

Suppression of the RAGE gene expression in RAW 264.7 murine leukemia cell line by ethyl acetate extract of *Mikania micrantha* (L.) Kunth.

Alex Zohmachhuana¹, Malsawmdawngliana Tlaisun², Vabeiryureilai Mathipi^{3*}, Lalrinzuali Khawlhing³,
Joyce Sudandara Priya⁴

¹Department of Botany, Mizoram University, Aizawl, Mizoram, India.

²Department of HAMP, Mizoram University, Aizawl, Mizoram, India.

³Department of Zoology, Mizoram University, Aizawl, Mizoram, India.

⁴Department of Botany, Madras Christian College, Chennai, Tamil Nadu, India.

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ABSTRACT

The aim of this study is to evaluate the *in vitro* anti-inflammatory effect of *Mikania micrantha* (L.) Kunth. leaf extract on RAW 264.7 murine leukemia cell line. The qualitative phytochemical analysis of the different extracts of the leaves of *M. micrantha* revealed the presence of carbohydrates, flavonoids, quinones, terpenoids, phenols, and coumarins, whereas the quantification revealed that the methanol extract contained the highest phenol content (259.88 mg catechol equivalent/g dried sample) as well as flavonoid content (156.55 mg quercetin equivalent/g of dried sample). The different extracts were tested for antioxidant activity using a DPPH scavenging assay. The antioxidant capacity of ethyl acetate extract at 100 µg concentration showed the highest DPPH scavenging ability with an IC₅₀ value of 40.34 µg/ml in comparison with the standard (39.92 µg/ml). *Allium cepa* assay and MTT assay were performed to assess the cytotoxicity effects. The fresh leaf extract increased the incidence of anomalous mitosis. Cytotoxicity study showed that ethyl acetate extracts exhibit the highest cytotoxicity with an IC₅₀ value of 47.68 µg/ml. Reverse-transcription polymerase chain reaction analysis exhibited the suppression of the RAGE gene. This is the first report on the effect of the anti-inflammatory activity of *M. micrantha* leaf extract on RAW 264.7 murine leukemia cell line. This study concluded that *M. micrantha* possesses antioxidant property and limiting RAGE gene expression suggests anti-inflammatory properties.

1. INTRODUCTION

One of the major traits of cancers in human is unresolved inflammation, which often occurs before the onset of the disease and orchestrates a favorable microenvironment for tumor growth. Over the years, several population-based epidemiological and experimental animal model systems and clinical reports emphasized a major rise in the prevalence of the disease leading up to 70% of cancer deaths globally [1]. Recent studies revealed that the mortality rate of cancer worldwide is increasing every year [2]. Medicinal plants have taken a crucial role in life and have been used by mankind since time immemorial for primary healthcare. They are still vital for treating several disorders, and numerous systems of medicine are especially dedicated to the application of medicinal plants for health care [3]. It is assumed that herbal-based products are nontoxic, safe, and even more reliable than the synthetic compounds used for health care [4]. At present, over 60% of anticancer agents are collected from their natural sources such as

plants, microorganisms, and marine organisms [5]. The World Health Organization projected that majority of the population rely on herbal preparations [6].

The production of reactive oxygen species is associated with the activation of RAGE in a variety of cells. RAGE gene has a large extracellular part, a transmembrane domain with 43 amino acid long cytoplasmic tail [7], having a cell surface receptor and is a part of the immunoglobulin superfamily, involved in the pathogenesis of cancer advancement and metastasis [8]. Cumulative evidence supports the use of RAGE level as clinical biomarkers in different types of cancers, such as breast, lung, colorectal, and prostate cancer [9]. The expression of the RAGE gene increases at the site of inflammation, mainly on inflammatory, endothelial, and epithelial cells, and elevates the expression of both cell surfaces [10]. Subsequently, the inflammatory cytokines expression increases, resulting in an inflammatory response with associated cellular migration and proliferation [11].

Mikania micrantha (L.) Kunth. is an exotic, neotropical perennial herbaceous vine belonging to the family Asteraceae. *M. micrantha* is also known as Mile-a-minute vine. It grows well in humid and fertile soil, though it can survive in less fertile soils. It is widely

*Corresponding Author:

Vabeiryureilai Mathipi, Department of Zoology, Mizoram University, Aizawl, Mizoram. E-mail: m.vabeiryureilai@gmail.com

distributed in the Pacific, Southeast Asia, and so on. In India, it is found in West Bengal, Orissa, Andaman, Nicobar Islands, and some parts of North East India [12]. *M. micrantha* is a plant that has been used for skin diseases, wound dressings, chickenpox, and so on and as folk medicine in Africa, Jamaica, and Guyana Patanoma [13]. In India, the plant is commonly used by various indigenous communities as medication for several ailments such as itches, body sprain, gout, snake bites, dysentery, diabetes gout, rheumatism, and cancer [14].

Ethnic people of Mizoram apply the juice of *M. micrantha* to cuts and wounds for clotting the blood as first aid [15]. The leaves decoction is given to the patients suffering from dysentery, as hemostatic [16]. As ethnoveterinary medicine in India, *M. micrantha* is used for treating diarrhea of veterinary animals and repelling body lice of poultry birds [17].

The present work aims to carry out a preliminary study on the phytochemicals, antioxidant, antiproliferative, and regulation of the RAGE gene of the crude leaf extracts of *M. micrantha*.

2. MATERIALS AND METHODS

2.1. Collection, Identification, Authentication, and Preparation of Sample

M. micrantha (L.) Kunth. sample was collected from Aizawl, Mizoram, during the month of November 2016 and identified by Dr. Sherry, Department of Plant Biology and Plant Biotechnology, Women's Christian College, Chennai, Tamil Nadu, and the herbarium was deposited in the Institutional Herbarium, Mizoram University, Aizawl, with voucher 00345. The leaf samples were washed with clean water, air-dried, and made into uniformly fine powder. The powdered sample was subjected to sequential extraction using three solvents based on increasing polarity such as hexane, ethyl acetate, and methanol for 72 h. The extracts were filtered and dried using a rotary evaporator. The extracts obtained were stored at -20°C until further used.

2.2. Qualitative Phytochemical Tests

The preliminary phytochemical analysis of the different extracts of *M. micrantha* was performed in standard procedures [18].

2.3. Quantitative Phytochemical Analysis

Phenols and flavonoids are the most important groups of secondary products and bioactive compounds in plants [19]. Phenols and flavonoids possess various biological activities, cardioprotective [20], antidiabetic [21], antioxidant [22], cytotoxic, and antitumor [23], in addition to the ability to modify gene expressions [24].

The selective quantitative analysis was carried out for phenol [25]. Different concentrations (100, 200, and 300 μg) of the samples were tested, and a calibration curve for catechol was obtained. The results were compared to a catechol calibration curve, and the total phenol content of the sample was expressed as mg of catechol equivalents per gram of extract.

For flavonoid content [26], the results were expressed as quercetin equivalents (mg quercetin/g dried extract):

Amount TFC = Sample OD/Standard OD \times Respective Amount of extract

2.4. Induction of Chromosomal Aberration

2.4.1. Method of treatment

Commercially available onions were used as the test material in the present study. The cytotoxicity and genotoxicity potential of the methanol extract of *M. micrantha* against *Allium cepa* was carried out according to Levan [27]. The roots of onions were treated with the plant infusion for three exposures of 1, 6, and 24 h duration, and the concentration was 100 ppm. The treated roots were fixed in freshly prepared acetic acid: ethanol (1:3).

2.4.2. Slide preparation

Each treated root sample was washed in distilled water and then hydrolyzed for 15 min at RT in 1 N HCl. About 4% ferric ammonium sulfate was prepared, and the roots were mordanted for 15–10 min. The roots were stained for 10–12 min using 0.5% hematoxylin after washing with distilled water. The roots were finally squashed on a slide using a drop of 45% acetic acid. DPX was used for sealing the slide.

2.4.3. Scoring

Around 2000 nuclei were recorded from the treated as well as the control samples for calculating the mitotic indices [28].

The incidence of the chromosomal aberrations present in the treated and normal root tips was calculated and classified according to Buckton and Evans [29]. Six root tips were collected from each bulb and used for the experimental point. The mitotic irregularities such as metaphasic clump, anaphasic bridge, prophasic clump, and lagging chromosome were considered.

2.5. DPPH Free Radical Scavenging Assay

The antioxidant activity of the leaf extracts of *M. micrantha* was carried out according to the method described by Shimamura *et al.* [30] with slight modifications. The antioxidant capacity was presented as a percentage scavenging effect and calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Acontrol OD} - \text{Atest OD}}{\text{Acontrol OD}} \times 100$$

Inhibitory concentration (IC_{50}) was calculated using a free online version of GraphPad Prism.

2.6. Cytotoxicity

The cytotoxicity was assessed according to Mosmann [31]. RAW 264.7 leukemia cell lines (NCCS, Pune) were cultured in DMEM medium added with 10% FBS at 37°C with 5% CO_2 . The cells were allowed to attach overnight at 37°C after plating in 96-well plates at a density of 1.2×10^4 cells/well. Different concentrations (25, 50, 75, 100, and 150 μg) of the samples were taken. Then, the medium was replaced, and the cells were incubated.

The media were discarded after 24 h of incubating. A 100 μl fresh medium and 10 μl of MTT (5 mg/ml) were added. The media were then discarded after 3–4 h, and 150 μl of DMSO was added for dissolving the formazan crystals. The absorbance was read at 570 nm in a microplate reader.

The positive control used was cyclophosphamide. Cell survival was calculated by the following formula:

Viability (%) = Test OD

Control OD

$$\text{Viability (\%)} = \frac{\text{Test OD}}{\text{Control OD}} \times 100$$

$$\text{Cytotoxicity (\%)} = 100 - \text{Viability (\%)}$$

2.7. Reverse Transcription Polymerase Chain Reaction (PCR) Analysis

Approximately 1×10^6 cells/ml were seeded into 6-well plates, allowed to adhere, and the ethyl acetate extract was added followed by 24 h incubation.

Tri-RNA reagent (Favorgen Biotech Corp, Taiwan) was used for extracting total RNA from the cells followed by the treatment of the RNA with deoxyribonuclease I (DNase I; Promega, USA). DNase I-treated RNA was reverse transcribed using EasyScript Plus™ Reverse Transcriptase. cDNA was amplified by PCR using these RAGE gene-specific pair primers.

Primer details are as follows:

RAGE F: 5'-GTGGGGACATGTGTGTCAGAGGGAA-3'

RAGE R: 5'-TGAGGAGAGGGCTGGGCAGGGACT-3'

β -Actin F: 5'-ACGGGTCACCACACTGTGC-3'

β -Actin R: 5'-CTAGAAGCATTTCGCGTGGACGATG-3'

Full-length cDNA amplification using Eppendorf Personnel Mastercycler, Germany, for RAGE gene was 35 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 7 min, and for β -actin gene was 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 7 min.

The amplified gene was separated on a 2% agarose gel, stained with ethidium bromide, and visualized with a gel documentation system (SynGene, UK).

2.8. Statistical Analysis

The data were presented as mean \pm SEM. Statistical analysis was performed using Excel 2010, and differences were determined by one-way ANOVA. Differences were considered statistically significant at $P < 0.01$.

Table 1: Phytochemical analysis of leaf extracts of *Mikania micrantha* *in vitro*.

Test	Extract		
	Hexane	Ethyl acetate	Methanol
Carbohydrate	++	+++	+++
Tannins	–	–	+
Flavonoids	+	+	++
Alkaloids	–	+	++
Quinones	+	++	++
Glycosides	–	+	+
Cardiac glycosides	–	+	+
Terpenoid	+	+++	+++
Phenols	+	+++	+++
Coumarins	+	+	+
Steroids	–	++	++
Phylobatannins	–	–	–
Anthraquinone	–	–	–

+++ : Strongly present, ++: Moderately present, +: Present in trace amount, –: Absent.

3. RESULTS

The qualitative phytochemical analysis showed the presence of carbohydrates, flavonoids, quinones, terpenoids, phenols, and coumarins [Table 1].

The quantitative estimation for phenols in hexane, ethyl acetate, and methanol extracts was 259.88 mg, 87.67 mg, and 104.70 mg catechol equivalent/g of dried sample [Table 2a], whereas the flavonoid content was 156.55 mg, ethyl acetate 106.73 mg, and hexane 87.35 mg quercetin equivalent/g of dried sample, respectively [Table 2b].

The antioxidant capacity of the different extracts showed an increase in concentration manner. The highest activity was observed at 100 μ g/ml. The IC₅₀ value for DPPH assay for leaf extracts of hexane, ethyl acetate, and methanol was 71.23 μ g/ml, 40.34 μ g/ml, and 52.45 μ g/ml, respectively, with respect to the positive control (ascorbic acid) with IC₅₀ value of 39.92 μ g/ml [Table 3]. The above results show a profound antioxidant activity in the ethyl acetate extract of *M. micrantha*.

The cytotoxicity of *M. micrantha* extract against RAW 264.7 murine leukemia cell lines showed increased toxicity with an increase in concentration, and its IC₅₀ was hexane (226.5 μ g/ml), ethyl acetate (47.68 μ g/ml), and methanol (212.3 μ g/ml) [Table 4 and Figure 1].

The mitotic indices of root meristem treated with leaves extract showed values of 4.12%, 4.7%, and 1.41% in 1 h, 6 h, and 24 h, respectively. The growing root tips exposed to the leaf extracts for 24 h lost their turgidity. The treated cells showed a considerable reduction in the frequency of division when compared to control (9.01%). The mitotic indices in the root tip cells treated for 1 h and 6 h brought about a similar effect [Table 5 and Figures 2a and c].

Infusion of leaves extracts of *M. micrantha* induced chromosomal damage and mitotic anomalies at various periods of exposure in onion root. Observations on the frequencies of aberration are presented in Table 5 and Figure 2b, where the leaves extract of *M. micrantha* increased the incidence of anomalous mitosis during all three durations of exposure. Toxicity of leaves extract on the induction of mitotic anomalies on the root meristems of onion was duration dependent. Cells treated for 24 h (55%) showed a significantly high increase in mitotic anomalies. An increase in chromosomal anomalies during 1 h (31.1%) exposure showed that the leaves extracts of *M. micrantha* had an immediate toxic effect on the root meristem. The types of chromosomal aberrations observed in the root meristems with leaves extracts were prophaseic clump, metaphasic clump, anaphasic bridge, anaphasic lag, and telophasic clump [Table 5 and Figure 2d]. Prophaseic clumps, metaphasic clump, and anaphasic bridge were found to be common during all three durations. They were significantly higher in cells exposed to leaves extracts for 1 h and 6 h. The percentage of anaphasic lag and telophasic clump was found to be of rare occurrence during the three durations [Table 5 and Figure 2d].

The IC₅₀ concentration of the ethyl acetate extracts of *M. micrantha* was treated, and the RAGE gene expression was determined. The treated showed suppression of RAGE gene compared with untreated control about 2-fold [Figure 3].

4. DISCUSSION

Phytochemicals are present naturally in plants and are biologically active chemical compounds. They provide multiple benefits for human health which macronutrients and micronutrients do not possess. They are involved in the inhibition of cancer cell

Table 2: Quantification of secondary metabolites content of the leaf extract of *Mikania micrantha*.

a. Total phenol content							
Concentration (µg)	Sample (OD) hexane	Sample (OD) ethyl acetate	Sample (OD) methanol	Catechol (OD)	Amount of phenol hexane (mg equiv/g)	Amount of phenol ethyl acetate (mg equiv/g)	Amount of phenol methanol (mg equiv/g)
100	0.107	0.206	0.328	0.457	23.551	45.090	71.747
200	0.298	0.409	0.697	0.891	66.988	91.831	156.552
300	0.485	0.592	0.863	1.665	87.355	106.736	155.475
b. Total flavonoid content							
Concentration (µg)	Sample (OD) hexane	Sample (OD) ethyl acetate	Sample (OD) methanol	Quercetin (OD)	Amount of flavonoid hexane (mg equiv/g)	Amount of flavonoid ethyl acetate (mg equiv/g)	Amount of flavonoid methanol (mg equiv/g)
100	0.097	0.214	0.38	0.514	18.860	41.609	73.886
200	0.153	0.261	0.669	0.796	38.413	65.528	167.963
300	0.332	0.278	0.824	0.951	104.709	87.678	259.882

Table 3: Antioxidant analysis of the leaf extracts of *Mikania micrantha* (DPPH radical scavenging assay).

Sample	Conc. (µg)	% inhibition	IC ₅₀ value
Ascorbic acid	25	36.95	39.92
	50	63.12	
	100	84.53	
Hexane	25	38.69	71.23
	50	44.24	
	100	57.39	
Ethyl acetate	25	44.57	40.34
	50	53.47	
	100	70.78	
Methanol	25	45.26	52.45
	50	48.23	
	100	60.17	

Table 4: Cytotoxicity analysis of leaf extracts of *Mikania micrantha* on RAW 264.7 murine leukemia cell lines.

Sample	Conc. (in µg)	% viability	% toxicity	IC ₅₀ value
Cyclophosphamide	5	7.47	92.53	90
Hexane	25	73.14	26.86	226.5
	50	68.22	31.78	
	100	62.04	37.96	
	125	59.27	40.73	
	150	51.13	48.87	
Ethyl acetate	25	57.02	42.98	47.68
	50	50.18	49.82	
	100	44.04	55.96	
	125	36.83	63.17	
	150	32.80	67.20	
Methanol	25	71.87	28.13	212.3
	50	68.49	31.51	
	100	64.03	35.97	
	125	58.37	41.63	
	150	48.57	51.43	

proliferation and protection from oxidative stress that can cause aging, cancer, cardiovascular disease, neurodegenerative diseases, metabolic disorders, and so on and can also inhibit and induce enzymes and gene expression [32,33]. The present phytochemical results are akin to the findings of Dev *et al.*, Lalrinzuali *et al.*, and Borkataky *et al.* [34-36] who reported the presence of different phytochemicals in the plant powder as well as different extracts of the *M. micrantha* leaves. *A. cepa* test performed in this study is used as an indicator of adequate cell proliferation by calculating the mitotic index and replication index [37]. Several other plants such as *Calotropis procera*, *Achyrocline satureioides*, *Paxia myriantha*, and *Paxia leiocarpa* have been demonstrated to inhibit cell division of *A. cepa* [38,39]. The present study of the crude leaves extract-treated cells revealed that the plant extract poses toxic effects on the dividing cells by reducing the mitotic index during 1 h exposure, and this observation is in agreement with the reports made on *Mikania cordifolia* infusions on the cell cycle of *A. cepa*. Results indicated that two populations of *M. cordifolia* exhibited a reduction of the mitotic index in all the treatments when compared with the negative control. In both populations, a decrease in the percentage of the mitotic index with increasing concentration of the infusions was observed [40]. The results of the present study demonstrate that root tip cells treated with fresh leaves extract of *M. micrantha* brought about cell death after 24 h of treatment. The genotoxicity

Table 5: Induction of chromosomal anomalies and mitotic indices in the root meristems of *Allium cepa* by fresh leaf extract of *Mikania micrantha*.

Treatment time (hra)	Cells count (N)	Dividing cells (n)	Mitotic index (n/N×100±SE)	Anomalies (r)	Mitotic anomalies observed					% anomalies r/n×100
					PC	MC	AB	AL	TC	
Control	2235.00±6.48	217.16±5.84	9.44±0.69	1.00±0.05	-	-	-	-	-	01.20±0.04
1	2143.33±4.45	88.16±2.51	4.13±0.54 [@]	25.83±1.98	7	15	6	-	-	29.51±2.99 [#]
6	2042.50±7.13	89.66±1.75 [*]	4.80±1.01 [@]	30.83±1.59	14	7	5	3	3	33.78±2.51 [#]
24	2035.66±4.95	26.66±2.15 [*]	1.30±0.42 [#]	13.00±1.31	5	4	1	2	4	55.29±2.57 [#]

PC: Prophasic clump, MC: Metaphasic clump, AB: Anaphasic bridge, AL: Anaphasic lag, TC: Telophasic clump. The results are presented as mean±SE, n=5. The *t*-test was performed to test the significant level between the control and treatment groups at a significant level of *<0.05, @<0.01, and #<0.001.

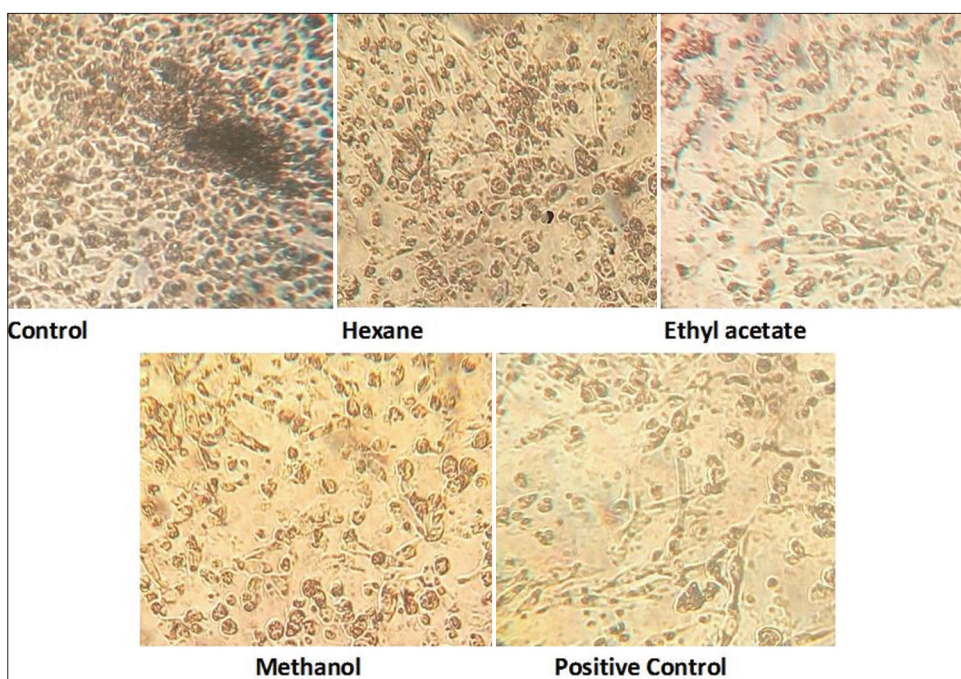


Figure 1: The cytotoxicity of different leaf extracts of *M. micrantha* against RAW 264.7 leukemia cell line.

study of *M. micrantha* extracts using root meristems of onion showed that the extract is toxic to the genetic material by inducing non-clastogenic chromosomal aberrations such as prophasic clump, metaphasic clump, anaphasic bridges, anaphasic lag, and telophasic clump. Prophasic clump, metaphasic clump, and anaphasic bridge were of common occurrence. Chromosome stickiness plate may be due to the action of the extract on the protein leading to the partial dissolution of nucleoprotein, which forms an integral part of the chromosome [41]. Spanish Jasmine extracts also induced mitotic anomalies such as sticky chromosome and C-mitosis on *Allium* root meristem [42].

In this study, the ethyl acetate and methanol extracts of *M. micrantha* leaves when tested for antioxidant activities using the *in vitro* system showed significant DPPH scavenging ability and antioxidant potential with an IC₅₀ value of 40.34 µg/ml and 52.45 µg/ml, respectively, when compared to the positive control 39.92 µg/ml. The different extracts as well as whole plants of *M. micrantha* have been shown to possess antioxidant activity in the previous reports [43]. The phenolic compounds were prominently found in this extract and could be attributed to the observed antiradical properties. The compounds isolaricresinol, caffeic acid, ethyl protocatechuate, and protocatechuic aldehyde isolated from *M. micrantha* plant have been shown to have higher DPPH scavenging activity than L-ascorbic acid,

which is a standard reference compound in an earlier study [44]. The present study on anticancer activity of hexane, ethyl acetate, and methanol leaf extracts of *M. micrantha* on Raw 264.7 murine leukemia cell lines suggests that the ethyl acetate extract of the leaves of *M. micrantha* possesses prominently high cytotoxic activity as the IC₅₀ value achieved was 59.14 µg/ml. The previous studies have shown the anticancer activity of aqueous extract of *M. micrantha* leaves against K562 and HeLa human cancer cell lines [45]. Different extracts of other species of *Mikania* have also been reported to show cytotoxic activities against HepG-2 and HeLa cell lines [46]. The ethanolic extracts of *M. cordata* leaves have also shown cytotoxic activity using Brine Shrimp Lethality Assay [47]. The essential oils extracted from *M. micrantha* plant, which contain mostly terpenoids, terpenes, and sesquiterpenes, have been shown to possess anticancer activity against the ovarian, pancreatic, and cervical cancer cell lines [48,49]. The ethanol extract of *Fimbristylis ovata* (Burm.f.) has also been shown to have significant inhibition of RAGE gene expression in human lung adenocarcinoma epithelial cell line [50].

The results from the study showed that the polarity of the solvent used is responsible for the variations in the flavonoid and phenol content of the different extracts. In addition, there was a correlation between the antioxidant capacity and the flavonoid and phenol content. The study revealed the downregulation of

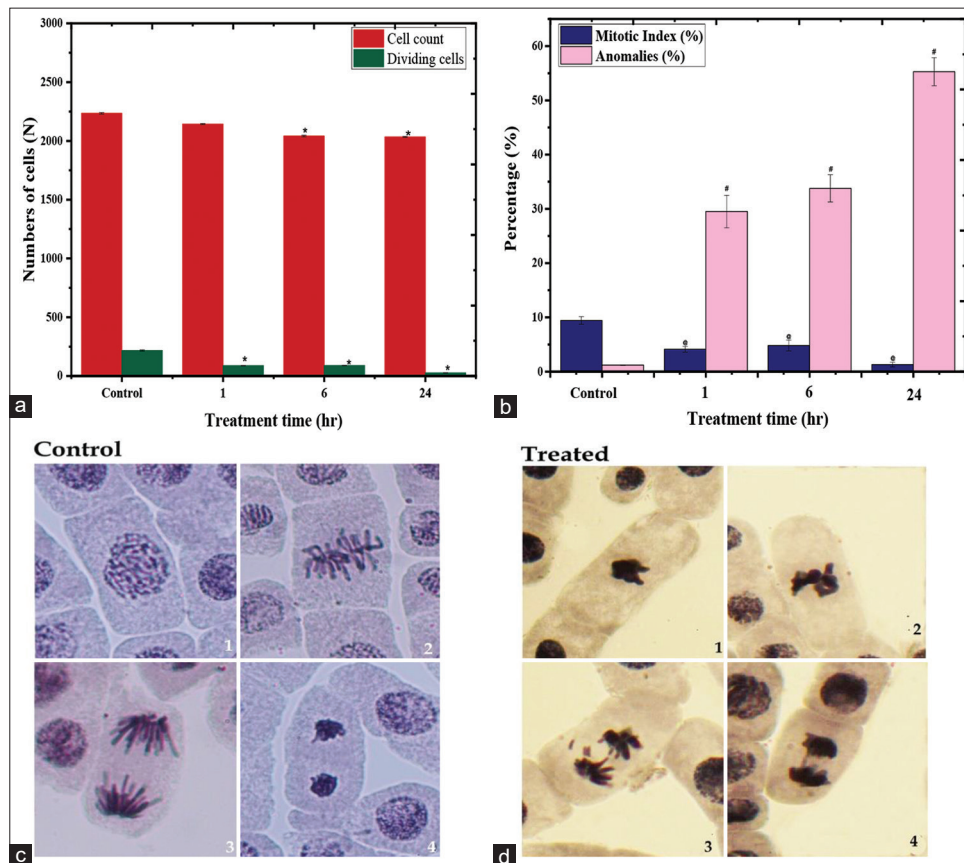


Figure 2: Induction of chromosomal anomalies and mitotic indices in the root meristems of *Allium cepa* by leaf extract of *Mikania micrantha*. (a) The total number of cell counts. (b) The total number of dividing cells. (c). Control: 1: Prophase, 2: Metaphase, 3: Anaphase, and 4: Telophase. (d) Treated: 1: Prophasic clump, 2: Metaphasic clump, 3: Anaphasic lag, and 4: Anaphasic bridge. The results are presented as mean \pm SE, $n=5$. The t -test was performed to test the significant level between the control and treatment groups at a significant level of * <0.05 , @ <0.01 , and # <0.001 .

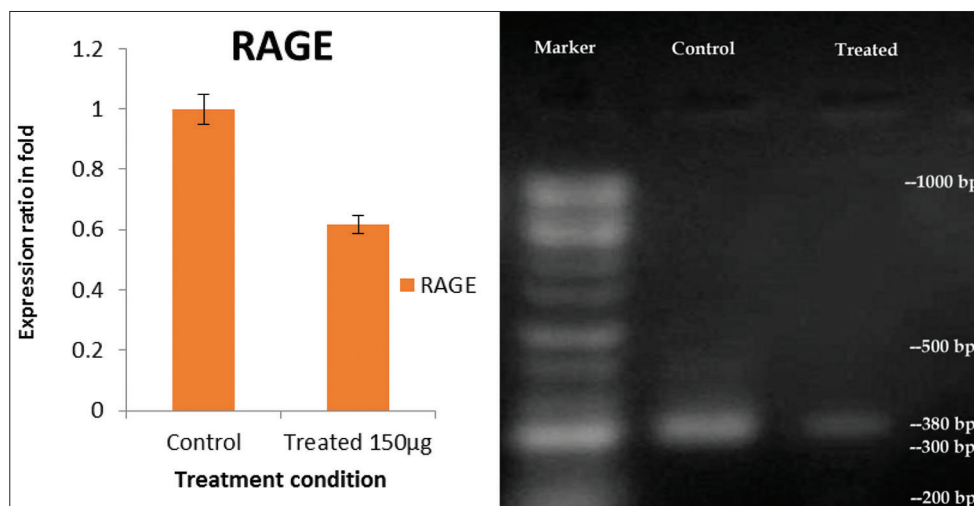


Figure 3: The effect of ethyl acetate leaf extracts of *Mikania micrantha* on RAGE gene expression. Expression ratio analyzed by ImageJ software. Lane 1, 100 bp marker; lane 2, untreated/control; and lane 3, treated, 47.68 μ g/ml. The result presented as mean \pm SE, $n=5$.

RAGE gene expression by *M. micrantha* leaf extracts. RAGE gene activation results in the transduction of the cell surface signals to various inflammatory pathways, including PI3K-Akt, MAPKs,

and NF- κ B [51]. Further studies can be done to investigate the inhibition of RAGE gene and the intracellular pathways involved in the regulation.

5. CONCLUSION

This study revealed that the crude extract of *M. micrantha* leaves is rich in phenol and flavonoid. The ethyl acetate leaf extract exhibited significant *in vitro* antioxidant and cytotoxic activities compared to hexane and methanol leaf extracts. The ethyl acetate extract suppresses the expression of the RAGE gene in the RAW 264.7 murine leukemia cell line. Our result suggested that the antioxidant property of *M. micrantha* could be involved in its inflammation-related inhibitory action through the RAGE gene signaling pathway. This is the first report on the suppression of the RAGE gene expression of *M. micrantha*.

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7. CONFLICTS OF INTEREST

Authors declared that they do not have any conflicts of interest.

8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

11. PUBLISHER'S NOTE

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