lotus* and *Luffa cylindrica* (NL) on *Allium cepa* cells.

Conc.(%)	No of dividing cells	MI	Disturbed spindle	Sticky chromosome	Distributed metaphase	Chromosome lag	C-mitosis	Bi-metaphase cells	Anaphase bridge	Total aberration	Frequency of aberration (%)/total cells scored
NC	263	66	8	8	6	5	-	-	-	27	0.68±0.41
1	121	30*	6	10	-	-	1	1	-	18	0.45±0.13
2.5	134	34*	15	3	-	3	-	1	1	24	0.60±0.32
5	143	36*	10	5	-	1	-	-	1	18	0.45±0.33
10	111	28*	3	7	-	-	-	-	-	10	0.25±0.10
20	85	21*	6	7	1	3	-	1	-	20	0.50±0.38
PC	172	43*	31	20	3	1	1	2	-	56	1.40±0.41*

*Values are significant compared to the negative control (tap water) at p<0.05. MI: Mitotic index NC = Negative control (tap water). PC = Positive control (10 ppm Lead nitrate).

0.90; NL, r = 0.05) to the root lengths. LNS and NS significantly induced chromosomal aberration (Tables 2 and 3), SL induced significant chromosomal aberrations at concentrations 1, 10 and 20% (Table 4), while NL did not induce significant (p<0.05) aberration at any of the tested concentrations (Table 5). In the anti-genotoxicity study, LNS reduced the frequency of chromosomal aberrations induced by lead nitrate to levels not significantly different from the negative control at concentrations 1, 10 and 20% (Table 6). The observed cytological aberrations include disturbed spindle, chromosome lag, sticky chromosome, distributed metaphase, C-mitosis, anaphase bridge, bi-nucleated cells, vagrant chromosome, non-disjunction at anaphase and bi-metaphase cells (Figure 1).

DISCUSSION

Medicinal plants have been widely used by both ancient and modern man of all cultures for treating different ailments. A single plant processed in different formulations can be used to cure a wide range of diseases (Adegbite and Sanyaolu, 2009). However, the historic role of medicinal herbs in the treatment and prevention of diseases and in the development of pharmacology do not assume their safety for uncontrolled use by an uninformed public (Mathews et al., 1999). Studies of genotoxicity and anti-genotoxicity can help evaluate the safety

Table 6. Cytological effects of the combination of aqueous extract of *Spondias mombin, Nymphea lotus* and *Luffa cylindrica* (NLS) on *Allium cepa* cells pretreated with lead nitrate.

Conc. (%)	No of dividing cells	MI	Disturbed spindle	Sticky chromosome	Distributed metaphase	Chromosome lag	C-mitosis	Bi-metaphase cells	Anaphase bridge	Total aberration	Frequency of aberration (%)/total cells scored
NC	263	66	8	8	6	5	-	-	-	27	0.68±0.41
1	257	64	60	2	9	33	-	1	2	107	2.68±0.76*
2.5	193	48*	27	4	7	18	-	-	1	57	1.43±0.13
5	321	80*	54	-	5	15	-	-	-	74	1.85±0.25
10	242	61	63	8	9	26	-	-	1	107	2.68±0.56*
20	229	57	58	1	6	22	-	-	-	87	2.18±0.16*
PC	172	43*	31	20	3	1	1	2	-	56	1.40±0.41*

*Values are significant compared to the negative control (tap water) at p<0.05. MI: Mitotic index NC = Negative control (tap water). PC = Positive control (10 ppm Lead nitrate)

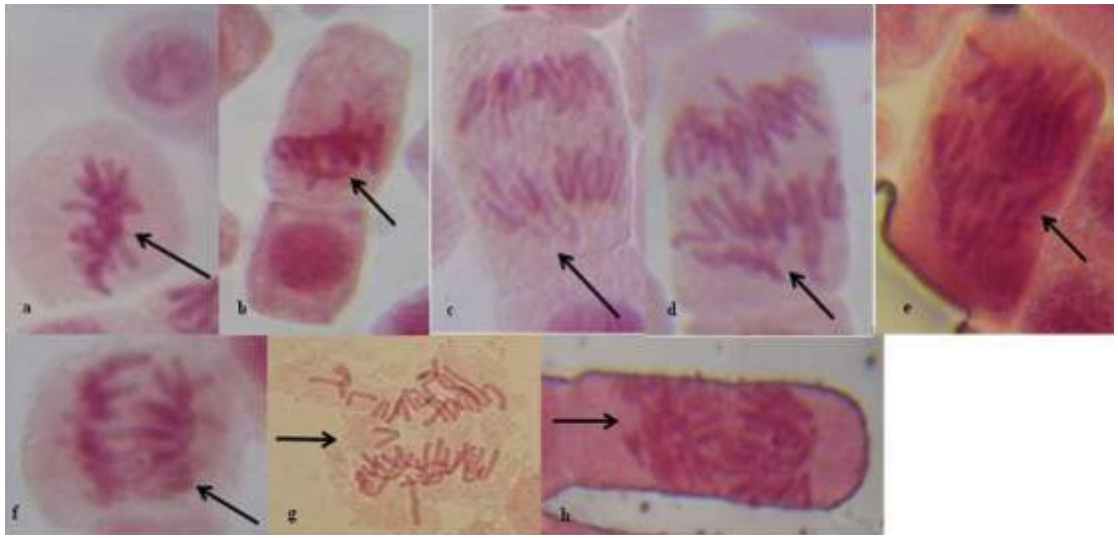

Figure 1. Chromosomal aberrations (arrowed) induced in *Allium cepa* root tips by the different combinations of aqueous extracts of *Spondias mombin, Nymphea* lotus and *Luffa cylindrica.* (a, b) sticky chromosomes at metaphase (c) multipolar division (d and e) spindle disturbance at anaphase (f) anaphase bridge (g) vagrant chromosome (h) c-mitosis Magnification: 1000×

and effectiveness of herbal health products (Bast et al., 2002).

All the recipes used in this study reduced root growth and were thus mitodepressive. The EC_{50}, in the order NS ≤ LNS < LNS+PbNO3 < NL < SL, showed the cytotoxic nature of each of the recipes. This indicates that one or more, or combination of the phytochemicals present in these recipes is/are toxic to *A. cepa* cells.

Preliminary phytochemical analysis showed the presence of tannins, saponins, sterol, glycosides and resins in *S. monbin*; tannins, saponins and sterols in *N. Lotus*; alkaloids, saponins, sterol and resins in *L. cylindrica* (Oyeyemi and Bakare, 2013). Reduction in root growth could be as a result of alteration in the duration of the mitotic cycle, resulting from direct interaction of the meristematic cells of *A. cepa* root tips with the phytochemicals present in plant materials.

MI is used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the M-phase of the cell cycle (Ping et al., 2012). It is an acceptable measure of cytotoxicity for all living organism (Smaka-Kınel et al., 1996). (MI significantly lower than the negative control indicates alteration in the growth and development of *A. cepa* (Hoshina, 2002); while MI higher than the negative control is as a results of increase in cell division leading to disordered cell proliferation and formation of tumor tissue (Leme and Marin-Morales, 2009). Decrease in MI observed herein is probably due to either chromatin dysfunction or disturbance in the cell cycle induced by the interaction of the phytochemicals with the DNA. This may be due to increase in the period of G2 (Van't Hoff, 1968), complete arrest of mitotic cycle at the G2 as observed by Bruneri (1971) or complete halt of metabolic activities preventing the cell to enter mitosis (Metin and Burun, 2010). Mitodepressive effect signifies that these recipes have the ability to block the synthesis of DNA and nucleoproteins (Schulze and Krischer, 1996). This action occurring in the interphase nucleus suggests that the recipes may not even allow the initiation of the biosynthesis of nucleoproteins and DNA (Akinboro and Bakare, 2007). The inhibitory and mitodepressive effects of the recipes may probably be part of the mechanism/mode of actions utilized in the management of cancer traditionally. Evidence in support of this has been documented on some plant extracts with anticancer therapy (Sheng et al., 2000; Kura´s et al., 2007).

Chromosomal aberrations are changes in the structure of chromosomes resulting from breaks or exchange of chromosomal materials (Sultan and Celik, 2009.). All the recipes utilized except NL induced chromosomal aberrations ($p < 0.05$) at one or more concentrations. These did not induce significant aberration at the 20% which was the highest concentration tested. A possible explanation for this is that there was a higher level of cytotoxicity at this concentration thus probably leading to fewer cells dividing, with most of them at prophase stage of cell division. Individually, the aqueous extract of *S. mombin* and *N. lotus* was reported to be non-genotoxic while that of *L. cylindrica* was genotoxic in *A. cepa* (Oyeyemi and Bakare, 2013). Herein, the aqueous extract of LNS (resulting from the combination of the three plants) was observed to be genotoxic; likewise the extract of NS. The actions of herbal products have been known to be due to the combined actions of many types of chemical compounds in the complex mixture (Wang et al., 2009). This suggests that the genotoxicity of LNS and NS in *A. cepa* was as a result of the interaction; synergistic, additive or antagonistic, of the phytochemicals present in the combined plants. Interestingly, the extract of NL was not genotoxic; which may mean that some phytochemicals present in *N. lotus* antagonized the phytochemical(s) responsible for the genotoxicity of *L. cylindrica* or perhaps blocks its binding site.

Most of the observed aberrations were due to spindle failure (such as disturbed spindle and distributed metaphase) which indicates the interaction of the phytochemical constituents of the recipes with the spindle apparatus. Alkaloids have been reported to inhibit mitosis and also bind to tubulin, preventing the formation of the mitotic spindle (Khakdan and Piri, 2012). The presence of alkaloids in the recipes might have contributed to the success acclaimed with these plants in the traditional management of cancer in Nigeria; however they may be genotoxic to normal cells if they are not selective in their mode of action.

Lead nitrate is a potent mutagen which has been reported to be mutagenic in *A. cepa* (Liu et al., 1994), wheat (Truta et al., 2011), mice (Madhavi et al., 2007) and human cultured cells (Yedjou and Tchounwou, 2007). In this study, it induced mostly disturbed spindle and chromosome lag. The extract of LNS showed anti-genotoxic effect at some of the tested concentrations by reducing the frequency of lead nitrate induced cytological aberrations to levels not significantly different from the negative control. It acted as a bio- antimutagen by suppressing the process of lead nitrate induced mutation in *A. cepa* and inhibited further DNA damage probably by inhibiting the binding of free radicals to DNA. The extract also probably induced cell death in cells with lead nitrate induced DNA damage. This was expressed as significant inhibition of cell division in lead nitrate pretreated cells which implies cytotoxic and anti-proliferative effect of the extract. Induction of apoptosis in *A. cepa* cells by plant extract have been previously reported (Celik and Aslanturk, 2010). Human exposure to genotoxic substances present in food and the environment is inevitable. Various genotoxic physical and chemical agents are known to act as mutagenic, co-carcinogenic and/or carcinogenic agents (Mitscher et al., 1986). Consumption of natural antigenotoxic and antimutagenic substances can protect from the effect of these genotoxic substances thereby protecting from life threatening diseases (such as cancer) caused by mutagens. The findings herein suggest that the tested recipes showed potential genotoxic and anti genotoxic activity not exhibited by the individual extract in *A. cepa*. We are currently assessing potential oxidative damage, mutagenic and anti-mutagenic effect of the extracts *in vivo* and *in vitro.* This is expected to provide further information on the mechanism of action of these plants and their recipes in biologic system.

Conclusion

The recipes tested in this study induced chromosomal aberrations which are due to spindle failure. This is a common feature of some orthodox medicine used in chemotherapy such as vincristine and vinblastine. Hence, this may be the mechanism underlying the success acclaimed by traditional healers in using these plants to treat cancer. However, caution should be taken in using these recipes since they may not be selectively mutagenic to cancer cells.

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Conflict of interest

Authors have none to declare.

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