

Effects of Thai medicinal plant, *Vernonia cinerea* Less. extract on catecholamine, oxidative stress and chromosome aberration in nicotine-treated rats

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Abstract

Background: *Vernonia cinerea* Less. (VC) is a Thai natural plant that can help stop smoking, as claimed previously by smoking clinics in Thailand, but there is lacked of scientific supporting its mechanism and toxicity.

Objectives: The aims of this study were to evaluate total phenolic content, total antioxidant capacity (TAC) and radical scavenging activity of separated extracts from stem, flower and leaf of VC *in vitro*, including levels of catecholamines (dopamine, adrenaline, and noradrenaline), oxidative stress, and acute toxicity in the chromosomes of nicotine-treated rats.

Materials and methods: *Vernonia cinerea* (VC) Less. was extracted and total phenolic was determined by standard methods. Antioxidant capacity and radical including, nitric oxide, superoxide, and hydroxyl were studied. Moreover, catecholamine oxidative stress, malondialdehyde were focused. Effects of extract on animal were explored compared to distilled water and chromosome aberration was determined.

Results: The results showed that leaf extract had the highest total phenolics and TAC. Stem extract showed higher activity than leaf and flower extracts in scavenging nitric oxide (NO) and superoxide radical ($O_2^{\cdot-}$). However, flower extract presented the highest activity on scavenging hydroxyl radical ($OH^{\cdot}$). After 20-day treated. statistical differences in levels of dopamine, noradrenaline, and adrenaline was demonstrated in nicotine-treated group. Co-treatment with bupropion showed a significant reduction in dopamine in all extract. Co-treatment with leaf extract showed significantly higher activity in increasing noradrenaline and adrenaline as well as bupropion, with reduced malondialdehyde (MDA) and increased TAC levels compared to flower or stem extract. In addition, there was no acute toxicity in any chromosomes from all directed extract treatments.

Conclusion: This result suggested that VC extracts possibly involved the catecholamine neurotransmitter in nicotine-treated rats, and may relate to antioxidant activity by radical scavenging *in vitro*, including non-acute toxicity in chromosomes from short-term direct treatment.

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Keywords: *Vernonia cinerea*, antioxidant, nicotine, oxidative stress, chromosome aberration

Introduction

Smoking is still a major problem worldwide because cigarette smoke contains an abundance of free radicals and pro-oxidant species and is known to cause serious health problems,^{1,2} such as coronary heart disease, chronic obstructive pulmonary disease (COPD), and cancers.^{3,4} Thousands of chemicals in tobacco smoke and over 4,000 chemical substances in cigarettes are toxic and carcinogenic and have as many free radicals as the toxicity from nicotine.⁵ In addition, basic knowledge of free radicals strongly claims that tobacco smoke has powerful risk factors of degeneration, mutation, aging, and several serious diseases.⁶ Previous study has shown that nicotine from cigarette smoke likely contributes to catecholamine release,⁷ especially on dopamine, a precursor of both adrenaline and noradrenaline secretion from adrenal medullar levels.⁸ Dopamine is an important neurotransmitter that affects to heart rate and blood pressure, as well as behavior and cognition, sexual performance, mood, learning capacity, sleep, memory, motivation, lactation, and involuntary movement.^{7,9} Some evidence has reported that nicotine can enhance motivation during prolonged exercise,¹⁰ and improve exercise endurance, but it also induces oxidative stress and chromosome aberration.¹¹ Interestingly, previous evidence shows that adverse effects of nicotine induce inflammatory status and oxidative stress via activated NF- κ B and apoptosis,¹² while decreasing glutathione (GSH) and increasing malondialdehyde (MDA).¹³ Therefore, these findings are still controversial and confirmation of toxicity or side effects from nicotine administered oxidative stress would possibly support the adverse effects of cigarette smoking on protein and lipid oxidation and cause of DNA damage.¹⁴⁻¹⁶

Many countries including Thailand launch campaigns to reduce smoking by introducing various rules, such as behavioral counseling and/or pharmacotherapy, with a successful long-term abstinence rate of approximately 30%,¹⁷⁻¹⁹ In case of heavy smokers, nicotine replacement therapy (NRT) is the best medicinal choice in smoking cessation protocol, and increases the chances of smoking cessation by 50 to 70%, when compared to a placebo or no treatment at all.²⁰ However, the major disadvantages of this approach are high cost and unwanted side effects

such as nausea, dry mouth, weight gain, and sedation.²¹

At present, some choices of traditional Thai herbs for the purpose of smoking cessation are challenging. It has been reported that some traditional Thai plants are able to reduce smoking rate; such as, Peep (*Millingtonia hortensis* Linn), Shallot (*Allium ascalonicum* Linn), Snow lotus (*Saussurea laniceps*) and Mor Noi (*Vernonia cinerea* Less).

Vernonia cinerea Less. (VC) is a natural plant that grows around the world. It is classified in the Asteraceae family.^{22,23} Dominant characteristic with slender stem, variable leaf shape, and pinkish-purple flowers can be seen. Previous studies reported that VC leaf or whole parts of plant are analgesic, antipyretic and anti-inflammatory in rats.^{24,25} VC plant has been documented as widely used for relieving cigarette craving, however, there is less evidence regarding human smoking. A previous study of whole dry VC plant carried out a clinical trial to stop smoking in a smoking cessation clinic at Thanyarak Institute, Pathumthani, Thailand, resulted in no significant difference between a placebo-controlled and parallel trial.²⁶ Moreover, it showed that benefits of VC supplementation combined with exercise helped to reduce oxidative stress by releasing endorphin in active smokers.²⁷ Some reports have studied the active compounds in VC; such as, flavonoid and terpenoid,²⁸ steroids, saponins, alkaloids, carbohydrates, flavonoids, phenols, tanins, and proteins, including N-hexadecanoic acid (42.88%), 1,2-benzene-dicarboxylic acid (23%), squalene (11.31%), caryophyllene oxide (2.31%), guaialol (1.75%), octadecanoic acid (4.41%) and 9,12-octadecanoic (9.38%).²⁹ In Thailand, VC was approved for its benefits in smoking cessation, and has been recorded as a drug from plants in the National List of Essential Medicines (NLEM) in Thailand (2013).³⁰ Moreover, it may be possible to utilize it in modern medicine, as currently used in NRT. Therefore, the aims of this study were to evaluate whether VC extracts have anti-oxidant properties and radical scavenging activities *in vitro* including on catecholamine neurotransmitters, oxidative stress related to nicotine-administration, and direct anti-toxicity in bone marrow of preliminary rat model.

Materials and methods

Chemicals: Folin-Ciocalteu phenol reagent, 2,2-Azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS), trolox, gallic acid, sodium nitrite (NaNO_3), nitroblue tetrazolium (NBT), hypoxanthine (99%), xanthine oxidase (from bovine milk), 2-deoxy-D-ribose (97%), ascorbic acid, 2-thiobarbituric acid (TBA), trichloroacetic acid (TAC), hydrogen peroxide (H_2O_2), sodium nitroprusside (SNP), standard tetramethoxypropane (TMP), and cyclophosphamide (CP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulfate, $\text{KH}_2\text{PO}_4\text{-KOH}$, ethylene diamine tetraacetic acid (EDTA), Ortho-phosphoric acid (85%) and Tris (hydroxymethyl)-aminomethan were purchased from Merck Ltd. (Darmstadt, Germany). *N*-1-naphthylethylene-diamine dihydrochloride (NED) (VWR, Prolabo, EC), sulfanilamide (Sulfa) (Fluka, Steinheim, China) and 3-CAT research ELISA™ Kit (Cat. No. BA E-5600, Labor Diagnostika Nord GmbH & Co. KG, Germany) were experimentally prepared in this study.

Animals: Male and female Wistar rats (aged 5-8 weeks, weigh 250-300 gm) were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand, and housed in individual cages under an ambient temperature of $24 \pm 1^\circ\text{C}$ at the Thailand Institute of Scientific and Technological Research (TISTR) Center (Pathum Thani, Thailand).

Preparation of *Vernonia cinerea* (VC) Less.

Naturally grown mature VC plants, of at least three months old, were collected from a local clean area, without toxic insecticide spraying, Chiang Mai Province, Thailand. VC characteristics were identified using voucher specimens deposited at CMUB herbarium, Department of Biology, Faculty of Science, Chiang Mai University. Flower, stem, and leaf of VC were separated and washed four times with clean water before cutting into small pieces of approximately one inch long and dry heating in an oven (Binder GmbH, Germany) at 60°C . Dried materials were kept in sterile bottle (20% of moisture) containing a small bag of anti-moisture silica-gel pills (Chemipan Corporation Co., Ltd, Thailand). VC extract was prepared as mentioned previously²⁷ with a water-extracted protocol, as generally used in an anti-smoking clinic. One-hundred and

thirty mL of VC condensed juice was prepared by mixing dried materials (stem, leaf, or flower part) with clean water (Nestle Pure Life, Nestle (Thai) Ltd, Thailand) (20:390, w:v) and boiling in a traditional pot until completely evaporated. Lyophilized extracts were prepared from each 130 mL of VC condensed juices of different parts by freeze dryer (LYOMASTER™, Germany).

Antioxidant and radical scavenging activity assay *in vitro*

Total phenolic assay

Total content of phenol in each VC extract was determined by Singleton and Rossi method³¹ in which 50 μL of extract solution (20 mg/mL) was mixed with 1.0 mL of Folin-Ciocalteu reagent and kept in the dark for 10 min before 500 μL of 20% sodium carbonate was added. Tube was then incubated for 2 hrs, and absorbance of clear supernatant from short high speed centrifugation for 3 min was read at 765 nm by spectrophotometry. Total phenolic content was calculated by comparison to standard gallic acid.

Total antioxidant capacity (TAC) assay

Total antioxidant capacity of each VC extract from stem, flower, and leaf was assayed by ABTS cation radical de-colorization method.³² Stock ABTS cation radical was produced by activating ABTS (14 mmol/L) with potassium persulfate (14 mmol/L), and kept overnight with light protection. Working ABTS cation radical was diluted from stock ABTS radicals with deionized water until starting absorbance at 0.7 ± 0.2 before adding of extract solution. Ten μL of extract solution; from the stem, flower, or leaf (10 mg/mL) was added to 990 μL of working ABTS cation radical solution in a plastic cuvette (size 1.5 mL), and gently alternated inversely 9 times before adding to the spectrophotometer at 734 nm by spectrophotometry. Decreased absorbance was recorded continuously every 1 min for 3 min and finally calculated to $\Delta A/\text{min}$, automatically by spectrophotometry. Total antioxidant capacity (TAC) of the VC extract was calculated by comparing with the $\Delta A/\text{min}$ of standard Trolox.

Nitric oxide scavenging assay

Nitric oxide was evaluated by a Griess reagent according to the company (Promega).³³ Briefly, 0.2 mL of

various concentrations of extract solution (20-100 mg/mL) was added to 1.8 mL of SNP and kept at 25 °C for 180 min. One mL of solution was then mixed with 1 mL of 0.1% of NED in water and left in dark for 5 min. Then, mixed with 500 µL of 1% sulfanilamide (Sulfa) in 5% phosphoric acid and kept in dark for 5 min. Pale pink color was produced with an absorbance reading of 540 nm. Nitric oxide was calculated compared to standard NaNO₃. The result presented an inhibitory extract concentration of 50% (IC₅₀).

Superoxide radical scavenging assay

Superoxide radical scavenging activity was determined following the previous protocol.³⁴ Firstly, 100 µL of various concentrations of extract solution (20-100 mg/mL) was added to 760 µL of hypoxanthine (1.1 mmol/L), 100 µL of NBT (300 µmol/L) and 40 µL of xanthine oxidase (1.67 U/mL). Absorbance (532 nm) was recorded continuously every 15 seconds. Data were represented as inhibitory concentration of 50% (IC₅₀).

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay was determined as mentioned previously.³⁵ Firstly, 200 µL of KH₂PO₄-KOH (100 mmol/L) was added to 200 µL of deoxyribose (15 mmol/L), 100 µL of EDTA (1 mmol/L), 100 µL of H₂O₂ (10 mmol/L), 100 µL of extract solution (20-100 mg/mL) and 100 µL of ascorbic acid (1 mmol/L). After being kept at room temperature (37 °C) for 60 min, 1 mL of TBA (1%) with TAC (2.8%) was added before boiling at 80 °C for 20 min. A pale pink color was produced and absorbance was read (532 nm). Data was represented as inhibitory concentration of 50% (IC₅₀).

Animal study

The animal study protocols on catecholamine, oxidative stress, and chromosome aberration were approved by the Ethics Committee at the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand [CODE: TS54001].

Catecholamine, oxidative stress assay

All rats were selected by a simple sampling method and identity numbers were marked on their tail. They were fasted 16 hours before the experiment. They were then divided randomly into six groups of 10 rats in each, of

which 5 were male and 5 female for (1) control with normal saline (0.9%), (2) only nicotine, (3) bupropion (150 mg/kg body weight), (4) leaf extract (10 gm/kg body weight), (5) flower extract (10 gm/kg body weight), and (6) stem extract (10 gm/kg body weight). All groups were injected each day subcutaneously with nicotine at 0.6 mg/kg body weight, except for control group. After 20 days of daily feeding, all rats (n=10) were water-deprived overnight before blood taking via right ventricle of heart to determine catecholamine neurotransmitters (dopamine, noradrenaline and adrenaline) in plasma by a 3-CAT research ELISA™ Kit. Moreover, fresh plasma sampling of 6 rats (3 males and 3 females) from each of the six groups above was evaluated for TAC32 and MDA.³⁶

Malondialdehyde (MDA) by TBARS

A modified version of original protocol of Chirico (1994) used high-performance liquid chromatography (HPLC) from which 200 µL of plasma was mixed with 750 µL of ortho-phosphoric acid (2.5%, v:v) and vortexed. Then, 500 µL of TBA (0.2 mol/L) in Tris (hydroxymethyl)-aminomethane (0.14 mol/L) was added. After incubation in a water bath (90°C) for 30 min, all samples were cooled and centrifuged at 10,000 rpm for 3 min. Twenty µL of samples was injected into the rheodyne valve with a 20 µL fixed loop, and peak of MDA-TBA adduct was measured at 532 nm (UV-VIS detector, 1100 Hewlett-Packard, Germany) with a C-18 reverse-phase column (250x4.6x5.0 mm: Phenomenex, Aschffenburg, Germany) under pure isocratic methanol (HPLC grade, LabScan, Bangkok, Thailand), with a flow rate of 1.0 mL/min. MDA (µmol/L) was calculated by comparing with standard TMP.

Chromosome aberration study

Male and female Wistar rats were divided randomly into five groups of 5 female and 5 male rats in each one. Distilled water, flower, stem, or leaf extract were single administered orally at 2,000 mg/kg body weight, whereas cyclophosphamide (CP) at 50 mg/kg body weight was injected intraperitoneal. Protocol for evaluating acute toxicity on chromosome aberration of bone marrow followed a previous protocol³⁷ under the guideline of the Organization for Economic Cooperation and Development (OECD, 2000).³⁸ After 24 hours of single dose treatment,

colchicine was injected to arrest chromosome. Rats were then sacrificed humanely by inhaling carbon dioxide. Bone marrow cells were removed from femur thigh bone to study the chromosomal aberration (gap, break, exchange and multiple aberrations). Briefly, bone marrow cells were washed in hypotonic solution (0.075 mol/L KCl) and fixed (glacial acetic acid: methanol, 1:3 v:v). Slides were coded and stained with 10% Giemsa. Normal cells were counted and compared in mitosis stage with a total of 1,000 cells on each slide to present percentage of mitotic index (%MI). Types of chromosome aberration in both male (n=5) and female (n=5) rats, such as gap, break, exchange and multiple aberrations, were scored and recorded.

Statistical analysis

Data were expressed as mean±SEM. All parameters were compared statistically by independent measurement in analysis of variance (ANOVA) and Bonferroni test

(statistical software package, SPSS for windows version 11). Level of significance was established at $p<0.01$.

Results

Antioxidant and scavenging activities in vitro

Table 1 shows leaf extract presented the highest total phenolic content and total antioxidant capacity (TAC) compared to stem and flower extracts. Stem extract presented the highest scavenging activity on nitric oxide (NO) compared to flower and leaf extracts. Stem and flower extracts present the significant highest activity on scavenging on superoxide radical ($O_2^{\cdot-}$) compared to leaf extract. However, flower extract had the highest activity for scavenging on hydroxyl radical ($OH^{\cdot}$) when compared to stem and leaf extracts. Results of all extracts on scavenging radicals or non-radicals were confirmed by standard ascorbic acid.

Table 1 Antioxidant and radical scavenging activities of stem, flower, leaf extracts, and standard ascorbic acid (VitC)

Test	Stem (n=5)	Flower (n=5)	Leaf (n=5)	Standard Vit C (µg/mL)
Total phenolics (mg GA/mg)	123.5±14.4	179.5±11.3	669.2±17.2	-
Total antioxidant capacity (TAC) (mmol of Trolox/mg)	0.97±0.22	1.34±0.12	3.12±0.45 [†]	-
Nitric oxide scavenging (IC50) (mg/mL)	0.91±0.23 [‡]	1.08±0.11	2.77±0.75	146.4±2.56
Superoxide radical scavenging (IC50) (mg/mL)	0.62±0.21 [*]	0.69±0.11 [*]	4.41±0.27	39.56±3.12
Hydroxyl radical scavenging (IC50) (mg/mL)	3.03±0.12	1.68±0.23 ^{**}	3.90±0.13	23.23±5.06

Data presents the mean±SEM, IC50 = inhibitory concentration at 50%. GA = gallic acid, [†] $p<0.01$ when compared to the stem and flower extracts, [‡] $p<0.01$ when compared to the flower and leaf extracts, ^{*} $p<0.01$ when compared to the leaf extract, and ^{**} $p<0.01$ when compared to the stem and leaf extracts.

Catecholamine and antioxidant control in rats

Table 2 and Figure 1 present the level of catecholamine neurotransmitters in plasma as dopamine (Figure 1A), noradrenaline (Figure 1B), and adrenaline (Figure 1C) in all Wistar rats. Results showed significant levels of dopamine, noradrenaline and adrenaline after treatment with nicotine at

0.6 mg/kg/BW, when compared to control group ($p<0.01$). After co-treatment with bupropion, dopamine level was reduced, whereas noradrenaline and adrenaline levels increased significantly ($p<0.01$), when compared to nicotine treatment. In addition, dopamine was reduced significantly after co-treatment with stem, flower, and leaf

extracts ($p<0.01$), as observed in bupropion co-treated group. A significant difference in stem treated group was demonstrated but not in flower or leaf co-treatment, when compared to bupropion co-treatment ($p<0.01$). Noradrenaline and adrenaline were significantly increased after co-treatment with leaf extract ($p<0.01$), when compared to nicotine

treatment, but was not different from bupropion co-treatment ($p>0.01$). Moreover, flower and stem extracts co-treated group did not increase noradrenaline and adrenaline levels, with significant difference from the bupropion co-treatment ($p<0.01$).

Table 2 Catecholamine parameters (dopamine, nor-adrenaline and adrenaline) in nicotine-treated Wistar rats.

Experiments	Dopamine (ng/mL)	Noradrenaline (ng/mL)	Adrenaline (ng/mL)
0.9% Normal saline (n=10)	0.54±0.004	3.79±0.011	3.26±0.038
Nicotine (n=10)	0.95±0.004	3.58±0.025	3.10±0.028
Nicotine+bupropion (n=10)	0.61±0.004	4.32±0.004	3.73±0.015
Nicotine+flower extract (n=10)	0.56±0.08	3.77±0.008	3.24±0.028
Nicotine+stem extract (n=10)	0.34±0.009	3.51±0.009	3.04±0.024
Nicotine+leaf extract (n=10)	0.53±0.005	4.69±0.024	4.03±0.023

Data are mean±SEM

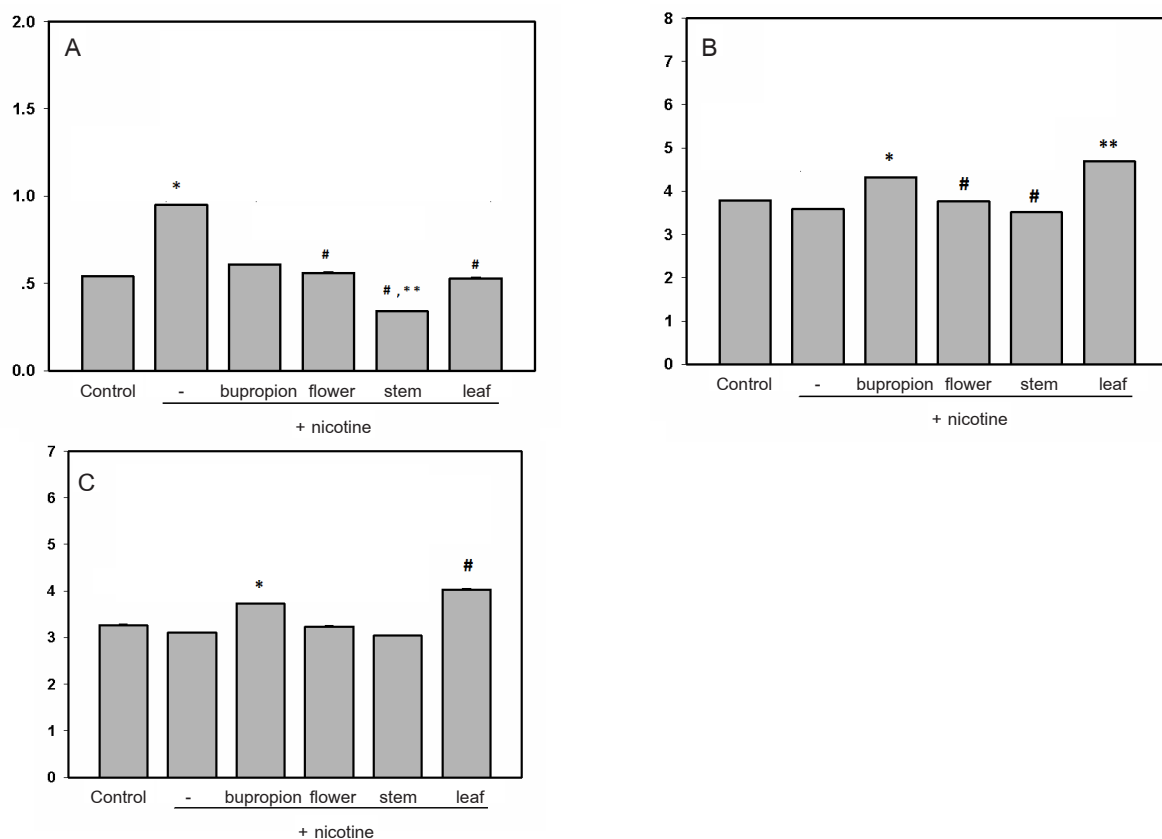


Figure 1. Catecholamine levels; dopamine (A), noradrenaline (B), and adrenaline (C) in plasma after treatment with VC extracts (flower, stem, and leaf), control with normal saline solution and bupropion in nicotine-treated rats ($n = 10$). * $p<0.01$ when compared to control and bupropion-treated groups. ** $p<0.01$ when compared to flower, leaf bupropion and nicotine-treated groups. # $p<0.01$ when compared to nicotine-treated group.

Study of TAC levels in plasma (Table 3, Figure 2) showed no significant difference between control and nicotine treated groups ($p>0.05$). After co-treatment with bupropion, TAC was non-significantly increased compared to either control or nicotine group ($p>0.05$). Moreover, TAC levels had no statistical difference after co-treatment with all extracts ($p>0.05$) when compared to nicotine administrated group. Regarding the results of MDA levels

(Table 3 and Figure 2) in control and nicotine treated groups, no statistical difference was shown ($p>0.01$), but they were significantly reduced when co-treated with bupropion ($p<0.01$). Interestingly, results of MDA levels from three extract co-treatments demonstrated that only co-treatment with leaf extract had statistical difference compared to flower and leaf extracts, but similar to bupropion co-treatment.

Table 3 Oxidative stress parameters; total antioxidant capacity (TAC) and malondialdehyde (MDA) in all groups.

	TAC (mmol/L)	MDA (μ mol/L)
Control (n=6)	12.04 $\pm$ 0.29	24.77 $\pm$ 0.79
Nicotine (n=6)	11.74 $\pm$ 0.06	29.17 $\pm$ 1.68
Nicotine+bupropion (n=6)	13.15 $\pm$ 0.38*	15.14 $\pm$ 0.60*,**
Nicotine+flower extract (n=6)	12.03 $\pm$ 0.42	28.67 $\pm$ 2.14
Nicotine+stem extract (n=6)	11.83 $\pm$ 0.21 [†]	23.45 $\pm$ 2.03 [†]
Nicotine+leaf extract (n=6)	12.34 $\pm$ 0.15	18.99 $\pm$ 0.89 [‡]

Data are mean $\pm$ SEM * $p<0.01$ when compared to nicotine-treated groups, [†] $p<0.05$ when compared to bupropion-treated group, ** $p<0.01$ when compared to control group, and [‡] $p<0.01$ when compared to flower flower-treated group.

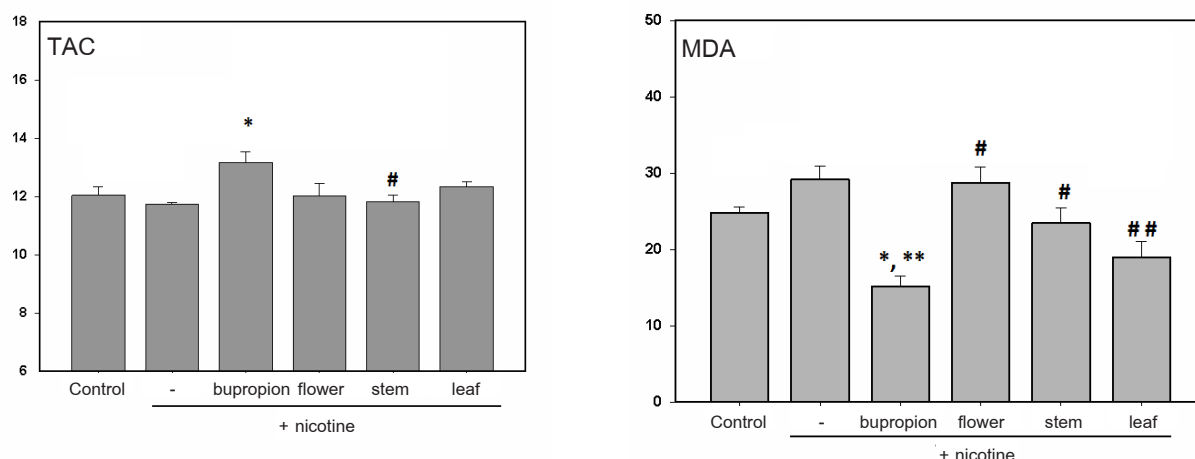


Figure 2. Total antioxidant capacity (TAC) levels and malondialdehyde (MDA) in plasma after treatment with VC extracts (stem, flower, and leaf), control with normal saline solution, and bupropion in nicotine-treated rats ($n = 6$; 3 male & 3 female). * $p<0.01$ when compared to nicotine-treated group. ** $p<0.01$ when compared to control group. # $p<0.05$ when compared to bupropion-treated group. ### $p<0.01$ when compared to flower-treated group.

Chromosome aberration

Chromosomal analysis at the metaphase stage of bone marrow cells in nicotine-treated and positive control (PC) male and female Wistar rats are summarized in Table 4, after VC stem, flower, and leaf extract had been administered orally and CP intraperitoneally injected at 2,000 mg/kg body weight and 50 mg/kg body weight, respectively. MI (%) presented a cell proliferation of no statistical difference in any of the VC extracts in the

treated groups compared to control ($p>0.01$). However, a significant decrease in %MI was seen after treatment with cyclophosphamide ($p<0.01$). Additionally, CP also induced chromosomal damage. Figure 3 shows chromosome characteristic after treatment with all extracts and nicotine but not in control. Chromosome breaking, exchange, and multiple aberrations of bone marrow in both male and female rats of CP treated group.

Table 4 Mitotic index (MI); types of chromosomal aberration and damage in Wistar rats.

Male Groups	M.I. (%)	Types of chromosomal aberration (%)			Chromosomal damage/cell
		Break	Exchange	Multiple Ab.	
Distilled water (n=5)	6.28±0.16	0	0	0	0
Flower extract (n=5)	6.34±0.13	0	0	0	0
Stem extract (n=5)	6.30±0.22	0	0	0	0
Leaf extract (n=5)	6.30±0.20	0	0	0	0
Cyclophosphamide (n=5)	3.96±0.23*	6.80±2.76*	1.00±0.77	1.20±0.49*	5.80±1.59*
Female Groups					
Distilled water (n=5)	5.80±0.33	0	0	0	0
Flower extract (n=5)	6.28±0.11	0	0	0	0
Stem extract (n=5)	6.02±0.19	0	0	0	0
Leaf extract (n=5)	6.12±0.19	0	0	0	0
Cyclophosphamide (n=5)	4.10±0.14*	21.20±1.62*	2.00±0.84*	1.40±0.51*	9.20±0.97*

Data are mean±SEM. Independent measurement in among of five groups was analyzed with ANOVA and Bonferroni test were used. *percentage of mitotic index (%MI). *significant differences: ($p<0.05$) compared to distilled water and VC extract groups.

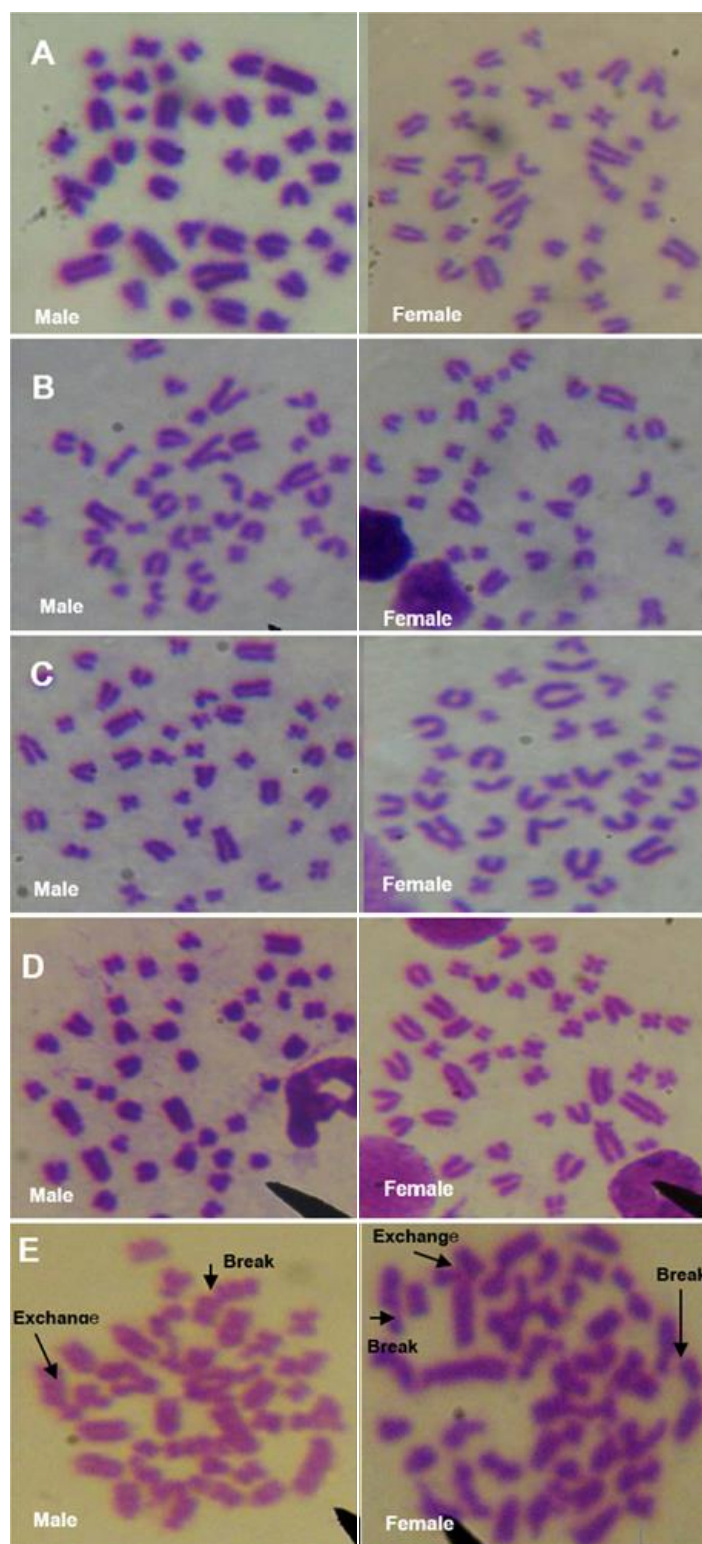


Figure 3. Characteristics of chromosomal aberrations from the bone marrow of male and female Wistar rats after treatment with distilled water (A), and VC extracts from the stem (B), leaf (C), and flower (D), compared to cyclophosphamide (Giemsa stain, 1000x).

Discussion

The results of this study possibly supported the efficiency of smoking cessation reported in previous clinical studies, including results of safety measures against chromosomal damage and better future distribution of information to clinics for users and physicians. In this study, the leaf extract presented the highest total phenol content and highest TAC, but the results of scavenging radicals; either NO non-radicals or radicals such as $O_2^{\cdot-}$ and $OH^{\cdot}$, was controversial even. Results possibly came from the extract preparation in water in this study. Some interesting results in update study on active and antioxidant activities from difference extracts from VC condense juices in 2017 showed that dominate flavonoid and catechins were in the leaf than flower or stem extract.³⁹ Moreover, other active compounds in VC extracts, such as terpenoid,²⁸ steroids, saponins, alkaloids, carbohydrates, phenols, tannins, and proteins²⁶ including N-hexadecenoic acid, 1,2-benzene-dicarboxylic acid, squalene, caryophyllene oxide, guaiol, octadecanoic acid, and 9,12-octadecanoic,⁴⁰ presented on radical scavenging activity. However, dry crude powder from the whole parts was contained in a small tea bag, which suggested the possible effect on smoking cessation.¹⁷ But, improvement in VC preparation, by condensed juice as in this study, also represented higher rate of smoking cessation.²⁷ From the previous data, unpleasant taste and smell of cigarette smoke were a direct influence from VC condensed juice that has been proposed as a first step to stop smoking.^{26, 27} Moreover, the efficacy of VC on stop smoking is possible related to the nicotine replacement because the nicotine and nitrate or nitrite were found in leaf extract.³⁹ In Wistar rat-animal protocol, pure nicotine at 0.6 mg/kg for 20 days was injected subcutaneously that was following the previous evidence that the LD50 of nicotine at 50 mg/kg in rats or 3 mg/kg in mice, or 0.5-1.0 mg/kg body weight can be a lethal dosage in adult humans.⁴¹ Moreover the maximum venous blood concentration was possibly varies from 10 to 30 minutes after used.⁴² Furthermore, at least 12 days of daily subcutaneous injection was able to induce nicotine tolerance, and affected locomotor activity from nicotine addiction.⁴³ Bupropion, which is an antidepressant drug

and used to relieve the withdrawal symptoms from stop smoking⁴⁴ was used to positive control that can interact with the nicotine receptor in different pathways, for example, a non-competitive antagonist agent blocks nicotine activation of $\alpha 3\beta 2$ -, $\alpha 4\beta 2$ -, and $\alpha 7$ -neuronal acetylcholine nicotinic receptors (nAChRs).

From the results of all catecholamine levels, pure nicotine increased dopamine level that indicated from an antagonistic mechanism as in a previous suggested.⁴⁰ When treated with all extracts, dopamine levels were reduced that the mechanism is still unclear. Possibly, in a previous research proved that phenolic monoterpenoid, as a carvacrol in many plants, was able to bind to AChRs,⁴⁵ and possibly stimulates the function of mitochondrial monoamine oxidases (MAOs), then increases dopamine degradation to noradrenaline and adrenaline.^{38, 43} Possibly hypothesis of total phenolic evaluation in different VC extracts can be explained on the catecholamine results. The results of noradrenaline and adrenaline levels after leaf treatment is similar to those in the bupropion treated group. Thus, the hypothesized pathway on inhibition of AChRs may be possibly. Part of the results from oxidative stress status, in nicotine-treated group showed the slightly non-significant increase of MDA and decrease of TAC levels that has been proposed from NF-kB and apoptosis activity.¹³ Whereas bupropion increased TAC and reduced MDA levels significantly. This mechanism is still unclear and controversial, because the latest evidence proved that bupropion is not an antioxidant agent, but has the distinguished function of inhibiting dopamine and noradrenaline reuptake or the post-synaptic acetylcholine nicotine receptor.⁴² Thus, the antioxidant mechanism of bupropion on co-nicotine treatment also is controversial and needs proving. The results between the three extract treatments, co-treatment with leaf extract reduced MDA level better than co-treatment with stem or flower extract which is possibly explained by higher phenolic active compound.

Finally, the acute toxicity protocol in the chromosome from all extracts was designed by comparing to a positive inducer, cyclophosphamide that damages chromosomes through generating free radicals and alkylating DNA.⁴⁶ Although, the standardized protocol in animal test has suggested to perform in male or female animal for exclusion

the con-founding factor as hormone, but this study selected both male and female rats that is possibly to apply in human of both gentle. A single dose at 2,000 mg of extract per kg body weight was performed following the OECD guideline (2000).³⁸ Results showed no acute toxicity in chromosomes when applied any VC extracts presenting possibly safety. In the other hand, a higher dose in human subjects needs more study or long term administration.

Conclusion

Vernonia cinerea (VC) that is a natural plant and used in clinical smoking cessation, involves the catecholamine neurotransmitter, especially regarding depression on dopamine and enhanced noradrenaline or adrenaline release, and the reduction on lipid peroxidation being the same as bupropion function. Finally, a high dose of all VC extracts has non-acute toxicity in chromosomes.

Conflicts of interest

There are no conflicts of interest.

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