Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Made Pharmawati, Ni Nyoman Wirasiti, Luh Putu Wrasiati (2022) Genotoxic and antigenotoxic potential of encapsulated *Enhalus acoroides* (L. f.) Royle leaves extract against nickel nitrate. *Caryologia* 75(2): 89-99. doi: 10.36253/caryologia-1571

Received: February 08, 2022

Accepted: March 24, 2022

Published: September 21, 2022

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

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Genotoxic and antigenotoxic potential of encapsulated *Enhalus acoroides* (L. f.) Royle leaves extract against nickel nitrate

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Abstract. Several environmental pollutants can cause damage to chromosomes, one of which is the heavy metal $NiNO_3$. Some plant extracts have antigenotoxic properties that result in a decrease in chromosomal damage. Member of flowering plants that need to be tested is seagrass. One seagrass species is *Enhalus acoroides* which was found to contain phytochemical compounds. This study aimed to analyse the genotoxic effect and the potential of encapsulated *E. acoroides* leaf extract as antigenotoxic against nickel nitrate $NiNO_3$. The extraction was conducted using a mixture of chloroform and ethanol, and crude extract encapsulated using maltodextrin and tween 80. Chromosomal aberrations were evaluated using the squash technique of *Allium cepa* var. aggregatum root tips. Triphenyltetrazolium chloride and Evans Blue staining were used to observe mitochondrial and apoptotic activities. The results showed that at higher concentrations (250 ppm and 500 ppm), the encapsulated *E. acoroides* extract decreased mitotic indices; however, no chromosome aberration observed. $NiNO_3$ itself induced a genotoxic effect as observed by low mitotic index and a high percentage of chromosome aberration. The modulation of $NiNO_3$ effect by adding the encapsulated *E. acoroides* extract at low concentration (100 ppm) increased mitotic index compared to treatment with Ni alone, but did not reduce chromosome aberration. Simultaneous encapsulated *E. acoroides* extract and Ni treatment, significantly reduced nuclear fragmentation and nuclear lesion. The encapsulated *E. acoroides* extract can repair several types of nuclear damage but cannot minimise chromosomal damage.

Keywords: chromosome aberration, *Enhalus acoroides*, heavy metal, nuclear abnormality, seagrass.

INTRODUCTION

Heavy metals are hazardous inorganic environmental pollutants due to their toxicity. However, when present in low amounts, several heavy metals such as Cu, Fe, Mn, Co, Zn, and Ni are required for plants and animals as

micronutrients (Singh et al. 2020). Recently, due to high industrial activities and the extensive use of fertilizer and pesticides, heavy metals are present in enormous amounts in the environment, posing a serious global environmental threat.

One of the most common heavy metal contaminants found in the environment is nickel (Ni), along with Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Mercury (Hg), Lead (Pb), and Zinc (Zn) (Huang et al. 2020). Nickel is widely distributed in the environment including air, water, soil, and biological materials. It is mainly derived from natural sources such as windblown dust, resulting from the weathering of rocks and soils, forest fires, and volcanic activity. Nickel is also present in the environment due to the combustion of coal, diesel oil, and fuel oil, as well as the incineration of trash and sewage (Cempel and Nikel 2006). In plants, high nickel concentrations can inhibit growth by causing oxidative damage and disrupting nutrient uptake and translocation (Amjad et al. 2020). Nickel has also been reported to have cytotoxic and mutagenic effects in plants (Gantayat et al. 2018).

Natural ingredients with bioactive compounds that can fight mutagenic and carcinogenic effects are now getting more and more attention. Compounds capable of reducing the mutagenicity of physical and chemical mutagens are referred to as antimutagens. However, considering that all mutagens are genotoxic, then compounds that reduce DNA damage caused by genotoxic agents are also called antigenotoxic agents (López-Romero et al. 2018). For example, aqueous extracts of medicinal plants, *Spondias mombin*, *Nymphea lotus* and *Luffa cylindrica* reduced chromosomal and nuclear aberration induced by $PbNO_3$ in *A. cepa* root tip cells (Oyeyemi and Bakare 2013). Butanol and ethyl acetate fractions of *Parkinsonia aculeata* L. leaf extract demonstrated the most significant reduction in chromosomal abnormalities in *A. cepa* cells treated with maleic hydrazide, indicating that they had chemo-preventive efficacy (Sharma et al. 2018). Previously, Sharma et al. (2012) reported that using the *A. cepa* root chromosomal aberration assay, the chloroform extract of *Brasicca juncea* seeds possesses antigenotoxic potential against mercury-induced genotoxicity.

Exploration of antitoxic properties from natural products has also been done at marine organisms such as *Ulva fasciata* (Rodeiro et al. 2015), *Sargassum* sp. (Kilawati and Islamy 2019). However, limited studies were conducted in seagrass. Seagrass are flowering plants that grow in a marine environment. One of the seagrass species is *Enhalus acoroides* (L.f.) Royle. *Enhalus acoroides* is tropical seagrass, a member of the Family of Hydrocharitaceae, found throughout the Indo-Pacific region, including southern Japan, Southeast Asia, northern Australia, southern India, and Sri Lanka (Short and Waycott 2010). In Indonesia, this species is distributed widely in Papua, North Maluku, Ambon, Sulawesi, Bali, Java, Borneo, and Sumatra in Indonesia (Kiswara and Hutomo 1985). Extract of *E. acoroides* leaves contains phytochemical compounds such as phenols, flavonoids, and tannins as well as several pigments including chlorophyll, lutein, pheophytin, and b-carotene (Pharmawati and Wrasiati 2020). It has been known that flavonoids, phenolic compounds and pigments have antioxidant activity. To extend self-life and protect oxidative stability of plant extract, microencapsulation is often applied (Yusop et al. 2017) using colloidal particles such as maltodextrin, Arabic gum, or chitosan (Özkan and Bilek 2014, Šturm et al, 2019).

The aim of this study was to analysed the genotoxic effect of encapsulated *E. acoroides* leaves extracts and its antigenotoxic potential against heavy metal nickel nitrate $Ni(NO_3)_2.6H_2O$ using *Allium cepa* var. *aggregatum* root tips assay.

MATERIALS AND METHODS

Sample Collection, Extraction and Encapsulation

Leaves of *E. acoroides* were collected from Semawang Beach, Denpasar, Bali, Indonesia. The methods of den Hartog and Kuo (2006) and McKenzie and Yoshida (2009) were used to identify *E. acoroides* based on morphological traits. The voucher specimen was deposited in the Herbarium Biology Udayana (HBU-MP10), Biology Study Program, Universitas Udayana. Leaves were washed in running water, cut into 10 cm long, and air-dried for three days. The leaves were then further dried for one day using an oven at 50°C. Using a blender, the dried leaves were mashed and sieved through a 60-mesh sieve. Using 200 mL of chloroform: ethanol at a 9:1 (v/v) ratio, up to 20 g of dried leaves powder was extracted. The extraction was done using a Soxhlet extractor. The solvent was filtered using Whatman filter paper, and the filtrate was vacuum evaporated using an IKA® RV10 rotary evaporator at 40°C and 100 mbar (Pharmawati and Wrasiati 2020)

The encapsulation of crude extract was conducted using a 20% maltodextrin solution. As much as 10% extract of *E. acoroides* and 2% tween 80 were mixed with the encapsulated solution and homogenized at 6000 rpm for 30 minutes. After that, the mixture was dried up to 8% moisture content, mashed with a blender and then sieved through a 60 mesh sieve (Sulistyadewi et al. 2014).

Treatment of Allium cepa var. aggregatum root

The base of *A. cepa* var. *aggregatum* bulbs were soaked in water to induce roots. When the length of the root was approximately 1 cm, bulbs were transferred to a glass jar containing treatment solutions. The treatments were 30 ppm Ni in the form of $Ni(NO_3)_2.6H_2O$, encapsulated *E. acoroides* extract at 100 ppm, 250 ppm, and 500 ppm, and combined 30 ppm Ni with each of 100 ppm, 250 ppm and 500 ppm of encapsulated *E. acoroides* extract. The treatments were given for 72 hrs. As controls, bulbs were soaked in H_2O. Three bulbs were used in each treatment.

Chromosome preparation

Modified procedures were used to prepare mitotic squash (Sharma and Sharma 1994). Following treatments, roots were rinsed in distilled water and cut in the morning. Roots were then soaked in Farmer's fixative containing of ethanol and acetic acid (3:1) for 24 hrs in the refrigerator. For hydrolysis, root tips were cut 2 mm long and treated with 1N HCl for 15 minutes. The root tips were cleaned in distilled water before being coloured with 2% acetoorcein for 20 min. Excess stain was absorbed using filter paper. Stained root tips were covered with cover glass and then squashed. Slides were inspected for mitotic chromosomes and aberration using microscope binocular XSZ 107BN (Nanjing BW Optics and Instrument Co.) with 400x total magnification. The photographs were taken using the top mount camera Optilab Advance (Miconos). The data was collected from a total of six roots of three bulbs for each treatment and six fields chosen at random from each root.

Metabolic Activity

Following the treatment and control procedures, 5 root tips were removed and soaked in 0.5% 2,3,5-triphenyl tetrazolium chloride (TTC) for 15 minutes in the dark at 35°C. The root tips were then analysed qualitatively after being rinsed with distilled water. Furthermore, the roots were soaked in 95% ethanol to extract the colourful triphenyl formazan complex. The absorbance was measured at 490 nm (Vazhangat and Thoppil 2017). Three replications were conducted in this experiment.

Apoptotic Activity

The Evans Blue staining method was used to investigate the loss of cell viability. After treatments, five roots with identical lengths were cut and dyed with a 0.25% (w/v) aqueous Evans Blue solution for 15 minutes before being rinsed with distilled water for 30 minutes. The experiment was conducted with three replications. The roots were then macro-imaged to determine cell death qualitatively. Roots then were soaked in 3 mL of N,N-dimethylformamide for 1 hour at room temperature for a quantitative estimation by measuring the absorbance of Evans Blue at 600 nm (Vazhangat and Thoppil 2017).

Data Analyses

The mitotic index (%) was computed as the number of dividing cells divided by the total number of cells ´ 100. The chromosomal aberrations were calculated by dividing the number of abnormal cells by the total number of cells counted × 100 (Sarac et al. 2019). Phase index (%) was determined by calculating the number of dividing cells in phases by the total number of dividing cells ´ 100 (Kumar and Thonger 2016).

The antigenotoxicity of encapsulated *E. acoroides* extract was determined by calculating the inhibitory activity of chromosomal aberration induced by Ni. The formula used was following Prajitha and Thoppil (2016). Inhibitory activity (%)= A–B: A–C × 100, where A: Number of aberrant cells induced by Ni, B: Number of aberrant cells induced by the mixture of Ni and encapsulated *E. acoroides* extract, C: Number of aberrant cells induced in the control

Statistical analyses were performed using Minitab 20, with randomised experimental design. The differences between treatments were analysed using the Tukey test with a 95% confidence level. The data were presented as mean ± standard deviation, except for the data of the types of aberration.

RESULTS

Mitotic Index

Using *Allium cepa* var. *aggregatum* root tips, the antigenotoxic potential of encapsulated extract of *E. acoroides* leaves was investigated. One of the metrics used to assess antigenotoxicity was mitotic activity as measured by the mitotic index. The mitotic indices were significantly affected by the treatments ($p<0.01$). Treatment of *A. cepa* root with Ni reduced mitotic index significantly. There are no differences between the mitotic index of control and treatment with 100 ppm encapsulated extract of *E. acoroides* leaves. The concentration of 250 ppm and 500 ppm encapsulated extract had a sig-

nificantly lower mitotic index than control, but significantly higher than nickel (Table 1).

Treatment with nickel resulted in the lowest mitotic index indicating genotoxic activity of nickel. When nickel and encapsulated extract of *E. acoroides* leaves were given simultaneously, the mitotic indices were higher than the mitotic index of Ni alone; however, statistical analysis showed that only the addition of 100 ppm encapsulated extract had a significant increase of the mitotic index. Table 1 shows the mitotic indices of control, treatment with Ni, encapsulated *E. acoroides* extract, and combined treatment of Ni and encapsulated extract.

Phase index

The distribution of mitotic phases was shown in Table 2. The treatments significantly affected prophase, metaphase and telophase indices ($p<0.05$), while anaphase index was not affected by treatments. The majority of chromosomes in all treatments and control were in metaphase. Treatment of Nickel resulted in the highest percentage of metaphase chromosomes, and Ni inhibited telophase as indicated by the significantly lowest index of telophase in Ni treatment. The addition of encapsulated *E. acoroides* extract to the Ni treatment increased the percentage of telophase.

Chromosomal Aberration and Nuclear Abnormality

Statistical analysis shows that the treatments affected chromosomal aberration ($p<0.01$) and nuclear abnormality ($p<0.01$). Mitotic chromosomal aberrations were detected in all treatments including control (Table 1) and control has a very low percentage of aberration. Treatment with Ni resulted in 1,677% of aberrant chromosomes. The percentage of the aberrant chromosome at root tips treated with encapsulated *E. acoroides* extract at the concentration of 100 ppm, 250 ppm and 500 ppm had no significant difference to control, suggesting that the encapsulated extract had no or very low genotoxic effect. Simultaneous treatments of Ni and encapsulated *E. acoroides* extract at concentrations 100 ppm, 250 ppm, and 500 ppm showed a similar percentage of chromosomal aberration to treatment with Ni alone. Modulation of Ni-induced genotoxicity with encapsulated *E. acoroides* extract showed no significant reduction of chromosomal aberration. The inhibitory activities of encapsulated extract to the genotoxic activity of Ni were only 4.9%, 6.5%, and 14.4% with simultaneous addition of 500 ppm, 250 ppm, and 100 ppm encapsulated extract.

Table 1. The Mitotic index and percentage of chromosomal aberration of *A. cepa* root tip cells induced by Ni, encapsulated *E. acoroides* leaves extract and mixture of Ni and encapsulated extract.

Treatment (ppm)	Mitotic index	Chromosome aberration (%)
Control	5.036± 0.497^{a}	0.091±0.1^{b}
30Ni	2.248±0.497^{c}	1.677±0.487^{a}
100Ea	5.048±0.864^{a}	0.553±0.462^{b}
250Ea	3.612±0.444^{b}	0.542±0.227^{b}
500Ea	3.383±0.418^{b}	0.551±0.2097^{b}
30Ni+100Ea	3.262±0.29^{b}	1.453±0.343^{a}
30Ni+250Ea	2.905±0.2176^{bc}	1.575±0.1974^{a}
30Ni+500Ea	2.779±0.489^{bc}	1.601±0.289^{a}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

Table 2. Phase index of mitosis of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and mixture of Ni and encapsulated extract.

Treatment (ppm)	Prophase Index	Metaphase Index	Anaphase Index	Telophase Index
Control	21.68±8.02^{a}	27.8±5.38^{b}	27.08±7.57^{a}	23.44±5.08^{a}
30 Ni	19.22±8.82^{a}	59.17±18.87^{a}	19.14±13.58^{a}	2.74±3.83^{b}
100 Ea	26.6±8.27^{a}	32.96±4.89^{b}	20.13±7.21^{a}	18.31±7.08^{a}
250 Ea	22.98±6.02^{a}	37.88±5.54^{b}	16.61±4.96^{a}	24.22±3.21^{a}
500 Ea	24.11±9.85^{a}	37.72±13.86^{b}	14.91±4.54^{a}	21.57±3.49^{a}
30 Ni+100 Ea	35.1±12.19^{a}	29.42±11.86^{b}	19.3±6.22^{a}	16.17±5.75^{a}
30 Ni+250 Ea	33.86±7.03^{a}	36.21±4.82^{b}	14.77±10.07^{a}	15.16±5.46^{a}
30 Ni+500 Ea	22.69±8.88^{a}	43.34±15.44^{ab}	17.97±2.83^{a}	16±9.7^{a}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

The types of chromosomal aberration at mitotic phases included prolonged prophase, stickiness, fragment, chromosomal break/fragmentation at metaphase, diagonal metaphase, diagonal telophase, chromosome bridge, star anaphase, fragment at anaphase, and vagrant telophase. Figure 1 shows normal mitotic phases, while Figure 2 shows types of aberrant chromosomes. Table 3 shows the percentage of each type of chromosomal aberration.

Nuclear abnormalities were observed in a different set of fields of view than that of chromosomal aberration. The nuclear abnormalities observed were micronuclei, nuclear fragments, and nuclear lesions (Figure

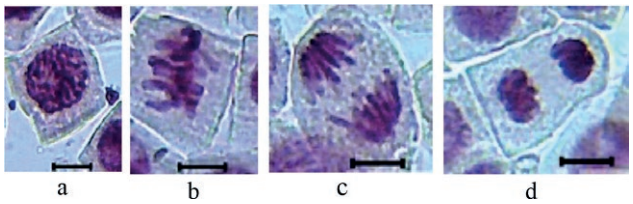

Figure 1. Normal mitosis of *A. cepa* root tip cells. a. Prophase; b. Metaphase; c. Anaphase; d. Telophase. Scale bar=10μm.

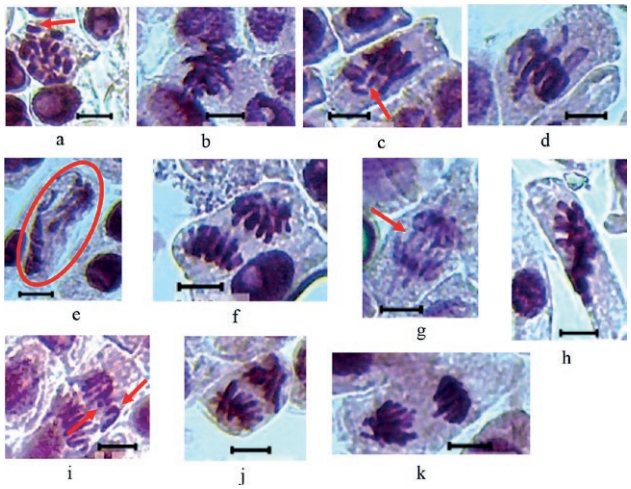

Figure 2. Types of chromosomal aberrations of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract. a=prophase abnormality with fragments; b= sticky metaphase; c= fragment at metaphase; d=chain metaphase; e=vagrant telophase (circle); f=diagonal anaphase; g=chromosome bridge; h=diagonal metaphase; i=fragment at anaphase; j=star anaphase; k=sticky anaphase. Scale bar=10μm. Arrows indicate abnormalities.

3, Table 4). Micronuclei were not detected in control and in treatment using 100 ppm, 250 ppm of encapsulated *E. acoroides* extract, while it was detected at 500 ppm encapsulated *E. acoroides* extract but not significantly different than control. Treatments with Ni alone and combined Ni and encapsulated *E. acoroides* extract resulted in the formation of micronuclei and statistically, they were not significantly different, although the percentage of combined treatments was much lower than Ni alone.

Fragmented nuclei were not observed in control and in treatment with 100 ppm, 250 ppm, and 500 ppm of encapsulated *E. acoroides* extract. The highest percentage of nuclear fragmentation was induced by Ni treatment only. The encapsulated *E. acoroides* extract also induced nuclear fragmentation in a significantly lower percentage than Ni treatment. The encapsulated *E. acoroides* extract given simultaneously with Ni, significantly reduced the percentage of fragmented nuclear (Table 4).

Another nuclear abnormality observed was nuclear lesion and Ni treatment showed the highest percentage. The addition of encapsulated extract to Ni treatments significantly reduced the percentages of nuclear lesions compared to Ni treatment (Table 4).

Metabolic activity

The triphenyl tetrazolium chloride (TTC) staining was used to examine the influence of Ni and encapsulated *E. acoroides* extract on mitochondrial function. Treatment of roots with Ni revealed a substantial decrease in mitochondrial activity. Visually, the encapsulated *E. acoroides* extract as well as combined encapsulated extract and Ni shows an increase in mitochondrial activity (Figure 4).

Based on the absorbance value of 490 nm, the encapsulated extract at concentrations of 100 ppm and 250 ppm has no effect, while 500 ppm extract reduced mitochondrial activity; however, the reduction was significantly less than treatment with Ni (Table 5). Simultaneous treatment of encapsulated extract at 100 ppm and Ni showed improvement of mitochondrial activity compared to Ni alone. In comparison, the addition of 250 ppm and 500 ppm encapsulated extract to Ni treatment did not show improvement of mitochondrial activity.

Apoptotic activity

Evans blue stain was used to analyse in situ cell death by assessing the cell membrane's integrity. Living cells keep the dye out due to the semipermeable nature of cell membranes. On the other hand, damaged cells are unable to remove the dye and are thus stained blue (Roy et al. 2019). Figure 5 shows the visualization of cell death using Evan's blue staining.

Evan's Blue staining method for in situ cell death revealed that the encapsulated *E. acoroides* extract at concentrations 100 ppm, 250 ppm, and 500 ppm showed less colour than treatment with Ni only. Simultaneous treatment of Ni and encapsulated *E. acoroides* extract also showed a reduction of blue colour indicating a reduction of cell death (Figure 5). Quantitative analysis using a spectrophotometer is shown in Table 5. Statistical analysis revealed that 100 ppm and 250 ppm of encapsulated extract had similar effect as control. The 500ppm extract showed higher absorbance than control, but significantly lower than Ni alone. The data of in situ cell death is similar to that of mitochondrial activity. The 100 ppm encap-

Table 3. The percentage of each type of chromosomal aberration of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract.

Treatment (ppm)	Frag.pro (%)	Sty.meta (%)	Frag.meta (%)	Ch.meta (%)	Diag.meta (%)	Diag.ana (%)	Star.ana (%)	Sty.ana (%)	Frag.ana (%)	Bridge (%)	Vr.telo (%)
Control	0^{c}	0.091^{b}	0^{c}	0^{a}	0^{a}	0^{a}	0^{b}	0^{a}	0^{a}	0^{a}	0^{a}
30Ni	0.256^{ab}	0.509^{a}	0.656^{ab}	0.062^{a}	0.062^{a}	0.042^{a}	0.1^{ab}	0^{a}	0.081^{a}	0.023^{a}	0^{a}
100Ea	0.069^{b}	0.180^{ab}	0.071^{c}	0.071^{a}	0.032^{a}	0^{a}	0.039^{ab}	0.026^{a}	0.027^{a}	0.039^{a}	0^{a}
250Ea	0.034^{bc}	0.156^{ab}	0.226^{c}	0.026^{a}	0.033^{a}	0^{a}	0^{b}	0.033^{a}	0.034^{a}	0^{a}	0^{a}
500Ea	0.029^{bc}	0.151^{ab}	0.233^{c}	0.032^{a}	0^{a}	0.037^{a}	0.034^{ab}	0^{a}	0.036^{a}	0^{a}	0^{a}
30Ni+100Ea	0.394^{ab}	0.221^{ab}	0.357^{bc}	0.034^{a}	0.069^{a}	0.029^{a}	0.225^{a}	0.059^{a}	0.064^{a}	0^{a}	0^{a}
30Ni+250Ea	0.224^{ab}	0.292^{ab}	0.73^{ab}	0.027^{a}	0^{a}	0^{a}	0.086^{ab}	0.1^{a}	0.027^{a}	0.027^{a}	0.065^{a}
30Ni+500Ea	0.229^{ab}	0.181^{ab}	0.758^{a}	0.034^{a}	0.029^{a}	0.029^{a}	0.13^{ab}	0.068^{a}	0.073^{a}	0.073^{a}	0^{a}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Frag.pro=prophase abnormality with fragments; Sty.meta= sticky metaphase; Frag.meta=fragment at metaphase; Ch.meta=chain metaphase; Diag.meta=diagonal metaphase; Diag.ana=diagonal anaphase; Star.ana=star anaphase; Sty.ana=sticky anaphase, Frag.ana=fragment at anaphase; Bridge=chromosome bridge; Vr.telo=vagrant telophase.
Means with same letters at the same column are not significantly different.

Table 4. The percentages of nuclear abnormalities of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract.

Treatment (ppm)	Micronuclei (%)	Nuclear lesion (%)	Nuclear fragmentation (%)
Control	0 ± 0^{b}	4.57 ± 5.75^{d}	0 ± 0^{b}
30 Ni	0.222 ± 0.192^{a}	90.67 ± 3.58^{a}	0.73 ± 0.609^{a}
100 Ea	0 ± 0^{b}	$20,11\pm2.84^{c}$	0 ± 0^{b}
250 Ea	0 ± 0^{b}	20.03 ± 3.78^{c}	0 ± 0^{b}
500 Ea	0.020 ± 0.063^{b}	21.76 ± 3.84^{c}	0 ± 0^{b}
30 Ni+100 Ea	0.083 ± 0.092^{ab}	60.43 ± 8.2^{b}	0.083 ± 0.091^{b}
30 Ni+250 Ea	0.082 ± 0.092^{ab}	62.73 ± 6.29^{b}	0.079 ± 0.087^{b}
30 Ni+500 Ea	0.091 ± 0.1^{ab}	67.48 ± 6.14^{b}	0.076 ± 0.084^{b}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

Table 5. Metabolic and apoptotic activities of *A. cepa* root tips after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract.

Treatment (ppm)	Metabolic activity	Apoptotic activity
Control	0.482 ± 0.034^{a}	0.166 ± 0.03^{c}
30 Ni	0.157 ± 0.006^{d}	0.448 ± 0.053^{a}
100 Ea	0.486 ± 0.05^{a}	0.268 ± 0.07^{bc}
250 Ea	0.479 ± 0.024^{a}	0.286 ± 0.071^{bc}
500 Ea	0.334 ± 0.026^{b}	0.301 ± 0.035^{b}
30 Ni+100 Ea	0.277 ± 0.022^{bc}	0.304 ± 0.015^{b}
30 Ni+250 Ea	0.242 ± 0.057^{bcd}	0.320 ± 0.011^{ab}
30 Ni+500 Ea	0.222 ± 0.024^{cd}	0.344 ± 0.053^{ab}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

sulated extract when given simultaneously with Ni demonstrated significantly less cell death than treatment with Ni.

DISCUSSION

As a result of increased urbanization and industrialisation, toxic metal poisoning has become a global issue. Moreover, accumulated heavy metal in plants has been known to induced chromosome abnormalities as shown by Sabeen et al. (2020).

This present study confirmed that nickel has a genotoxic effect by significantly decreasing mitotic index and inducing chromosomal aberration. At the concentration of 30 ppm $Ni(NO_3)_2.6H_2O$ the reduction of the mitotic index was 44.53% in comparison to control. The decline of the mitotic index below 50% has sub-lethal effects and is known as the limit value of cytotoxicity (Madike et al. 2019). The genotoxic effect of nickel has been studied using nickel chloride ($NiCl_2$) (Ganesan and Panneerselvam 2013), nickel sulfate ($NiSO_4.6H_2O$) (Pavlova 2017) and nickel nitrate $Ni(NO_3)_2$ (Sarac et al. 2019). The concentration of 30 ppm Ni was used in this study, to evaluate a lower concentration than that used by Sarac et al. (2019) which was 50 ppm.

Nickel at 30 ppm induced chromosomal aberration where chromosome stickiness and chromosome break

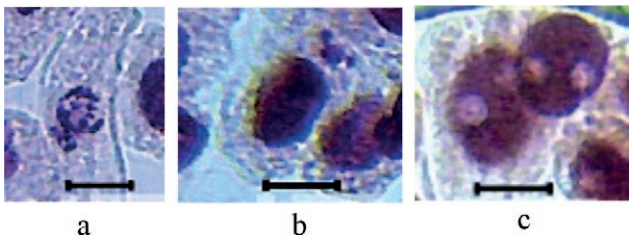

Figure 3. Types of nuclear abnormalities of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract. a. nuclear fragmentation; b. micronucleus; c. nuclear lesion. Scale bar=10μm.

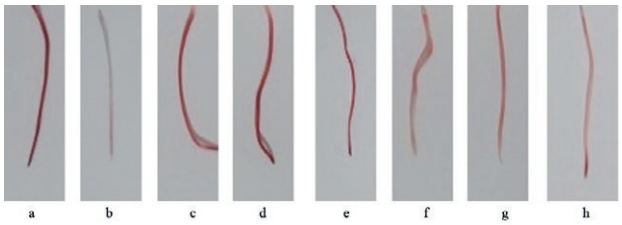

Figure 4. Analysis of metabolic activity using TTC staining of root of *A. cepa*. a=control, b=30 ppm Ni, c=100 ppm Ea, d=100 ppm Ea, d= 250 ppm Ea, e=500 ppm Ea, f=30ppmNi+100 ppm Ea, g=30 ppm Ni+250 ppm Ea, h=30 ppm Ni+500 ppm Ea. Ea=encapsulated *E. acoroides* leaves extract.

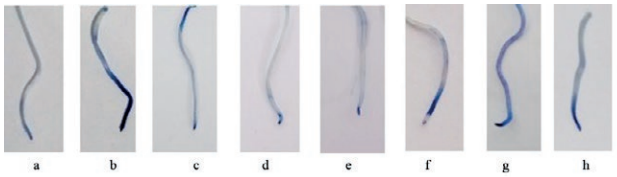

Figure 5. Analysis of apoptotic activity using Evans Blue staining of root of *A. cepa*. a=control, b=30 ppm Ni, c=100 ppm Ea, d=100 ppm Ea, d= 250 ppm Ea, e=500 ppm Ea, f=30ppmNi+100 ppm Ea, g=30 ppm Ni+250 ppm Ea, h=30 ppm Ni+500 ppm Ea. Ea=encapsulated *E. acoroides* leaves extract.

were at a high percentage. This agrees with Sarac et al. (2019) and Kaur et al. (2019) who observed that chromosome break and chromosome stickiness were the major types of chromosomal aberration found due to heavy metal treatments. Nickel promotes the generation of a large quantity of reactive oxygen species (ROS) which is a factor of nickel toxicity. Reactive oxygen species harm all cellular components in plants, including cell membranes, lipids, pigments, enzymes, chloroplasts, and nucleic acids (Gopal and Nautiyal 2012). Excessive ROS promotes DNA break which can be observed through the chromosomal break (Ganesan and Panneerselvam 2013). Chromosome stickiness is caused by chromosome loss of physical identity due to physical attachment of chromatin material or inter-chromosomal connections (Asita et al. 2017). Heavy metal complexes are very reactive, and their complexes interact directly or indirectly with DNA, histone, or non-histone proteins, causing chromosomal surface properties to change, making them sticky (Kumar and Srivastava 2015).

The cytotoxicity of encapsulated *E. acoroides* extract was tested by calculating the mitotic index. The mitotic index of encapsulated *E. acoroides* extract at a concentration of 100 ppm was similar to control. Higher concentrations of encapsulated extract decreased mitotic index but were still higher than the mitotic index of Ni treatment. This suggests that at 250 ppm and 500 ppm, the encapsulated *E. acoroides* extract was less toxic than Ni. The reduction in the mitotic index shows that the encapsulated *E. acoroides* extract inhibits mitotic activity in *A. cepa*. The reduction in mitotic index is attributable to compounds in the aqueous extracts that have cytotoxic effects, as the mitotic index is a quantitative measure of mitotic activity in an organism or a particular organ (Sreeranjini and Siril 2011).

When Ni and encapsulated extract were given simultaneously, only 100 ppm encapsulated extract resulted in a significant increase of the mitotic index. This indicates that the encapsulated *E. acoroides* extract had the potential in modulated Ni-inhibited mitotic activity by increasing the proliferative activity of cells. The antigenotoxic activity of low concentrations of plant extract was also reported by Prajitha and Thoppil (2016). The lower concentration (5 ppm) of *Amaranthus spinosus* employed in the antigenotoxicity experiment was beneficial in reversing the genotoxicity.

Treatment with higher concentrations of encapsulated *E. acoroides* extract combined with Ni did not significantly increase mitotic index. A study using *Chenopodium album* extract found that at low concentration, the extract reduced the genotoxic effect induced by EMS (ethylmethane sulfonate). At higher concentrations, *C. album* extract showed synergistic action with EMS, resulting in an increased genotoxic effect (Asita et al. 2015). In the present study, encapsulated *E. acoroides* extract did not have a synergetic effect with Ni since the addition of encapsulated extract together with Ni, had no significantly different with Ni alone on the chromosomal aberration.

Based on analysis of phase index, Ni treatment had a significantly higher metaphase index, while the anaphase indices were not significantly different between treatments. This means that in Ni treatment the anaphase index was low. The telophase index of Ni treatment was significantly lower than other treatments.

According to Asita et al. (2017), a decrease in the proportion of dividing cells in A + T indicates that the chromosome spindles were poisoned, resulting in metaphase arrest. Low anaphase and telophase indices can cause daughter cells to be damaged, limiting plant growth. The simultaneous addition of Ni and encapsulated *E. acoroides* extracts at all concentrations significantly increased the telophase indices.

The percentage of chromosome abnormality between control and treatment with all concentrations of encapsulated *E. acoroides* extract were not significantly different, indicating the possibility of the non-toxic effect of encapsulated *E. acoroides* extract. This result is important since the encapsulated *E. acoroides* extract had an antiproliferative effect by reducing the mitotic index. Therefore, it can be further explored in anticancer research.

Chromosome aberrations were detected in the combined treatment of Ni and encapsulated *E. acoroides* extract at all concentrations tested. Although there were decreases in percentages of chromosome aberration in combined Ni and encapsulated extract treatments, the percentages were not significantly different from that of Ni treatment alone. This result indicates that the concentrations of encapsulated *E. acoroides* extract used unable to effectively suppress chromosome aberration induced by Ni. However, it is worth noting that there was 14.4% inhibitory activity of Ni when 100 ppm encapsulates *E. acoroides* extract was given simultaneously with 30 ppm Ni. This suggests that the mixtures were less genotoxic than Ni alone.

Nickel induced the formation of micronuclei and nuclear fragmentation at low levels but formed nuclear lesions in extremely high percentages. Nuclear lesions provide cytological evidence of DNA biosynthesis inhibition (Sajitha and Thoppil 2018). At all concentrations tested, the encapsulated *E. acoroides* extract did not induce micronuclei and chromosome fragmentation. However, it induced nuclear lesions at low percentages, significantly lower than induced by Ni. The control group had a low level of nuclear lesion, which could be due to unintentional DNA changes. According to Nefic et al. (2013), root tip cells show a very low frequency of spontaneous abnormalities.

In TTC analysis, the roots treated with Ni were unable to convert TTC to red coloured TF, indicating a significantly lower activity of the mitochondrial respiratory chain compared to control. The roots treated with encapsulated *E. acoroides* extract at 250 ppm and 500 ppm demonstrated no effect on mitochondrial activity. The addition of lower concentration of encapsulated *E. acoroides* extract (100 ppm) to 30 ppm Ni increased mitochondrial activity compared to treatment with Ni alone.

Apoptotic activity was highly induced in Ni treated root, while encapsulated *E. acoroides* extract showed lower apoptotic activity than Ni, but higher activity than control. This suggests that encapsulated *E. acoroides* extract was less toxic than Ni. Supplementation of encapsulated *E. acoroides* extracts to Ni, visually resulted in lower apoptotic activity than Ni alone as observed as less blue colour. However, when measured using a spectrophotometer, there were no differences between the apoptotic activities at Ni treatment and Ni supplemented with 250 ppm and 500 ppm encapsulated *E. acoroides* extract. Lower concentration of encapsulated *E. acoroides* extract (100 ppm) when given together with 30 ppm Ni, induced reduction of apoptotic activity.

The effects of simultaneous addition of Ni and encapsulated *E. acoroides* extract at a lower concentration to metabolic activity and apoptotic activity agreed with their effect on the mitotic index. According to Prajitha and Thoppil (2016), a higher concentration of an extract can have mutagenic effect and a lower concentration can have an antimutagenic effect or *vice versa*. In mice, lower levels of b-carotene increased the anticlastogenic activity of cyclophosphamide-induced clastogenicity, but there was no protective impact at higher concentrations. This finding implies distinct processes of b-carotene modulation and a probable shift in the balance of the promutagen activation/detoxification mechanism (Salvadori et al. 1992). Similar reasoning may apply to the effect of simultaneous addition of Ni and low concentration encapsulated *E. acoroides* extract on increasing mitotic index and metabolic activity and reducing apoptotic activity.

Enhalus acoroides leaves extract contained phytochemical compounds, including phenols, tannins, and flavonoids. The FTIR analysis confirmed the presence of flavonoid and polyphenols as a high C-H out-of-plane bending (oop bend) vibration for the substituted benzene ring was identified in the extract (Pharmawati and Wrasiati 2020). These phytochemical components in the plant extracts may be responsible for the reduced mitotic index in *A. cepa* root meristematic cells when roots were treated with encapsulated *E. acoroides* leaves extract. On the other hand, these phytochemical compounds may contribute to the increasing mitotic index, lowering nuclear abnormalities when the encapsulated extract is present together in the Ni treatment. This kind of result where plant extract showed the opposite effect was also observed by Prajitha and Thoppil (2016) in *Amaranthus spinosus* extract. The encapsulated *E. acoroides* leaves extract also contained pigments such as chlorophyll b,

ethyl-chlorophyllide a, Mg-free chlorophyll b, lutein, Mg free chlorophyll a, pheophytin, and β-carotene (Pharmawati and Wrasiati 2020). It is well known that phenolic compounds, tannins, flavonoids, chlorophyll, and carotenoids have antioxidant properties (Aryal et al. 2019). Antioxidants containing phenolics can prevent the generation of free radicals and/or stop the spread of autoxidation. At the same plant pigments can chelate metals and transfer hydrogen to oxygen radicals, delaying oxidation (Brewer 2011).

In the present investigation, the encapsulated *E. acoroides* extract was found to have preventive activity, as evidenced by the reduction and reversion of nuclear damages (nuclear lesions and nuclear fragmentations) caused by Ni. However, the encapsulated extract cannot reduce chromosomal aberration. Preincubation with the encapsulation extract before Ni treatment needs to be evaluated to test the ability of encapsulated extract to suppress chromosomal abnormalities. Further study is also needed to test the protective activity of the encapsulated extract on animal cells.

ACKNOWLEDGMENT

The authors thank Universitas Udayana for supporting this study through the Study Program Flagship Research Scheme No. B/773/UN14.2.8.II/PT.01.03/2021

FUNDING

Universitas Udayana through the Study Program Flagship Research Scheme No. B/773/UN14.2.8.II/PT.01.03/2021

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