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Evaluation of leaf extracts of *Artemisia afra* Jacq. ex Willd. and *Leucosidea sericea* Eckl. & Zeyh. for genotoxicity and the modulation of EMS-induced genotoxicity

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A battery of short-term *in vitro* assays for apoptosis and/or cell cycle arrest, cytotoxicity, genotoxicity and antigenotoxicity are used to screen and establish the efficacy of medicinal plants. This study evaluated three concentrations (0.1, 0.2 or 0.4 mg/ml) of methanolic leaf extracts of *Artemisia afra* and *Leucosidea sericea* and their individual mixtures with Ethyl methanesulfonate (EMS) (0.15 mg/ml) for induction of those end points using the *in vivo Allium cepa* assay. Cytotoxicity was measured by the mitotic index, genotoxicity was expressed as the number of aberrant mitotic cells per 100 mitotic cells and modulatory effect (ME) was calculated as: ME = (B - C) - (A - C) / (A - C) and the value, positive or negative, indicated the number of units of the mutagen-induced genotoxicity (A) that equaled the mixture-induced genotoxicity (B). The three concentrations of *A. afra* extract tested did not induce cell cycle arrest and were not cytotoxic. The 0.4 mg/ml concentration and its mixture with EMS were genotoxic. The concentrations of *L. sericea* extract tested did not induce cell cycle arrest, were not cytotoxic nor genotoxic to the *A. cepa* root tip cells. The mixture of either 0.2 or 0.4 mg/ml *L. sericea* extract with EMS was genotoxic. The mixture of 0.4 mg/ml *L. sericea* extract with EMS was significantly more (ME = 4.40>2) genotoxic than EMS alone. Leaf extracts of *A. afra* and *L. sericea* lacked cell-cycle arrest activity, were non-toxic but lacked antigenotoxic activity against EMS-induced genotoxicity. High concentrations of *A. afra* were genotoxic whereas high concentrations of *L. sericea* interacted synergistically with EMS. Chromosomal abnormalities observed included sticky chromosomes, c-mitosis, chromosome largards, chromosome fragments, anaphase and telophase bridges.

Key words: Anti-genotoxicity, cell cycle arrest, safety of medicinal plants of Lesotho.

INTRODUCTION

Different human civilizations have depended for many centuries on plants and plant products for their medicinal (Balandrin et al., 1985) needs. The scientific basis for the use of plants in traditional medicine, in many ancient

communities has been attributed largely to secondary metabolites which have been shown to possess various biological activities (Bourgaud et al., 2001). Advances in analytical techniques such as chromatography allowed the recovery of more and more of these molecules, which eventually formed the basis of the discipline of phytochemistry (Hussein and El-Anssary, 2018). Some of the phytochemicals to which the protective and therapeutic benefits of plants have been attributed include alkaloids, terpenoids, tannins and phenolics among others (Hertog et al., 1993; Harborne, 1998; Zhang et al., 2001). Herbal traditional medicines are therefore, in most cases, concoctions of crude extracts of plant materials in water, alcohol or distillates or essential oils, which contain many secondary metabolites (SMs) from various structural groups; so that the therapeutic activity of the concoction is often due to synergistic interactions of the SMs present (Mulyaningsih et al., 2010; Eid et al., 2012). The synergistic interactions of the SMs, such phenolic compounds and polysaccharides, present in the concoctions have also been credited with the apparent broad-spectrum activity of the concoctions used in traditional medicine (Wink, 2015). At high concentrations, SMs change membrane fluidity and increase permeability. Therefore, many lipophilic SMs (especially those in essential oils) exhibit antimicrobial and cytotoxic activities (van Wyk and Wink, 2015).

In Lesotho, as in many other countries in the world, a system of traditional medicine based on the use of plants, birds, animals, their products and their combinations to treat a broad spectrum of communicable and noncommunicable diseases is still being practiced, especially in areas with limited access to clinics or other health facilities (Shale et al., 1999; Padmanabhan and Sujana, 2008). Lesotho has approximately 2000 recorded species of flowering plants which have been used for medicinal purposes for many generations and the knowledge of such plants has been carried from generation to generation through oral tradition (Moteetee and Van Wyk, 2011). Two examples of such plants used for traditional medicinal purposes in Lesotho are *Artemisia afra* (Asteraceae) and *Leucosidea sericea* (Rosaceae). *A. afra* Jacq. Ex. Willd', also called "African wormwood", is a well-known medicinal plant in South Africa, where it is known as "wilde als." (van Wyk, 2008; Abad et al., 2012) and in Lesotho as "*Lengana*" in Sesotho. The roots, stems, and leaves of *A. afra* are used to treat numerous ailments including colds, coughs, diabetes, heartburn, bronchitis, asthma acne, boils, headaches, stomach disorders, sprains and rheumatic swellings. It is widely used for numerous ailments including colds, coughs, diabetes, heartburn, bronchitis, and asthma (van Wyk, 2008; Abad et al., 2012). The various uses (broad-spectrum effects) reveal that it possesses antiviral, antibacterial, and anti-inflammatory activities (Thring and Wertz, 2006; Liu et al., 2009; Moteetee and Van Wyk, 2011). The leaf extracts of the plant species *L. sericea* (known as "*Cheche*" in Sesotho) are used by indigenous peoples of Southern Africa as a vermifuge, astringent and anti-inflammatory agent and a paste made from the crushed leaves is used to treat ophthalmia (Van Wyk and Van Wyk, 1997; Nair et al., 2012).

The therapeutic benefits of plants notwithstanding, investigations have revealed that many plants which are used in traditional medicine have mutagenic, cytotoxic and genotoxic effects in *in vitro* and *in vivo* assays (Higashimoto et al., 1993; Schimmer et al., 1994; Kassie et al., 1996; Fennell et al., 2004; Çelik and Aslantürk, 2007; Kuete, 2014) and contain mutagenic and/or carcinogenic substances (Ames, 1986; de Sá Ferreira and Ferrão Vargas, 1999) and their use has been correlated with high rate of tumor formation in some human populations (Wynder et al., 1983; Ames, 1986; Nagao et al., 1986; Nguyen et al., 1989; Brito et al., 1990).

Medicinal herbs have also been shown to cause adverse effects or have the potential to interact with other medications (Zink and Chaffin, 1998; Markowitz et al., 2003), modify the mutagenic and carcinogenic effects of environmental contaminants and induce both mutagenic and antimutagenic effects on known mutagens in different test systems (Debisri et al., 1996; Gimmler-Luz et al., 1999). These observations raise concern about the potential mutagenic or genotoxic hazards that could result from the long-term use of such plants as food or medicine, especially because not enough information is available on the potential risk to health of many of these plants (Basaran et al., 1996).

The interest in plant-based pharmaceuticals intensified due to the development of methods of screening for anticarcinogenic drugs determined by their cytotoxic effects represented by inhibitory concentration (IC_{50}) and Selectivity index (SI), using a panel of different independent cancer cell-lines as well as normal cell-lines (Hartwell, 1971, Cassady and Douros, 1980; Mbaveng et al., 2017) and through a battery of *in vitro* and *in vivo* short-term genotoxicity tests, and long-term rodent carcinogenicity assays (Moreira et al., 2014). The development resulted in the extension of the survey to include plant extracts and plant products able to modify the process of mutagenesis, because of the observation of a correlation between carcinogenesis and mutagenesis (McCann et al., 1975; Debisri et al., 1996) and medicinal plants usage gradually abandoned the anecdotal framework and became founded on empirical and explicatory facts (Petrovska, 2012). Whereas many in *vivo tests* on aqueous extracts largely support the safety of herbal medicines, most *in vitro* tests on isolated single cells mostly with extracts other than aqueous ones however, show contrary results and thus continue the debate on herbal medicine safety (Mensah et al., 2019).

The relationship between apoptosis and cell cycle arrest has been a recent focus and many

chemotherapeutic agents exert their anticancer effects by inducing apoptosis and/or cell cycle arrest in cancer cells (Schuchmann and Galle, 2004; Belayachi et al., 2017). In *in vitro* studies, plant extracts can be evaluated for cell cycle arrest potential where, cancer cell lines are treated with various concentrations of plant extracts for 24 h, stained with propidium iodide (PI), and subsequently analyzed by flow cytometry to detect and quantify the amount of cells undergoing apoptosis as measured by the level of DNA fragmentation as well as the increase in the percentages of cells in the sub G1 phase compared to the untreated or solvent-treated cells (Pumiputavon et al., 2017). A decrease in the proportion of dividing cells in anaphase (A) and telophase (T) is an indication of metaphase arrest due to the poisoning of the spindle fibers, akin to the action of the well documented spindle poison, colcemid (Parry et al., 1999). The chemotherapeutic agents taxol, vincristine, vinblastine and nocodozole act in a similar manner (Alberts et al., 2008) to colcemid. These compounds act by binding to and stabilizing microtubules, inhibiting their dynamic instability and cuasing various genetic disruption, including the induction of cell cycle arrest (Alberts et al., 2008; Zhang et al., 2015). Drugs suppress microtubule dynamics by binding to different sites of tubulin heterodimer, disturb the assembly of the mitotic spindle apparatus and arrest cell cycle progression through M-phase, leading to eventual cell apoptosis (Chen et al., 2010). Therefore, cell cycle arrest is one of the targets for many anticancer drugs. Among them, taxanes, colchicines and vinca alkaloids are well-known examples that induce G2/M phase arrest leading to subsequent apoptosis (Kim et al., 2011). The anti-cancer activity of plant extracts and isolated compounds is often evaluated by assessing their ability to induce cell cycle arrest and apoptosis (Pumiputavon et al., 2017; Abu-Darwish and Efferth, 2018). Certain types of alkaloids, widely distributed in plants such as jerantinine B (Qazzaz, 2016), liriodenine (Nordin et al., 2015), and vinoreline (Rabbani-Chadegani et al., 2015), exhibit the ability to induce apoptosis and block the cell cycle in the G1 phase (Pumiputavon et al., 2017).

Screening medicinal plants for biological activity to harness their therapeutic potential involves cytotoxicity studies as a first step to evaluate the toxicity of plant extracts or plant-derived compounds and to establish their safety or lack thereof (McGaw et al., 2014; Mafole et al., 2017). Minimal to no toxicity is essential for the successful development of a pharmaceutical or cosmetic preparation and in this regard, cellular toxicity studies play a crucial role (McGaw et al., 2014). The toxicity of plant extracts has been attributed to the presence of cytoxic secondary plant metabolites in the solvent extracts (Asche, 2005; Hussein and El-Anssary, 2018; Priyanka et al., 2019). Since the observation of a high correlation between carcinogenicity and mutagenicity: 90% (156/ 174) of carcinogens are mutagenic (McCann

et al., 1975) and the indication of epidemiological studies that many cancers are dependent on multiple mutational etiology, as well as on inherited mutator phenotype (Verschaeve and Van Staden, 2008), carcinogenic potential of environmental chemicals and plant extracts is assessed through a battery of *in vitro* and *in vivo* short-term genotoxicity tests, and long-term rodent carcinogenicity assays during research and development (R&D) of conventional drugs and to screen and establish the efficacy of medicinal plants (Moreira et al., 2014; Atanasov et al., 2015). Researchers often use a battery of *in vitro* assays to screen and establish the efficacy of medicinal plants (Atanasov et al., 2015). The advantage of this approach is that it takes into consideration the often complex and interwoven nature of the disease state (Houghton et al., 2005). However, Rank and Nielsen (1994) found an 82% correlation between the *Allium cepa* test and the mouse carcinogenicity test, concluding that *A. cepa* was even more sensitive than the Ames test. Teixeira et al. (2003) found the same results in tests comparing meristematic cells of onion roots, bone marrow cells of rats, and human lymphocytes as bioindicators, validating the safety of *A. cepa* for cytogenetic studies.

Antimutagen is described as an agent that reduces the apparent yield of spontaneous and or induced mutations in organism genome (Bhattacharya, 2011). Seaweeds were found to contain naturally occurring antimutagens and antioxidants compounds which demonstrated great protective effect against oxidative damage caused by free radicals (Arumugama et al., 2017). Therefore the Antimutagenic properties of plant extracts are considered of particular interest as it may be assumed that these antimutagenic natural substances are able to lower the risk of occurrences of mutation and cancer risk from everyday exposures to environmental mutagens as well as to mutagenic pharmaceuticals (Verschaeve and Van Staden, 2008; Nagarathna et al., 2013) The mechanism by which plant extracts exhibit antigenotoxic effects is either by inducing or inhibiting enzyme such as glutathione- stransferase or CYP1A1 respectively as well as antioxidant and scavenging properties of polyphenolics contained in it. The most important mechanism in antimutagenesis and anticarcinogenesis is the scavenging of bio active molecule (Mersch-Sundermann et al., 2006)

During research and development (R&D) of conventional drugs, carcinogenic potential is assessed through a battery of *in vitro* and *in vivo* short-term genotoxicity tests, and long-term rodent carcinogenicity assays (Moreira et al., 2014).

Studies on agents that modulate carcinogen-induced genotoxic effects in experimental animals provide end points that can be used for assessing the antimutagenic or anticarcinogenic properties of putative chemopreventive compounds and for predicting their protective efficacy in humans (Khaidakov et al., 2001).

The *A. cepa* L assay is an *in vivo* assay and one of the established plant bioassays, validated by the international programme on chemical safety (IPCS, WHO), as an efficient and standard test for chemicals screening, *in situ* monitoring of the genotoxicity of environmental substances (Leme and Marin-Morales, 2009) and to evaluate the genotoxic potential of medicinal plants (Camparoto et al., 2002; Knoll et al., 2006; Fachinetto et al., 2007; Lubini et al., 2008; Fachinetto et al., 2009). The *A. cepa* L assay is also a short-term *in vivo* genotoxicity test that tests genotoxicity using chromosomes and therefore detects chromosome structural and numerical alterations (Tedesco and Laughinghouse, 2012; Bonciu et al., 2018). The assay is relatively inexpensive, fast, give reliable results and chemicals which cause chromosomal aberration (CA) in plant cells also produce CA in cultured animal cells that are frequently identical (Grant, 1978; Ma et al., 1994). Ethyl methanesulphonate (EMS) is a direct-acting mutagen, tetratogen, and brain carcinogen (Stubbs et al., 1997).

In view of the foregoing therefore, the aim of this study was to evaluate the methanol extracts of the leaves of *A. afra* and *L. sericea* for cytotoxicity, genotoxicity and the modulation of EMS- induced genotoxicity using the *A. cepa* chromosome aberration assay system.

MATERIALS AND METHODS

Test organism

Onion (*A. cepa*) seeds of the variety, Texas Grano 502 P.R.R., a product of Sakata seeds, Lanseria 1748, Republic of South Africa were purchased from Maseru Garden Centre, Lesotho, Southern Africa.

Mutagens and chemicals

EMS was a product of Fluka (Biochemika, Germany). Methanol (absolute) was a product of Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa); hydrochloric acid glacial and acetic acid were products of UNILAB (Krugerdorp, South Africa); acetocarmine stain was obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA.

Plant material collection and crude extract preparation

The aerial parts of *L. sericea* and *A. afra* were collected from the outskirts of the National University of Lesotho, with the following geographical coordinates: Latitude: 29° 22′ 49″ S, Longitude: 27° 33′ 13″ E. with altitude of 1600 m. The two plant species were authenticated by the curator of the Herbarium at the Department of Biology of the university, where the voucher specimens of these plants have been deposited. The leaves were dried in a fanned Labcon oven at 37°C to a constant weight and brittle, about 48 h. Thereafter, the dried leaves were ground to fine powder using a pulveriser (Kenwood) and the powder was stored in sealed amber bottles in the refrigerator at 4°C until they were used. Extraction was carried out according to the method of Magama et al. (2013). Briefly, the ground leaf material was soaked in 95% methanol (v/v

in distilled water) and the mixture was shaken on the orbital shaker for 24 h and filtered through Whatman No.1 filter paper using suction. The filtrates obtained were then concentrated under vacuum in a rotary evaporator and oven dried at 35°C for 12 h to a constant weight and were stored at 4°C in the refrigerator until they were used.

Genotoxicity experiments

The preliminary assay to select concentrations of EMS and leaf extracts to use and Genotoxicity assay (including the treatment of *A. cepa* seedlings with test agents, root harvest, slide preparation and scoring of slides) were conducted according to the methods of Asita et al. (2017). From the results of the preliminary assays to select the concentrations of EMS and plant extracts to use, the following concentrations of leaf extracts (in mg/ml) of *A. afra* (0.1, 0.2 and 0.4) and *L. sericea,* (0.1, 0.2 and 0.4) and EMS (0.15) were assessed for cytotoxicity that is, mitotic index (MI), genotoxicity (GT) and the modulatory effect (ME) of plant extracts on EMS-induced genotoxicity.

The aberrations observed and scored were: sticky chromosomes (S), C-metaphase (C-Mit), lagging chromosomes (L), chromosome bridges at anaphase and telophase (A.B) and chromosome fragment (F). For calculating the GT, only aberrant mitotic cells were considered.

In each assay, three root tips (triplicate) were assessed at each concentration. On each of three slides (n = 3) per treatment, a total of 2000 cells, classified into interphase or dividing cell, that is, prophase (normal, N or aberrant, ABN), metaphase (N or ABN), anaphase (N or ABN) or telophase (N or ABN) were scored; that is, a total of 6000 cells each for the control and treatment groups. The aberrations assessed were sticky chromosomes (S), C-metaphase (C-Mit), lagging chromosomes (L), chromosome bridges at anaphase and telophase (A.B) and chromosome fragment (F). For calculating the GT, only aberrant mitotic cells were considered.

Analysis of slide preparations

Cytotoxicity, as determined by the mitotic index (MI) was expressed as the number of dividing cells per 100 cells scored according to the formula:

MI = Number of dividing cells/Total number of cells scored x 100.
(1)

The MI was used as a measure of cytotoxicity (CT). The MI of each treatment group was compared with that of the negative control group using t-test at a probability level of 0.05, using the SPSS for windows, version 11.0 software.

Genotoxicity (GT) was expressed as the number of aberrant mitotic cells (AMC) per 100 mitotic cells [*i.e* AMC + normal mitotic cells (NMC)] scored according to the formula:

Frequency of GT = AMC/ (AMC + NMC) x 100 (2)

The mean GT of each group of three slides per concentration of test agent was compared with that of the negative control group using t-test. P values less than 0.05 (P < 0.05) were considered as indicative of significance. The modulatory effect (ME) of plant extract on EMS-induced genotoxicity (GT) was calculated using the formula of Asita et al. (2017):

ME = (B – C) – (A – C) / (A – C) (3)

Where 'A' is the genotoxicity induced by the mutagen (CP or EMS) alone that is, mutagen-induced genotoxicity; 'B' is the genotoxicity

induced by mixture of plant extract and mutagen, that is, mixture-induced genotoxicity and 'C' is the genotoxicity induced by negative control, such as tap water alone.

The modulatory effect (ME) was thus obtained by comparing the mutagen-induced genotoxicity (A) with the mixture-induced genotoxicity (B). The ME value indicated the number of units of the mutagen-induced genotoxicity (A) that equaled the mixture-induced genotoxicity (B). ME was significant only if ME was ≥ 2, that is, mixture was at least twice (200%) more (+) or less (-) genotoxic than mutagen alone.

A positive ME (+ME) indicated that the mixture was more genotoxic (increased GT) than the mutagen and if mixture is also more genotoxic than the genotoxic plant extract alone then a synergistic interaction is inferred. But mutagen-potentiation is inferred if mixture is less genotoxic than the non-genotoxic plant extract alone.

A negative ME (-ME) indicated that the mixture was less genotoxic (reduced GT) than the mutagen alone. If mixture is less genotoxic than the mutagen and the genotoxic plant extract then antagonism is infered. However, if mixture is less genotoxic than mutagen and also, more or less genotoxic than the non-genotoxic plant extract then it is antimutagenicity.

Data analysis

The mean value of each group of three slides per concentration of test agent was compared with that of the negative (solvent) control group using student's t-test. P-values less than 0.05 (P < 0.05) were considered as significant.

RESULTS

Cytotoxicity and genotoxicity figures and tables

Photographs of the most representative pictures of normal mitotic cells and cells containing the different types of chromosome aberrations that were observed and scored are presented in Figure 1. The results of the cytotoxicity and genotoxicity experiments with methanol extract of leaves of *A. afra* and *L. sericea* are presented in Tables 1 and 2 respectively.

Results of experiments with *A. afra* leaf extract

(P+M)/ (A+T) Ratio

Examination of the (P+M)/(A+T) ratio in column 8 of Table 1 shows that the treatment with EMS (0.15 mg/ml), the different concentrations of *A. afra* leaf extract and the mixtures of AA extract with EMS did not induced a significant change in the (P+M)/(A+T) ratio, when compared with water treatment negative control group (P < 0.05).

Cytotoxicity

Examination of the MI in column 9 of Table 1 shows that the concentration of EMS (0.15 mg/ml) used was not toxic, none of the three concentrations of *A. afra* leaf

extract (0.1, 0.2 or 0.4 mg/ml) tested was toxic and the mixtures of *A. afra* leaf extract with EMS were also not toxic to the *A. cepa* root meristem cells, when compared to the water treated negative control (P<0.05).

Genotoxicity (GT)

Examination of induction of genotoxicity in column 10 of Table 1 shows that only EMS, highest concentration (0.400 mg/ml) of *A. afra* leaf extract and the mixture of EMS and the 0.40 mg/ml *A. afra* leaf extract was genotoxic when compared to the water treatment negative control (P<0.05).

Modulatory effect (ME) of A. afra leaf extract on EMS-induced Genotoxicity (GT)

Examination of the modulatory effect (ME) in column 11 of Table 1 shows that the mixture of each of the three concentrations of *A. afra leaf* extract (0.1, 0.2 and 0.4 mg/ml) with EMS was insignificantly (< twofold or 200%) less genotoxic than EMS alone.

Results of experiments with *Leucosidea sericea* leaf extract

(P+M)/ (A+T) Ratio

Examination of the (P+M)/(A+T) ratio in column 8 of Table 2 shows that the treatment with EMS (0.15 mg/ml), the different concentrations of *L. sericea* leaf extract and the mixtures *L. sericea* leaf extract with EMS did not induced a significant change in the (P+M)/(A+T) ratio, when compared with water treatment negative control group (P < 0.05).

Cytotoxicity

Examination of the MI in column 9 of Table 2 shows that the concentration of EMS (0.15 mg/ml) used was not toxic, non of the three concentrations of *L. sericea* leaf extract (0.1, 0.2 or 0.4 mg/ml) tested was toxic and the mixtures of *L. sericea* leaf extract with EMS were also not toxic to the *A. cepa* root meristem cells, when compared to the water treated negative control (P<0.05).

Genotoxicity (GT)

Examination of induction of genotoxicity in column 10 of Table 2 shows that only three treatments, EMS, and the mixture of EMS with each of the two highest concentrations (0.2 and 0.4 mg/ml) of *L. sericea* leaf extract, were genotoxic when compared to the water treatment negative control (P<0.05).
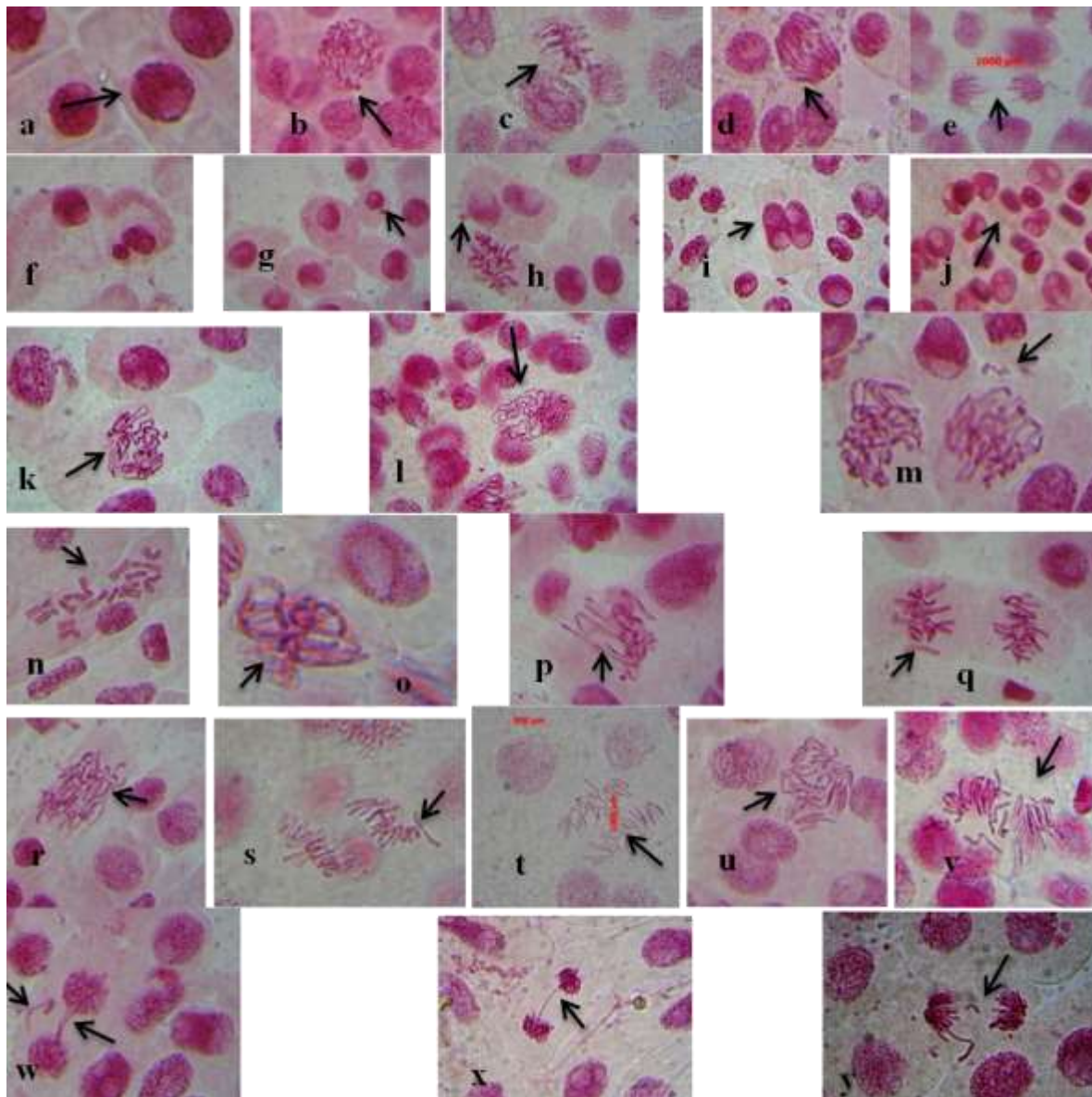
Figure 1. Photographs of cells of *Allium cepa* captured with light microscope (Olympus CX 21) using the 100X objective lens with oil immersion, showing untreated cells in normal division stages and chromosomal aberrations (arrowed) in cells treated with EMS, plant extract or mixture of plant extract with EMS. (a) Normal interphase (b) Normal prophase (c) Normal metaphase (d) Normal anaphase (e) normal telophase (f) Interphase nuclear bud (g) Interphase micronucleus (MN) (h) Interphase micronucleus (MN) (i) Interphase binucleated cell (BN) (j) Interphase pyknotic nuclei (k) Prophase sticky chromosomes (l) Prophase with sticky chromosomes (m) Prophase with chromosomal stickiness and loss (n) Metaphase C-Metaphase (o) Metaphase with chromosomal stickiness (p) Metaphase with chromosomal stickiness (q) Metaphase with chromosomal loss or dislocation (r) Anaphase with chromosome stickiness (s) Anaphase with chromosome loss (t) Multipolar Anaphase (u) Anaphase with chromosome stickiness and loss (v) Anaphase with chromosome stickiness, displaced poles and scattered chromosomes (w) Telophase with chromosome loss (shorter arrow), and chromosome bridge (x) Telophase with bridge (y) Telophase with lagging chromosome (L). Magnification is 1000 X.

Modulatory effect (ME) of leaf extract of *L. sericea* on EMS-induced genotoxicity (GT)

Examination of the modulatory effect (ME) in column 11 of Table 2 shows that only the mixture of the top concentration (0.4 mg/ml) of *L. sericea* leaf extract with EMS was significantly (> twofold or 200%) more genotoxic than EMS or *L. sericea* leaf extract alone (synergism), with a positive ME value of 4.40. This observation suggested a synergistic interaction between

Table 1. Cytotoxic and genotoxic effects of methanolic extract of *Artemisia afra* leaves and EMS on meristem cells of onion root tip and the modulatory effects (ME) of the *Artemisia afra* extract on EMS- induced genotoxicity.

Treatment (TC concentration in mg/ml)	Statistics	Inter. Cells	Total number of cells in the stages of mitosis in 2000 cells scored			Total number of cells scored	(P+M)/(A+T)	MI	Genotoxicity	Modulatory effect
			N	ABN	Total (N + ABN)					Extract on EMS
Water	Mean	1890.33	105.00	4.67	109.67	2000	3.13	5.48	4.10	
	SD	44.75	42.44	2.31	44.75	0.00	1.24	2.24	0.57	
EMS (0.15)	Mean	1908.67	79.00	12.33	91.33	2000	2.52	4.57	12.78 #	
	SD	31.88	24.98	7.77	31.88	0.00	0.41	1.59	4.09	
A. afra (0.1)	Mean	1863.00	127.33	9.67	137.00	2000	2.14	6.85	7.66	
	SD	30.27	33.98	4.04	30.27	0.00	0.54	1.51	4.47	
A. afra (0.2)	Mean	1867.00	126.33	6.67	133.00	2000	1.86	6.65	4.99	
	SD	4.58	6.11	5.51	4.58	0.00	0.36	0.23	4.09	
A. afra (0.4)	Mean	1884.33	100.67	15.00	115.67	2000	2.29	5.78	14.00 #	
	SD	34.96	35.16	1.00	34.96	0.00	0.81	1.75	5.13	
A. afra (0.1)) + EMS	Mean	1921.00	72.33	6.67	79.00	2000	3.03	3.95	8.50	-0.49
	SD	19.08	17.95	2.89	19.08	0.00	1.08	0.95	2.88	
A. afra (0.2) + EMS	Mean	1894.33	104.00	1.67	105.67	2000	2.41	5.28	1.56	-1.29
	SD	18.77	18.33	0.58	18.77	0.00	0.25	0.94	0.42	
A. afra (0.4) + EMS	Mean	1920.33	72.00	7.67	79.67	2000	3.02	3.98	10.28 #	-0.29
	SD	26.58	26.06	0.58	26.58	0.00	0.31	1.33	2.96	

Key: TC = Test compound; N = Normal mitotic cells (comprising prophase, metaphase, anaphase and telophase); ABN = Aberrant mitotic cells; SD = Standard deviation; EMS = Ethylmethane sulphonate; ABN=aberrant mitotic cells; # = TC is genotoxic (significant difference from negative control, $P<0.05$ in the t-test, n = 3).

L. sericea leaf extract and EMS at high concentration of *L. sericea* leaf extract.

DISCUSSION

In this study, methanolic extracts of the leaves of *A. afra* and *L. sericea* used in traditional medicine in Lesotho to treat various diseases were evaluated for cytotoxicity, genotoxicity and modulatory effects on, the direct acting mutagen, EMS-induced genotoxicity using the *in vivo* onion (*A. cepa* L.) root tip meristem chromosome aberration assay system. The results of genotoxicity tests are presented in Tables 1 and 2.

As shown in Tables 1 and 2, the concentration of EMS (0.15 mg/ml) used in the present study did not reduce the mitotic index (MI) of meristem cells of the treated roots of *A. cepa* compared with the negative control and was adjudged not cytotoxic. The concentration of EMS used however induced genotoxicity in the root meristem cells of *A. cepa*. In a study by Çelik and Aslantürk (2010), EMS

Table 2. Cytotoxic and genotoxic effects of methanolic extract of *Leucosidea sericea* leaf and EMS on meristem cells of onion root tip and the modulatory effects (ME) of the *Leucosidea sericea* extract on EMS- induced genotoxicity.

| Treatment (TC concentration in mg/mL) | Statistics | Inter. Cells | Total number of cells in the stages of mitosis in 2000 cells scored | | | Total number of cells scored | (P+M)/ (A+T) | MI | Genotoxicity | Modulatory effect |
			N	ABN	Total (N + ABN)					Extract on EMS
Water	Mean	1890.33	105.00	4.67	109.67	2000	3.13	5.48	4.10	
	SD	44.75	42.44	2.31	44.75	0.00	1.24	2.24	0.57	
EMS (0.15)	Mean	1908.67	79.00	12.33	91.33	2000	2.52	4.57	12.78 #	
	SD	31.88	24.98	7.77	31.88	0.00	0.41	1.59	4.09	
L. sericea (0.1)	Mean	1889.00	105.00	6.00	111.00	2000	2.28	5.55	5.64	
	SD	68.42	65.28	6.93	68.42	0.00	0.63	3.42	4.04	
L. sericea (0.2)	Mean	1918.67	78.33	3.00	81.33	2000	2.05	4.07	3.72	
	SD	6.81	7.09	1.00	6.81	0.00	0.24	0.34	1.40	
L. sericea (0.4)	Mean	1879.00	113.00	8.00	121.00	2000	2.49	6.05	6.23	
	SD	17.00	11.00	6.00	17.00	0.00	0.21	0.85	4.12	
L. sericea (0.1)) + EMS	Mean	1933.33	63.33	3.33	66.67	2000	2.73	3.33	7.28	-0.64
	SD	46.20	44.84	1.53	46.20	0.00	1.25	2.31	5.27	
L. sericea (0.2) + EMS	Mean	1936.00	54.00	10.00	64.00	2000	7.11	3.20	22.83#	1.16
	SD	49.69	46.13	3.61	49.69	0.00	8.57	2.48	15.97	
L. sericea (0.4) + EMS	Mean	1960.00	28.67	11.33	40.00	2000	1.55	2.00	50.96#	4.40+†
	SD	42.93	41.88	5.03	42.93	0.00	0.57	2.15	32.60	

Key: TC = Test compound; N = Normal mitotic cells (comprising prophase, metaphase, anaphase and telophase); ABN = Aberrant mitotic cells; SD = Standard deviation; EMS = Ethylmethane sulphonate; ABN=aberrant mitotic cells; MI= mitotic index; *L. sericea* = *Leucosidea sericea*; # = TC is genotoxic (significant difference from negative control, P<0.05 in the t-test, n = 3); +† = *L. sericea* extract + EMS mixture significantly more genotoxic than EMS or *L. sericea* extract alone (synergism)

at a concentration of 2×10^{-2} M (0.2484 mg/ml) was both toxic and mutagenic to root meristem cells of *A. cepa*.

The results of the assessments of the cytotoxic and genotoxic effects of the methanolic extracts of *A. afra* leaves are presented in Table 1. The concentrations tested did not induce any changes in the (P+M)/(A+T) ratio. In the present in vivo study, no such observations were made and it can be concluded that methanolic extract of *A. afra* leaves, at the concentrations (0.1, 0.2 or 0.4 mg/ml) used, did not induce or demonstrate any cell cycle arrest capabilities. However, in an *in vitro* experiment, ethanol extract of *A. afra* was cytotoxic against U937 and HeLa cancer cells *via*

the decrease in mitochondrial membrane potential after G2/M arrest (Spies et al., 2013; Venables et al., 2016). Some Artemisia species and their active components that have been shown to arrest cells in *in vitro* experiments with cell lines include, *A. sphaerocephala* (Wang et al., 2017), *A. princeps*, cell cycle arrest at G2/M (Lee et al., 2013), *A. monosperma*, cell cycle arrest at S+G2/Mphase (Whelan and Ryan, 2004) and *A. annua* (Mizushina et al., 2010).

All three concentrations (0.1, 0.2 or 0.4 mg/ml) of the methanol extract of *A. afra* leaves and their individual mixtures with EMS (Table 1) tested in the present *in vivo* study were not cytotoxic to the *A. cepa* root meristem cells as determined by the reduction of mitotic index (MI) compared to the negative control treatment. But the methanol extract of another *Artemisia* species, *A. annua* showed cytotoxic effect on the root meristem cells of Allium cepa at >450 mg/ml (Karaismailoglu, 2014). The concentrations tested in that experiment were however, much higher than the ones tested here and the solvent for extraction was also methanol. In an *in vitro* experiment with U937 and HeLa cells, ethanol extract of *A. afra* was cytotoxic (Spies et al., 2013; Venables et al., 2016). Some *Artemisia* species and their active components that were cytotoxic in *in vitro* experiments with cell lines include, *A. sphaerocephala* (Wang et al., 2017), *A. princeps* (Lee et al., 2013) and *A. monosperma* (Whelan and Ryan, 2004).

Only the highest concentration (0.4 mg/ml) of the methanol extract of *Artemisia afra* leaves and its mixture with EMS (Table 1) were genotoxic. Dichloromethane and methanol extracts of *A. afra* leaves were not mutagenic in the *in vitro* Ames test with and without metabolic activation, using strains TA98 and TA100 (Elgorashi et al., 2003; Aremu et al., 2013). Dichloromethane and methanol extracts of leaves of *A. afra* were not toxic nor mutagenic when tested for genotoxic activity in human peripheral blood lymphocytes using the micronucleus test and the Ames test with TA98 and TA100 (Taylor et al., 2003; Verschaevea and Van Staden, 2008). However, some *Artemisia* species and their active components that have been shown to induce DNA damage and/or genotoxic effects in *in vitro* experiments with mammalian cell lines include, *A. turanica* (Tayarani-Najaran et al., 2013), *A. indica* (Zeng et al., 2015), *A. capillaris* (Masuda et al., 2015), *A. annua* (Mota et al., 2011; Alcântara et al., 2013) and *A. armeniaca* (Mojarrab et al., 2013). In a recent experiment with human peripheral blood lymphocytes (PBLs), methanol extracts of *Artemisia vulgaris* L. and *Artemisia alba* Turra significantly increased micronucleus (MN) frequency in the PBLs at concentrations of 50, 100 and 250 mg/ml (Jakovljević et al., 2020).

The mixtures of all three concentrations (0.1, 0.2 or 0.4 mg/ml) of the methanol extract of *A. afra* leaves with EMS (Table 1) tested in the present in vivo study were insignificantly less genotoxic than the EMS alone to the

A. cepa root meristem cells. With the modulatory effects (ME) below 2 (-0.49, -1.29, -0.29) it was concluded that the leaf extract, at the concentrations tested (0.1, 0.2 or 0.4 mg/ml), did not intereact in any significant way with the mutagen to reduce the EMS-induced genotoxicity, that is, the extract was not antigenotoxic. In tests of dichloromethane and 90% methanol extracts of leaves of *A. afra* for antimutagenicity using the Ames test with TA 100 and TA 98, with or without metabolic activation against the mutagenic responses of the well-known mutagens sodium azide (SA), 4-Nitroquinoline-N-oxide (4-NQO) or 2-amino-anthraceen (2-AA), the extracts did not indicate any antimutagenic activity (Verschaevea and Van Staden, 2008). The lack of antimutagenic activity obtained in the present *in vivo* test is in agreement with those of the *in vitro* Ames tests with bacteria, against the direct-acting mutagen-induced mutagenicity.

In treatment against known mutagen (mitomycin C, MMC), methanol extracts of two other *Artemisia* species, *A. vulgaris* L. and *A. alba* Turra dose-dependently reduced MMC-induced micronucleus (MN) frequencies in human peripheral blood lymphocytes (PBLs) in comparison with the positive control (Jakovljević et al., 2020). The results of the assessments of the cytotoxic and genotoxic effects of the methanolic extracts of *L. sericea* leaves are presented in Table 2. The concentrations tested did not induce any significant changes in the (P+M)/(A+T) ratio. It can be concluded that methanolic extract of *L. sericea* leaves, at the concentrations of (0.1, 0.2 or 0.4 mg/ml) used in the present *in vivo* experiments, did not induce or demonstrate any cell cycle arrest effects. We are not aware of studies conducted to determine cell cycle arrest activities of the extract of these plants in the *Allium cepa* assay.

Whereas we could not find any study on cell cycle arrest by extracts of *L. sericea* but, in *in vitro* studies dichloromethane (DCM)/methanol (MeOH) (ratio 1:1) extract of *L. sericea* leaves, showed weak anti-cancer activity when assayed in a three-cell line panel consisting of renal TK10, breast MCF7 and melanoma UACC62 at the concentrations of (0.1, 0.2 or 0.4 mg/ml) (Fouche et al., 2008). However, hexane and methanol extract of flowers of another plant of the Rosaceae family, *Rosa damascena* Mill (Damask rose), induce apoptosis and blocked (arrested) the cell cycle in the G1 phase in cervical (HeLa) cancer cells (Al-Oqail et al., 2021). The crude extract of *R. canina*, another Rosaceae also induced cell cycle arrest at the G_1 phase, apoptosis and exhibited a selective cytotoxic effect in treated human lung (A549) and prostate (PC-3) cancer cells compared to normal fibroblast cells (Kilinc et al., 2020). Crude methanolic extracts of leaves of four genera (four species) of the Annonaceae family namely *Uvaria longipes* Craib, *Dasymaschalon* sp., *Artabotrys burmanicus* A.DC., and *Marsypopetalum modestum* Pierre B., exhibited the ability to induce apoptosis and

block (arrest) the cell cycle in the G1 phase when subjected to this cell cycle analysis by PI staining using three groups of cancer cell lines, cervical carcinoma, human hepatocellular carcinoma, and human hematopoietic cell lines (Pumiputavon et al., 2017)

All three concentrations (0.1, 0.2 or 0.4 mg/ml) of the methanol extract of *L. sericea* leaves and their individual mixtures with EMS (Table 2) tested in the present in vivo study were not cytotoxic to the *A. cepa* root meristem cells as determined by the reduction of mitotic index (MI) compared to the negative control treatment. However, in *in vitro* studies, ethyl acetate and methanol crude extracts and fractions obtained from *L. sericea* leaves exhibited weak antiproliferative or cytotoxicity activity, measured in IC_{50} (in µg/ml) against different cancer cell lines as well as demonstrating cytotoxicity towards the normal human fibroblasts (BJ) cell line (Pendota et al., 2018). Ethanol extracts of leaves of *L. sericea* exhibited moderate cytotoxicity on B16-F10 mouse melanocytes cells (EC_{50} mg/ml/(mM) of 55.51) and comparatively higher toxicity on U937 human macrophage cells (EC_{50} mg/ml/(mM) of 26.03) (Sharma et al, 2014). Also, in the *in vitro* Cytotoxicity assay that used the tetrazolium-based colorimetric MTT (3-5-dimethyl thiazol-2-yl-2, 5-diphenyl tetrazolium bromide) assay with Vero monkey kidney cells, acetone extract of *L. sericea* leaves was toxic with LC_{50} (in mg/ml) of 0.0515, (Adamu et al., 2013). Based on the findings by Sharma et al., (2014), *L. sericea* ethanol extract demonstrated moderate toxic effects on B16-F10 cells and comparatively higher toxicity on U937 cells based on MTT cytotoxicity assay. The crude methanol extracts of flowers of *Rosa damascena* Mill, another Rosaceae reduced the cell viability (was toxic) of three carcinoma cell lines, MCF-7, A-549, and HeLa, when assessed by MTT assay at 0- 1000 µg/ml for 24 h (Al-Oqail et al., 2021).

All three concentrations (0.1, 0.2 or 0.4 mg/ml) of the methanol extract of *L. sericea* leaves (Table 2) tested in the present *in vivo* study were not genotoxic to the *A. cepa* root meristem cells compared to the solvent negative control treatment. However, only the mixture of the 0.2 or 0.4 mg/ml concentration of the *L. sericea* leaf extract with EMS, were genotoxic. In vitro mutagenic evaluation of organic solvent extracts of stem and leaves of *L. sericea*, in terms of the number of histidine independent (His+) revertants per plate using Salmonella typhimurium tester strains (TA98, TA100, TA102 and TA1535) with and without S9 metabolic activation, the extracts did not exert mutagenic effects (Aremu et al., 2011). On the basis of the non-mutagenic effects of the extracts, in the Ames test Aremu et al. (2011) concluded that it indicated that *L. sericea* extracts are safe but suggested that, it might be appropriate to use some other in vivo and *in vitro* tests to further confirm the safety of these extracts. In the present *in vivo* test, the *L. sericea* extracts were not genotoxic. Pitso and Ashafa (2015) reported the presence of β-thujone, a compound known

for its genotoxicity, neurotoxicity, reproductive toxicity and chronic toxicity, in essential oil obtained from oven-dried leaves of *L. sericea*. However, methanol extracts of fruit of *Crataegus oxyacantha* (Rosaceae) exerted genotoxic and clastogenic/aneugenic effects in cultured human cells, and in the Ames, test using TA98 with metabolic activation, +S9 (de Quadros et al., 2017)

The mixture of the lowest concentration (0.1 mg/ml) of the methanol extract of *L. sericea* leaves with EMS (Table 2) tested in the present *in vivo* study was insignificantly less (ME = -0.64<2) genotoxic than the EMS alone to the *A. cepa* root meristem cells. The mixture of the middle concentration (0.2 mg/ml) of the methanol extract of *L. sericea* leaves with EMS was insignificantly more (ME = 1.16<2) genotoxic than the EMS alone. The mixture of the highest concentration (0.4 mg/ml) of the methanol extract of *L. sericea* leaves with EMS was significantly more (ME = 4.40>2) genotoxic than the EMS alone. It can be concluded that low concentrations of the extract did not have appreciable interaction with the EMS and did not modulate EMS-induced genotoxicity. High concentrations of *L. sericea* extract interacted with EMS in a mixture of the two in a synergistic manner, such that the mixture was more genotoxic than either EMS or extract alone. It can be concluded that at high concentrations, the methanol extract of *L. sericea* leaves interacted synergistically with EMS and was not antigenotoxic to EMS-induced genotoxicity in the *in vivo*, *A. cepa* assay. The methanol extracts of the leaves of another Rosaceae, *Prunus africana* lacked any antimutagenic effect against mutagen-induced mutagenicity in the Ames test (Verschaeve and Van Staden, 2008). However, methanol extracts of the fruit and bark of *Cotoneaster integerrimus (Rosaceae)* were reported to exhibit antimutagenic potential against sodium azide (SAZ), 2-aminoanthracene (2-AA), 2-aminofluorene (2-AF) and 4-nitro-o-phenylenediamine (4-NODP) induced mutations in the Ames test using TA98, TA100 with and without metabolic activation at 500, 1,000, 5,000 mg/plate (Uysal et al., 2016).

The chromosomal abnormalities observed following treatment of the root tip cells of *A. cepa* with the methanolic leaf extract of *A. afra* and *L. sericea* alone or in mixture with EMS included sticky chromosomes, c-mitosis, chromosome largards, Chromosome fragments, and anaphase and telophase bridges. Genotoxic effects of different medicinal herbs in the *A. cepa* root meristematic cells assay have been demonstrated (Soliman, 2001; Bidau et al., 2004; Çelik and Aslantürk, 2007; Akinboro and Bakare, 2007; Akintonwa et al., 2009; Oyedare et al., 2009).

Conclusion

In treated *A. cepa* root meristem cells, leaf extract (0.1,

0.2 or 0.4 mg/ml) of *A. afra* did not induce cell cycle arrest and was noncytotoxic nor antigenotoxic against EMS-induced genotoxicity. High concentration (0.4 mg/ml) of extract of *A. afra* alone or its mixture with EMS was genotoxic. In treated *A. cepa* root meristem cells, leaf extract of *Leucosidea sericea* did not induce cell cycle arrest, was noncytotoxic and nongenotoxic. Mixtures of high concentrations (0.2 or 0.4 mg/ml) of *L. sericea* with EMS were genotoxic. The mixture of 0.4 mg/ml concentration of extract of *L. sericea* with EMS was significantly more (ME = 4.40>2) genotoxic than the EMS alone which indicated a synergistic interaction with EMS instead of antigenotoxicity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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