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ALLELOPATHIC EFFECTS OF *Cynometra ramiflora* L. (FABACEAE) IN THE SEED GERMINATION OF *Brassica rapa* L. (BRASSICACEAE) AS MODEL ORGANISM

Salumayag D. Linsahay[1]*, Chris Rey M. Lituañas[2], Reggie Y. Dela Cruz[1,3], Aprille Joy M. Luceño-Tenizo[4]

[1]Molecular Biology, Biotechnology, Genetics, and Microbiology Division, Institute of Biological Sciences, College of Arts and Sciences
[2]Plant Biology Division, Institute of Biological Sciences, College of Arts and Sciences
[3]Tuklas Lunas Development Center
[4]Animal Biology Division, Institute of Biological Sciences, College of Arts and Sciences
Central Mindanao University, Musuan, Maramag, Bukidnon, 8714, Philippines
*Corresponding author; E-mail: ayagdanluyan@gmail.com

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ABSTRACT. The study evaluated the potential allelopathic effects of the aqueous extract of a native plant, *Cynometra ramiflora* L. in the seed germination of *Brassica rapa* L. Seed germination was observed for 12, 24, and 48 hours after treatment (HAT) and radicle lengths were measured after 48 HAT. Results showed that exposure to 50% and 100% aqueous extract concentrations significantly reduced the germination percentage (GP) compared to the control. Moreover, cytogenetic observations showed intensive chromosomal fragmentation and decondensation in the 50% and 100% concentrations, respectively. Consequently, cellular aberrations were observed such as multinucleated cell, membrane damage, micronuclei, stickiness, huge-nucleated cell, nuclear lesion, giant cell, plasmolyzed cell, ghost cell, and lobulated cells showing no significant difference compared to the positive control. These suggest that native plants such as *C. ramiflora* demonstrated negative allelopathic effects impacting *B. rapa* development.

Keywords: abnormal cell, allelopathy, *Cynometra ramiflora,* germination, karyogram

INTRODUCTION

Plant species are diverse, and their dispersal and distribution are dynamic, partly influenced by allelopathy making it important in fostering competition forming plant communities (KUMAR *et al*., 2024). Allelopathy is one factor that greatly impacts biodiversity. It is defined as the chemical interaction among plant and microorganism, or between plants, which influences the establishment of coexisting species and affecting self-regeneration (THIÉBAUT *et al*., 2019). Allelopathy involves the interaction of plants by producing

ORCID:
S.D. Linsahay - 0009-0009-1402-7785; C.R.M. Lituañas, - 0000-0002-8584-3757;
R.Y. Dela Cruz - 0000-0002-4253-0988; A.J.M. Luceño-Tenizo - 0009-0006-6801-2763.

allelochemicals which can have detrimental and/or beneficial effects (CHENG *et al.,* 2015). Allelopathy is known to be more negative if allelopathy plant residues are used, but, has fewer negative effects when allelopathy plant will be used to condition soil (ZHANG *et al.,* 2021).

The phenomenon of allelopathy has been considered as a concern by farmers for many years now, but for around 80 years now, research has proven that it also could be applied in agriculture and forestry (QASEM *et al.,* 2001). The allelochemicals produced are plant secondary metabolites that are non-nutritive but were reported to have potential effects in growth of the target organism, could be used as herbicides, insecticides, and can be used for antimicrobial crop protection (CHENG *et al*., 2015) which generally mediate interspecific interactions (VOKOU, 2007). In major field crops, important allelochemicals includes, phenolic compounds, benzoxazinoids, glucosinolates, sorgoleone, terpenes, momilactones and alkaloids which can also be used on controlling weeds (JABRAN, 2017). According to FAROOQ *et al*. (2011), allelopathy has two options when used for pest management, where (1) essential oils will be used as an alternative for the commonly used methyl bromide controlling the growth of both weeds and parasitic plants, (2) there is also a *C*-glycosylflavonoid called isoschaftoside responsible for the allelopathic suppression, (3) leguminous cover crops such as *Mucuna pruriens* (velvet bean) can be beneficial for the cultivation of *Oryza sativa* (rice) increasing yield, and (4) crop weed interactions can also be affected by germination stimulants and other plant regulatory signals produced by allelopathy plants.

Pesticide application is the widely adopted application of allelopathy (FAROOQ *et al.,* 2011), allelochemicals can be very involved as well in the defense mechanisms of the plants under stress condition but can matter as well to their concentration (QASEM, 2010). Furthermore, invasive species has been reported to release more allelochemicals making them more competitive than the growth of the native plant species disabling allelopathic legacy effect decreasing competitiveness, but study results showed that native or invasive species also manifests allelopathy (DEL FABBRO *et al.,* 2015). It was also found that non-native and native species have somewhat similar allelopathic potential (GRUTTERS *et al.,* 2017).

Cynometra ramiflora, commonly known as "balitbitan" is a plant native to the Philippines. It belongs to the family Fabaceae and genus *Cynometra* which, accordingly, has been distributed widely and grows up to 3-10 meters tall (LILLO *et al*., 2023). Several studies reported that it has antioxidant, antihyperglycemic, cytotoxic, antibacterial and antinociceptive activity (AFRIN *et al*., 2016) and elicits broad spectrum of phytochemical compounds from its leaves SAMADD *et al.* (2024). Furthermore, since data about the allelopathic effects of native plants species remains limited, this study intends to investigate whether *C. ramiflora* contains adverse effects in the seed germination of *B. rapa*. Moreover, it is hypothesized that the *C. ramiflora* has significant inhibitory effects on the seed germination, chromosome organization and confers cytological effects in *B. rapa.* Thus, the present study aims to evaluate the potential allelopathic effects of the aqueous extract of a native plant, *C. ramiflora* in the seed germination of *B. rapa* providing a baseline information about the effects of its leaf extract up to the cellular level. This will also encourage the propagation of more native plant species given that there are much more applications that it holds.

MATERIALS AND METHODS

Fresh leaves of *C. ramiflora* were collected and transported to Tuklas Lunas Development Center Annex. These were washed simultaneously with tap and distilled water and were subjected for 36 hours oven-drying at 40˚C. After drying, the samples were powdered using blender and was used for the aqueous extraction. Twenty (20) grams of the sample were mixed with distilled water with a ratio of 1:10 (v/w) and was soaked for 24 hours. After the suspension has been made, the extract was then filtered using Whatman filter paper. To obtain 50% aqueous extract, a filtrate volume was added with the same volume of distilled water with

1:1 ratio. Meanwhile, 100% aqueous extract was a direct concentration of the filtrate. These were then stored in sealed test tubes and refrigerated at 4 ˚C for further utilization (Figure 1).

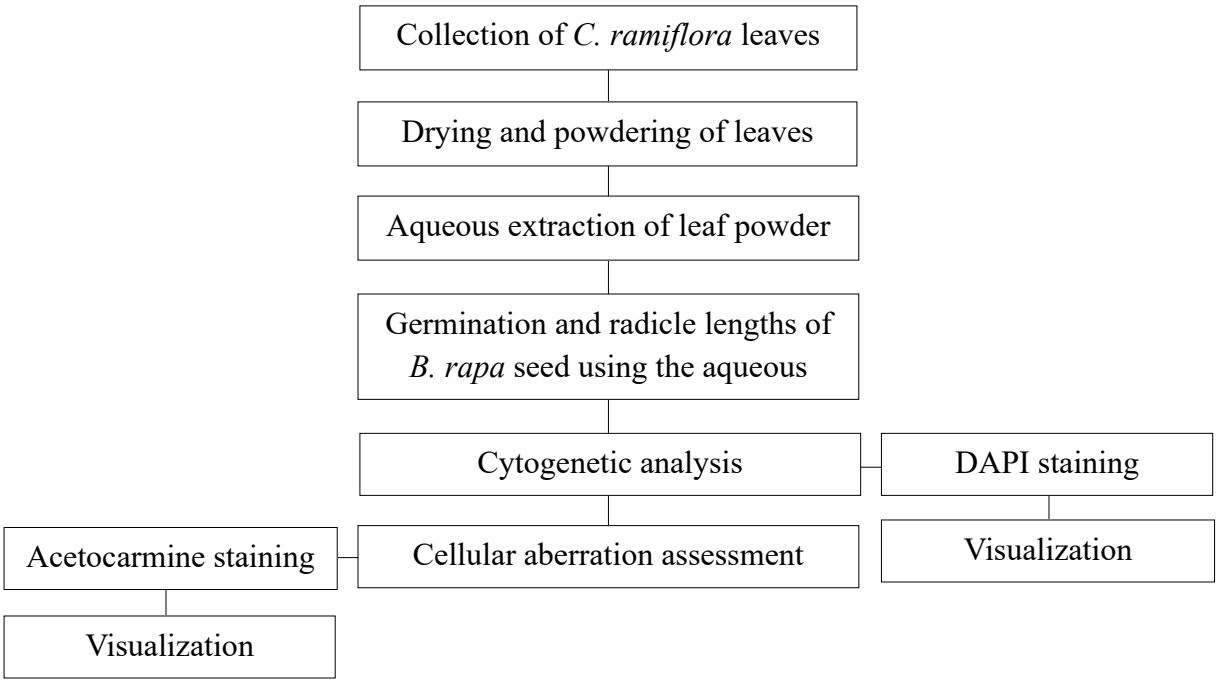

Figure 1. General experimental procedure

Germination and Radicle lengths of B. rapa seeds

Adapted from CASAS *et al.* (2024) with minor modifications, fifteen (15) seeds of *B. rapa* seeds were randomly selected and germinated on separate petri plates using the aqueous plant extract of *C. ramiflora* containing the concentrations 50% and 100% together with the positive control, water (Figure 2). Each of the treatment was performed in triplicates. The germinated seeds showing visible emergence of radicle were then counted at 12, 24 and 48 hours after treatment (HAT) and germination percentage (%) were calculated.

$$Germination\ percentage\ (\%) = \frac{Total\ number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ sown}\ x\ 100$$

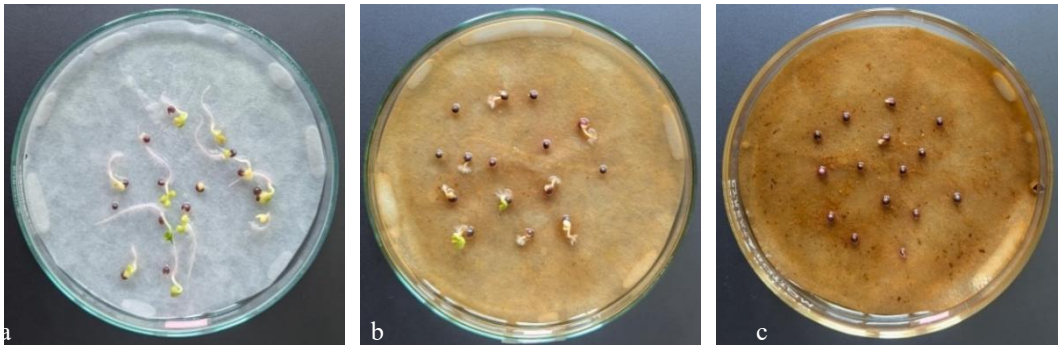

Figure 2. *B. rapa* seeds 48 hours after treatment with (a) water, (b) 50% aqueous leaf extract, and (c) 100% aqueous leaf extract

Using ImageJ version 1.54k (NIH, Maryland, USA) software, the radicle lengths were then measured 48 HAT and Analysis of Variance (ANOVA) using Microsoft Excel was employed for statistical analysis to determine level of significance of GP and radicle length of *B. rapa*.

Cytogenetic Analysis

Adapted from the methods of WAMINAL *et al.* (2018) with modifications, the root tips of the pretreated seeds were cut with a length of about 2 mm after washing. For further assessment, the seeds were grown in water and different concentrations of aqueous leaf extracts, separately. Roots were then harvested and soaked in 8-Hydroxyquinoline, Carnoy's solution and 70% ethanol, respectively, and were dissected as illustrated in Figure 3. The roots were transferred to different microcentrifuge tubes containing 2:1 ratio of enzyme cellulose and pectolyase before incubation at 37 ˚C for 90 mins. After incubation, the enzymes were removed, and roots were added with 3:1 Carnoy's solution for homogenization. For 3 minutes the tubes were centrifuged at 13,000 rpm. Supernatants were then discarded while the pellets were suspended with acetic acid:ethanol solution. The samples were then mounted in moist glass slides and air dried. For visualization, 1% DAPI (4'6-diamidino-2-phenylindole) fluorescent stain was added before viewing under a fluorescence microscope under oil immersion objective (OIO) to search for well spread chromosomes. Chromosomes were then measured using ImageJ for karyogram arrangement.

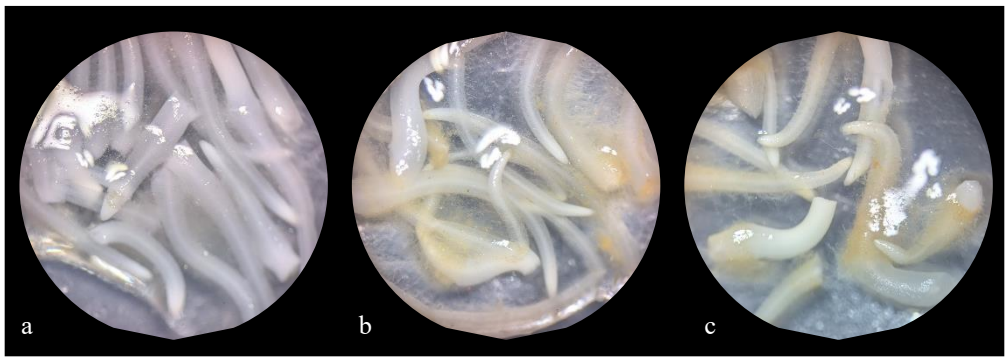

Figure 3. 10x magnification of roots from the germinated seeds: (a) water, (b) 50% aqueous leaf extract, and (c) 100% aqueous leaf extract

Cellular aberration assessment

To assess cellular aberrations in the root tips of *B. rapa*, same concentrations of *C. ramiflora* leaf extracts were used in the germination process with the addition of a positive control (3% hydrogen peroxide [H_2O_2]) and a negative control (water) following the procedure described by OWOLARAFE *et al*. (2020) with modifications. The grown roots were placed in a petri dish and were fixed with acetic alcohol (ethanol: glacial acetic acid in a 3:1 ratio) for 4 hours and were hydrolyzed in 1 N HCl (9:1) at 60˚ for 15 minutes followed with 1% acetocarmine staining. The root tips were then examined under a light microscope. Aberrations were then determined based on the abnormalities described by AKPAN *et al.* (2017) and SABEEN *et al.* (2020). Two-tailed t-test was then performed using Microsoft Excel to know the significant difference between positive control and the different concentrations of aqueous extract.

RESULTS AND DISCUSSION

Germination and Radicle lengths of B. rapa seeds

With the treatments (water, 50% and 100% aqueous leaf extracts), number of germinated seeds were recorded after 12-, 24- and 48-hours growth (Table 1) highlighting delayed germination of seeds treated with *C. ramiflora* aqueous leaf extract suggesting that the aqueous extracts exhibited possible allelopathic effects on *B. rapa* germination at 50% and 100% concentrations.

Table 1. Germination of *B. rapa* seeds 12, 24, and 48 hours after treatment (HAT) with different concentrations of *C. ramiflora* aqueous leaf extract

Treatment	Number of seeds germinated								
	12 HAT			24 HAT			48 HAT		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
+control (Water)	9	12	14	12	14	14	12	15	15
50% aqueous extract	0	0	1	6	4	3	9	7	6
100% aqueous extract	0	0	0	0	0	1	2	0	1

Results also revealed that there was a significant decrease in the germination percentage of *B. rapa* seeds (Figure 4) highlighting the suppressive effects of 50% (48.89%) and 100% aqueous leaf extract (6.67%) which are ~44.44% and ~86.66% less than the control (93.33%) (Table 2), respectively. Meanwhile, the radicle length (Figure 5, Table 3) measurements suggest statistical difference, where 50% (0.728) and 100% aqueous leaf extract (0.035) are 4.8102 and 4.848 less than the control (4.883), respectively. These implies significant inhibition of the germination and radicle growth of *B. rapa* after 48 HAT.

Table 2. Germination percentage (%) of *B. rapa* seeds at 12, 24 and 48 hours after treatment (HAT) with different concentrations of *C. ramiflora* aqueous leaf extract representing mean, standard deviation, and p-value

Treatment	Germination Percentage (%)		
	12 HAT	24 HAT	48 HAT
+ control (Water)	77.78 ± 16.78d	88.89 ± 7.70e	93.33 ± 11.55e
50% aqueous extract	2.22 ± 3.85a	28.89 ± 10.18b	48.89 ± 10.18c
100% aqueous extract	0 ± 0a	2.22 ± 3.85a	6.67 ± 6.67a
p-value	0.05		

Means with the same letter are not significantly different at $p<0.05$

Table 3. Radicle length of *B. rapa* at 48 hours after treatment with different concentrations of *C. ramiflora* aqueous leaf extract, representing mean, standard deviation, and p-value

Treatment	48 HAT
+ control (Water)	4.883 ± 2.537b
50% Aqueous extract	0.728 ± 0.775a
100% aqueous extract	0.035 ± 0.142a
p-value	0.05

Means with the same letter are not significantly different at $p<0.05$

As shown in Figure 4 and Table 2, at 12 HAT, all the concentrations successfully suppressed the germination of *B. rapa* seeds where 100% aqueous extract even showed no signs of germination. At 24 HAT, germination rate increased for the treated seeds, but it was still significantly different from the control. Significant suppression was still exhibited by 50% and 100% concentrations after 48 HAT.

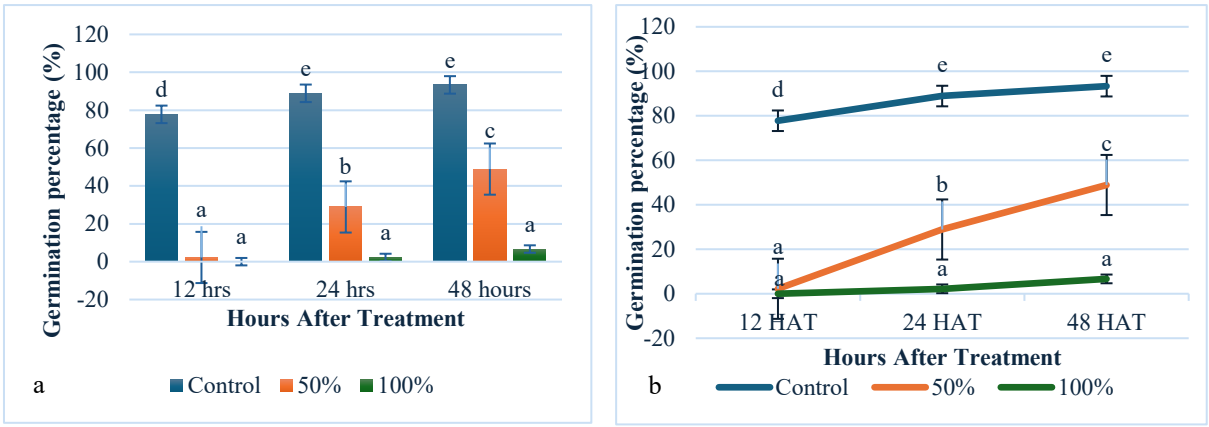

Figure 4. Germination percentage (%) of *B. rapa* seeds at 12, 24, and 48 HAT with different *C. ramiflora* aqueous leaf extract concentrations represented in a (a) bar graph of germination average with statistical groupings and (b) line graph showing the trend of germination percentage using different treatments (means with the same letter indicate no significant difference at $p<0.05$ represented with standard deviation lines)

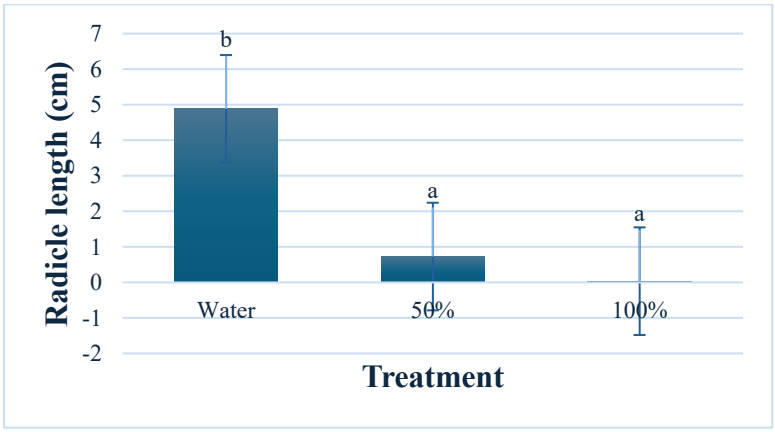

Figure 5. Radicle length of *B. rapa* at 48 HAT with different concentrations of *C. ramiflora* aqueous leaf extract (means with the same letter indicates no significant difference at $p<0.05$ represented with standard deviation lines)

Furthermore, after 48 HAT as illustrated in Figure 5 and Table 3, all *C. ramiflora* aqueous leaf extract used to treat *B. rapa* seeds caused significant decrease in the radicle growth of the seeds compared to the control. Due to random seed selection and sudden germination 48 HAT, results became variable resulting to a relatively higher SD when compared to the mean.

Cytogenetic Analysis

Through DAPI staining, all properly condensed cells from chromosome spreads treated with water confirmed that the chromosome number of *B. rapa* was *2*n=20 (Figure 6a) with 10 homologous pairs (Figure 6b) under fluorescence microscope as documented by GUAN et al. (2023). For further confirmation of the allelopathic effects of *C. ramiflora* aqueous leaf extract in the seed germination of *B. rapa,* the 50% and 100% aqueous extract concentrations were used to grow seeds and assess its proper condensation activity during metaphase stage. Results of chromosome spread replicates showed that 50% aqueous leaf extract treatment failed to properly condense most chromosomes of *B. rapa* cells and instead led to intensive chromosomal fragmentation (Figure 7b) which is thought to be a result of incomplete replication (AYUDANTE *et al.*, 2021). Meanwhile, 100% aqueous leaf extract treatment led to the intensive decondensation of chromosomes (Figure 7c) when compared to the control (Figure 7a). Aqueous leaf extract treatments possibly affected segregation of mitotic and meiotic

chromosomes causing dysfunction to genes and deregulation of its expression, reduces methylation (Bolaños-Villegas, 2021), ultimately affecting proper growth and development of the *B. rapa* seeds. The concentration-dependent results of *C. ramiflora* aqueous leaf extract remained consistent across chromosome spread replicates rendering its mentioned effects relative to other plant extracts.

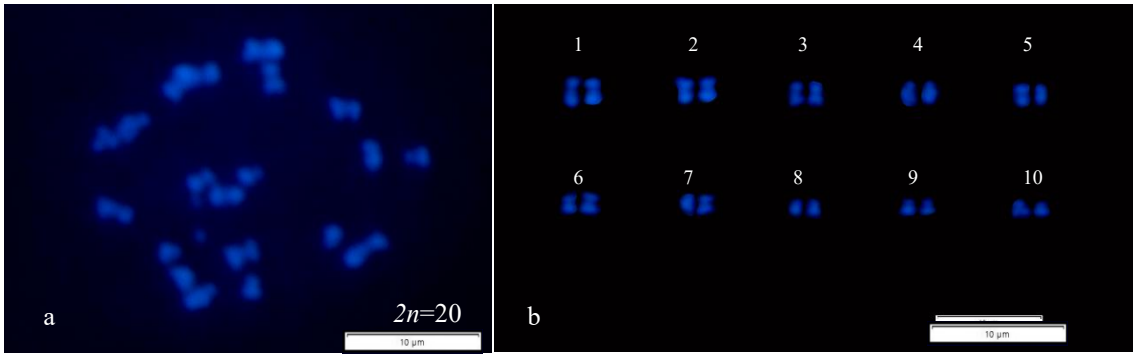

Figure 6. Karyogram of *B. rapa* showing the (a) chromosome spread and (b) chromosome in homologous pairs

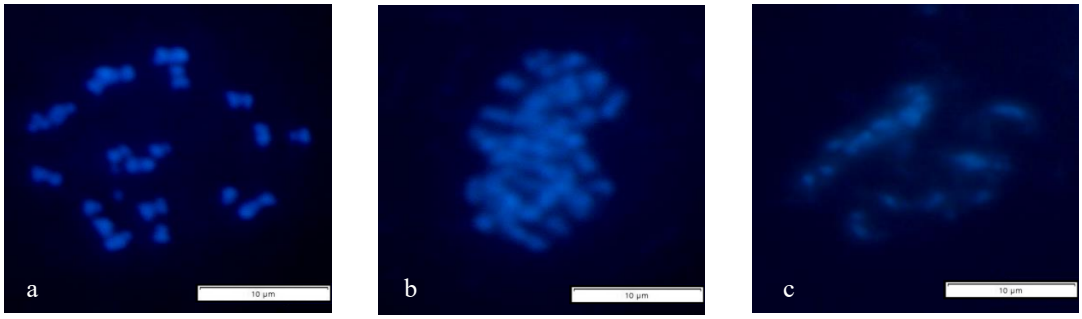

Figure 7. Chromosome spread treated with (a) water, (b) 50% aqueous leaf extract, and (c) 100% aqueous leaf extract

When compared with the results of GUPTA & KUMAR (2021) in evaluation of *Butea monosperma* Lam. (palash), higher extract concentration led to lesser number of germinated seeds and decrease length of radicle due to the lethal effects of the present allelochemicals that caused fragmentation and other chromosomal abnormalities. As reported by CELIK (2012), even the known herbal medicines can be potentially toxic to cells which can result to increase of chromosomal aberrations caused by damage to DNA, enzymes and structural proteins but effects could differ based on concentration. Additionally, leaf extracts of the plants such as *Nicotiana tabacum* L. (tobacco), *Spinacia oleracea* L. (spinach), *Aster amellus* L. (aster), *Ricinus communis* L. (castor), and *Zea mays* L. (corn) can have mutagenic effects inducing chromosome fragmentation and spindle disturbance in the root meristem of *Allium sativum* L. (garlic). Similarly, *Nerium oleander* L. (rose laurel) leaf extract was also found to cause interruption in cell division causing chromosomal breaks and abnormalities (BAKIR ÇILESIZOĞLU *et al.,* 2022) while *Parquetina nigrescens* Afzel. (african spider flower), particularly its aqueous leaf extract also confers chromosomal aberration and fragmentation to the roots of *Allium cepa* L. (onion) despite its ethnomedical utilization (ALABI *et al*., 2022).

Cellular aberration assessment

The results of cellular aberration assessment are presented in Table 4. Excluding the positive and negative controls, the highest total number of cell aberrations was observed in the cells treated with 100% aqueous extract concentration totaling 79 aberrant cells which is higher compared to the aberrant cells detected in 50% aqueous extract treatment.

Table 4. Cellular aberrations detected 50% and 100% aqueous extract treatment with water as the negative control and hydrogen peroxide as the positive control, representing percent aberrations

Treatment	Cell counted	Multinucleate cell	Membrane damage	Micronuclei	Stickiness	Huge-nucleated cell
50% aqueous extract	752±32.53	0	3	1	0	7
100% aqueous extract	705±22.63	6	2	4	4	18
- control (water)	828.50±53.03	0	0	0	0	0
+ control (H_2O_2)	472.50±28.99	13	3	3	7	9

Nuclear lesion	Giant cell	Plasmolyzed cell	Ghost cell	Lobulated cell	Total	Percent aberration (%)	T-test
0	9	0	17	0	37	4.92	0.282217914
2	5	9	27	2	79	11.21	0.486937117
0	0	0	0	0	0	0	
0	8	0	111	1	157	33.02	

Results correspond to 4.92% and 11.21% percent aberrations caused by 50% and 100% aqueous leaf extracts, respectively. Through two-tailed t-test, significant difference between the positive control, H_2O_2, and the treatments were determined with 0.282217914 for 50% aqueous leaf extract treatment and 0.486937117 for 100% aqueous leaf extract treatment ($p>0.05$). Since the results showed no statistically significant difference, these possibly suggest that cytological aberrations caused by the two treatments are comparable to the effects of the positive control, H_2O_2. These aberrations include, multinucleated cell, membrane damage, micronuclei, stickiness, huge-nucleated cell, nuclear lesion, giant cell, plasmolyzed cell, ghost cell, and lobulated cells (Figure 8) which were predominantly observed in the roots grown with 100% *C. ramiflora* aqueous extract.

The obtained results suggest negative allelopathic effects to the seed germination of *B. rapa* implying a possible active production of different allelochemicals involved in development suppression. Similar to the allelopathic activities of invasive plant species such as *Eucalyptus* sp. which was found to suppress seed germination of *B. rapa* affecting root elongation, stem elongation and fresh weight elongation (DENG *et al*., 2024), *C. ramiflora* exhibited the same effects. Another study written by IRFAN *et al*. (2022) about the effects of *Raphanus raphanistrum* (wild radish) to *B. rapa* which exhibited the same effects using its aqueous extract in assessing shoot development, root length, fresh biomass and dry biomass suggesting decreased development which were clearly cause by the produced allelochemicals. General utilization of aqueous extracts in allelopathic effects studies were attributed to change and pH and osmotic potential which is associated with the production of large amount of phenolics especially when extracted from the leaves (SISODIA & SIDDIQUI, 2010). GULZAR & SIDDIQUI (2017) utilized an invasive plant, *Calotropis procera* to study allelopathic effect that led to negative effects in growth and antioxidant activity of *B. oleracea* observing less peroxidase activity.

According to HUSSAIN *et al*. (2021), wide range of allelochemicals such as benzoic acid, vanillic acid, p-hydroxybenzoic acid, ferulic acid, chlorogenic acid, p-coumaric acid, m-coumaric acid, gallic acid, caffeic acid, p-hydroxybenzaldehyde, sorgoleone, dhurrin, and protocatechuic acid can be produced and has been reported to help in weed control making it a good alternative to the utilization of agrochemicals. Allelochemicals produced by plant is another mechanism for its survival which targets the few cellular activities such as fatty acid

biosynthesis, fatty acid elongation, fatty acid degradation, cutin biosynthesis, suberine and wax and linoleic acid metabolism that eventually cause changes is seed germination plant growth, antioxidant enzyme activity and chlorophyll content of plant (SHI *et al*., 2024).

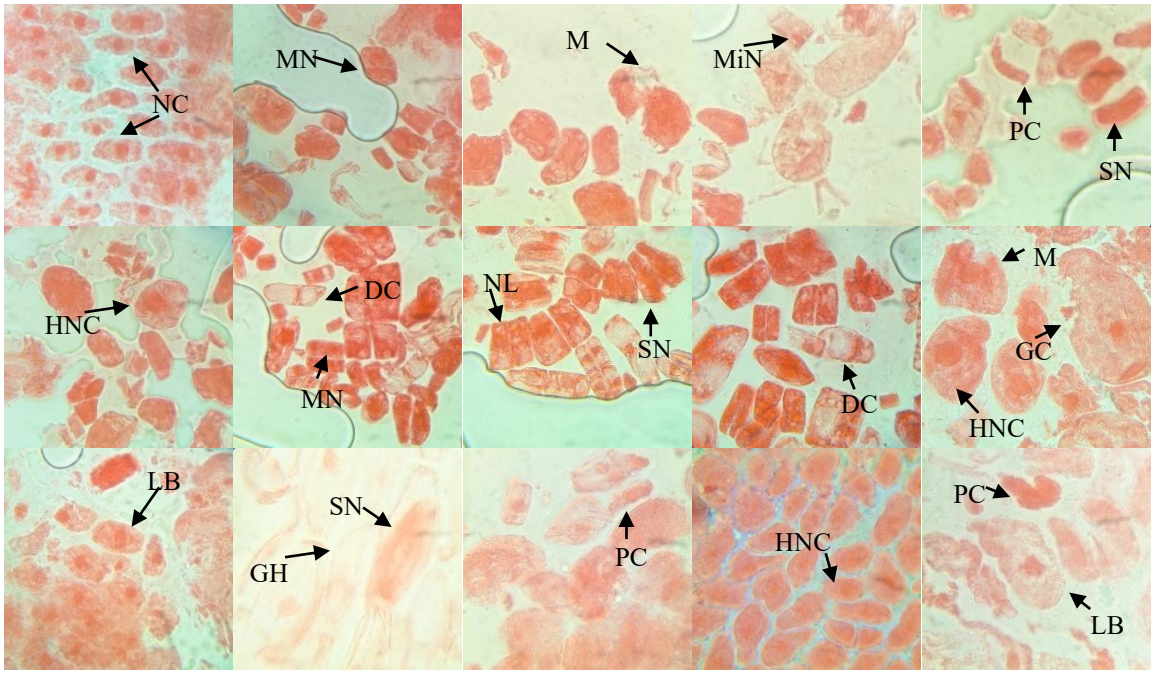

Figure 8. Cytological aberrations observed in the roots treated with 50% and 100% *C. ramiflora* aqueous leaf extracts showing the normal cell (NC), multinucleated cell (MN), membrane damage (MD), micronuclei (MiN), stickiness (SN), huge-nucleated cell (HNC), dead cell (DC), nuclear lesion (NL), giant cell (GC), plasmolyzed cell (PC), ghost cell (GH) and lobulated cell (LC) (indicated by arrows) under 100x magnification

Native plant species have also been reported to be capable of producing allelochemicals that can also inhibit seed germination of invasive species (YUAN *et al*., 2021) possibly resisting invasion. For the case of *C. ramiflora,* studies reported that it showed strong antioxidant and anti-lipid peroxidation activities (SABIHA *et al*., 2022) that will inhibit ROS accumulation inducing competitiveness when grown. When observing response to allelochemicals, ROS levels accumulation led to disturbance in redox homeostasis monitored through root growth inhibition and cell death stimulation due to the arrest in cell division and microtubule disruption that can result to severe abnormalities in the morphology of a plant. When severely affected, plants also exhibit less expression of photosynthetic pigment and destructive activity towards chlorophylls (STASZEK *et al*., 2021).

In most studies regarding the effects of leaf aqueous extract in allelopathy, chromosomal aberrations are usually observed, and anomalies are higher compared to the untreated ones (PARVEEN *et al*., 2023). Toxic effects by allelochemicals also induce reactive oxygen species (ROS) leading to oxidative stress due to lipid peroxidation and protein modifications (ŠOLN *et al*., 2022) inducing chromosomal abnormalities affecting cell division (HEIVACHI *et al.,* 2023) consequently leading to more abnormalities in the cells. With *Allium cepa* as the usual test organism, chromosomal and cellular anomalies are observed such as disrupted polarity, elongated cells, sickle-shaped cells, stickiness, chromosomal displacement, chained chromosomes, anucleated cells, ghost cells, malformed nuclei, multinucleated cells, and chromosome bridges may be observed pinpointing the phytotoxic effect that leads to growth retardation and cell death (KHAN *et al*., 2024). Furthermore, cellular changes because of phytochemicals such as quercetin can demonstrate mutagenic and genotoxic effects (AKPAN *et al*., 2017).

CONCLUSION

In this study, the aqueous leaf extract of *C. ramiflora,* a native plant species in the country, affected the seed germination and radicle length of *B. rapa* seeds with a concentration-dependent trend. Higher concentration significantly influenced proper chromosome condensation and cellular structure of *B. rapa*. Moreover, the extract was found to have potential phytotoxic effects interrupting normal cell cycle. Therefore, *C. ramiflora* aqueous extract showed negative effects towards *B. rapa* indicating possible allelopathic potential with relevance to biocontrol for weed management under controlled conditions.

This study recommends confirming the efficacy and non-target effects of *C. ramiflora,* the utilization of other extraction methods such as ethanolic and methanolic extraction to obtain more phytochemicals, and elucidate the compounds present in the leaves. Further studies should also be conducted adding more parameters considering seedlings fresh and dry matter, vigor test in the seedling experimentation and focus more on the cellular aspect involving the utilization of more concentrations of *C. ramiflora* leaf extract and its effect to the existing invasive plant species as a biocontrol agent under field conditions.

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