CHAPTER 4

RESULTS

For VC material preparation in this study, when fresh VC parts; stem, flower and flower at 1,000 g were dried in oven with controlled temperature under 60°C, the dry yields were 246, 258, and 183 g approximately. Then applied with lyophilization technique, the yields of VC extracts from each 130 mL of different dried parts of stem, flower and leaf at 20 gram in distill water at 390 mL were 0.82, 0.90 and 1.89 g, respectively.

4.1 Antioxidant activities and active compound *in vitro*.

4.1.1 Antioxidant activities

The results in **Table 4.1** show that leaf extract presented the significant highest total antioxidant capacity (TAC) (3.12 ± 0.45 mmol Trolox/mg), when compared to flower extract (1.34 ± 0.12 mmol Trolox/mg) and stem extract (0.97 ± 0.22 mmol Trolox/mg) ($p < 0.01$). The stem extract has the significant highest scavenging activity on nitric oxide (NO) (0.91 ± 0.23 mg/mL), when compared to the flower extracts (1.08 ± 0.11 mg/mL) and leaf extracts (2.77 ± 0.75 mg/mL) ($p < 0.01$). The stem and flower extracts present the significant highest activity for scavenging on superoxide radicals ($O_2^{\bullet -}$) (0.62 ± 0.21 mg/mL and 0.69 ± 0.11 mg/mL), when compared to the leaf extracts (4.41 ± 0.27 mg/mL) ($p < 0.01$). Whereas the scavenging activity on hydroxyl radical ($OH^{\bullet}$) was the significant highest from the flower extract (1.68 ± 0.23 mg/mL) when compared to that from the stem extracts (3.03 ± 0.12 mg/mL) and leaf extract (3.90 ± 0.13 mg/mL), respectively ($p < 0.01$).

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Table 4.1 Antioxidant activities of stem, flower and leaf extracts

	Stem	Flower	Leaf
Total antioxidant capacity (TAC) (mmol of Trolox/mg)	0.97 ± 0.22	1.34 ± 0.12	3.12 ± 0.45$^{\#}$
Nitric oxide (IC50) (µg/mL)	0.91 ± 0.23$^{\#\#}$	1.08 ± 0.11	2.77 ± 0.75
Superoxide radical (IC50) (µg/mL)	0.62 ± 0.21*	0.69 ± 0.11*	4.41 ± 0.27
Hydroxyl radical (IC50) (µg/mL)	3.03 ± 0.12	1.68 ± 0.23**	3.90 ± 0.13

Data presents the mean ± SEM. IC50 = Inhibitory concentration at 50%. GA = gallic acid (n = 5). # $p < 0.01$ 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 stem and leaf extracts.

4.1.2 Active compounds

Total phenolics

From total phenolics content has been reported in **Table 4.2**. the leaf extract presented the significant highest total phenolic (669.2 ± 17.2 mg GA/mg), when compared to flower (179.3 ± 11.5 mg GA/mg) and stem extracts (123.5 ± 14.2 mg GA/mg).

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Total tannin

From total tannin content has been reported in **Table 4.2**. the flower extract also presented the highest content of total tannin (66.2 ± 1.37 mg/g) when compared to leaf (59.3 ± 0.95 mg/g) and stem extracts (47.7 ± 0.47 mg/g)

Catechin

From the HPLC analysis results, the peaks of each standard five catechins (ECG, EC, EGCG, C and EGC) were represented at different retention time, and the individual peaks were repeated after mixed the standards and each VC extracts

(**Figure 4.1**). **Table 4.2** shows the results of each catechins that the leaf extract had the significant highest content of all catechins compounds; C (165.23 ± 1.22 mg/g), EC (35.12 ± 1.34 mg/g), EGCG (16.11 ± 0.98 mg/g) and ECG (12.42 ± 1.13 mg/g) respectively ($p < 0.01$), compared to the stem extract; EC (29.12 ± 1.23 mg/g), ECG (4.56 ± 0.98 mg/g), EGCG (0.87 ± 0.04 mg/g) and flower extract (0.89 ± 0.04 mg/g of EGCG). Whereas, EGC could not be detected in all extracts, as same as the catechin peak in stem and flower extracts or ECG and EC peaks in flower extract. Moreover, ECG, EC, C and EGC were not detected in flower extract as same as the C and EGC in stem extract.

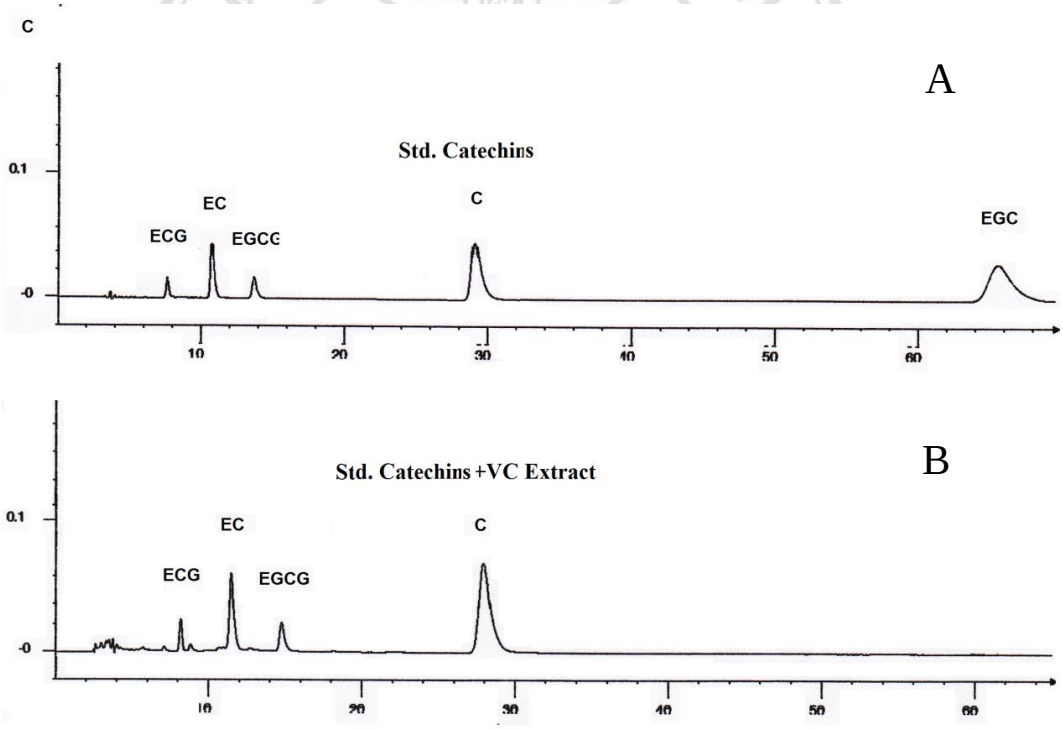

Figure 4.1 Chromatography peaks between pure standard five catechins (ECG, EC, EGCG, C and EGC) (A) and mixed standard catechins and VC leaf extract (B).

Flavonoid

For flavonoid content as kaempferol, myricetin, and quercetin has been reported in **Table 4.2**. The individual peaks from HPLC analysis results were represented at different retention time, including these peaks were repeated after mixing the standard and VC extract. The results in **Table 4.2** show that leaf extract had the

significant highest of myricetin (197.07 ± 4.05 mg/g), when compared to the flower (63.33 ± 2.12 mg/g) and stem extracts (47.65 ± 3.29 mg/g) ($p < 0.01$). Moreover, the leaf extract also had the significant highest content of quercetin (113.6 ± 5.67 mg/g) when compared to the flower extract (68.89 ± 2.56 mg/g) ($p < 0.01$) (**Figure 4.2**). But the kaempferol could be detected only in the flower extract (13.76 ± 1.56 mg/g).

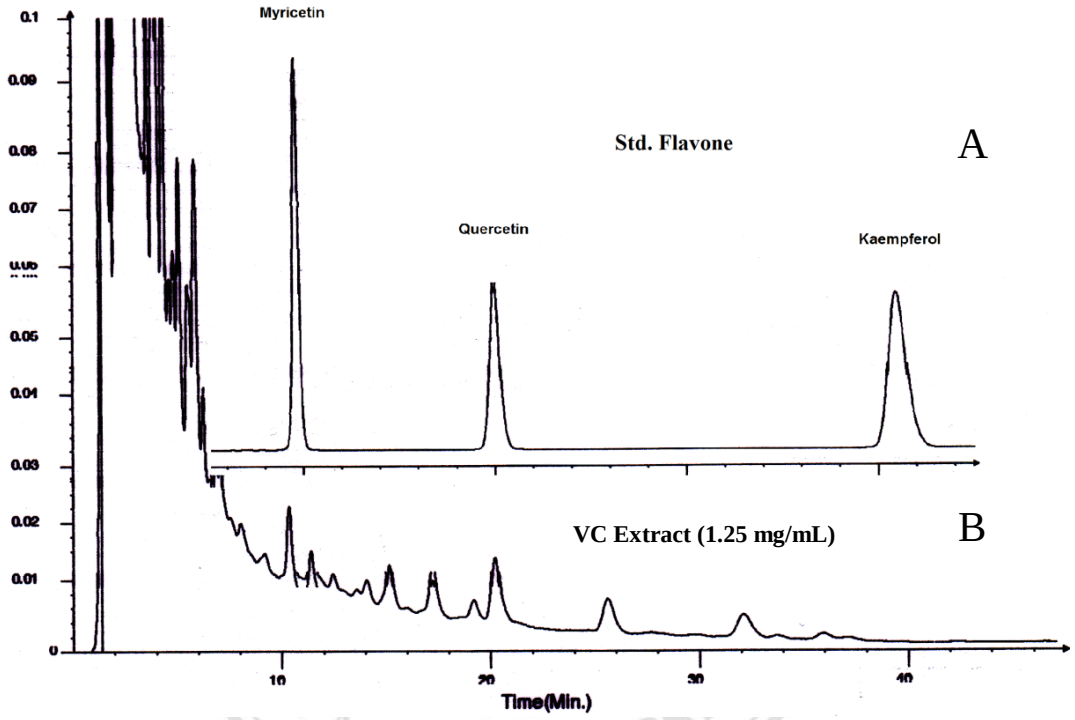

Figure 4.2 Chromatography peaks between pure standard myricetin, quercetin and kaempferol (A) and VC leaf extract at 1.25 mg/mL (B).

Isoflavone

For isoflavone content that is composed of daidzin and genistin. The leaf extract contained the significant highest of daidzin (50.87 ± 2.30 mg/g), when compared to the flower extract (27.29 ± 1.23 mg/g) and stem extract (13.36 ± 2.12 mg/g) ($p < 0.01$). In addition, the leaf extract had the significant highest content of quercetin (80.51 ± 2.34 mg/g) when compared to flower (39.43 ± 1.56 mg/g) and stem extract (9.42 ± 1.89 mg/g) ($p < 0.01$) (**Table 4.2**).

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Nitrite and Nitrate

The results found the nitrate and nitrite in leaf extract (0.19 ± 0.05 g/g & 0.25 ± 0.05 g/g) and stem extract (0.21 ± 0.11 g/g & 0.24 ± 0.01 g/g) (**Table 4.2**). Whereas nitrate and nitrite in flower extract could not detected in this study.

Nicotine

In the experiment, the peak of nicotine was identified with HPLC system. **Figure 4.3** shows the peak of standard nicotine and a mixture of standard nicotine and VC extract. The results showed that the leaf extract had the higher nicotine (1.54 ± 0.14 mg/g), when compared to the flower extract (1.23 ± 0.11 mg/g) (**Table 4.2**). But could not detected in stem extract.

Caffeine In this study, caffeine could not identified or detectable in any extracts (**Table 4.2**).

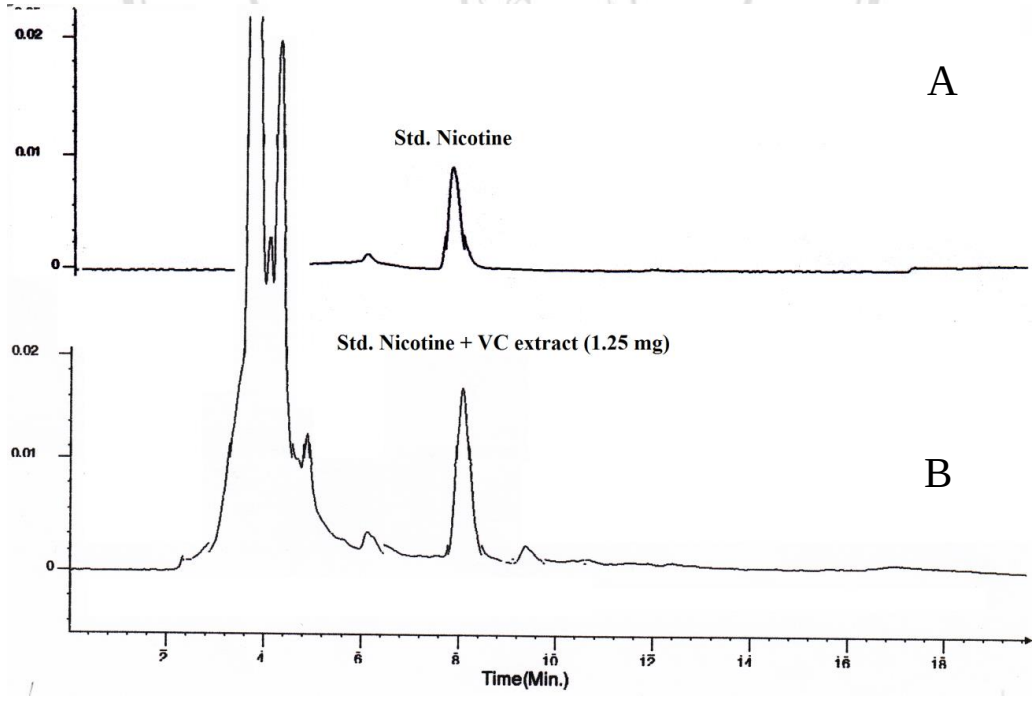

Figure 4.3 Chromatography peaks between pure standard nicotine (A) and mixed standard nicotine and VC leaf extract (B).

Table 4.2 All active compounds of stem, flower and leaf extracts.

	Stem extract	Flower extract	Leaf extract
Total phenolics (mg GA/mg)	123.5 ± 14.2	179.3 ± 11.5	669.2 ± 17.2#
Total tannin (mg/g)	47.7 ± 0.47	66.2 ± 1.37#	59.3 ± 0.95
Catechins			
ECG (mg/g extract)	4.56 ± 0.98	ND	12.42 ± 1.13#
EC (mg/g extract)	29.12 ± 1.23	ND	35.12 ± 1.34#
EGCG (mg/g extract)	0.87 ± 0.04	0.89 ± 0.04	16.11 ± 0.98#
C (mg/g extract)	ND	ND	165.23 ± 1.22#
EGC (mg/g extract)	ND	ND	ND
Flavonoid			
Kaempferol (mg/g extract)	ND	13.76 ± 1.56	ND
Myricetin (mg/g extract)	47.65 ± 3.29	63.33 ± 2.12	197.07 ± 4.05#
Quercetin(mg/g extract)	ND	68.89 ± 2.56	113.6 ± 5.67#
Isoflavone			
Daidzin (mg/g extract)	13.36 ± 2.12	27.29 ± 1.23	50.87 ± 2.30#
Genistin (mg/g extract)	9.42 ± 1.89	39.43 ± 1.56	80.51 ± 2.34#
Nitrate and Nitrite			
Nitrate (g/g extract)	0.21 ± 0.11	ND	0.19 ± 0.05
Nitrite (g/g extract)	0.24 ± 0.01	ND	0.25 ± 0.05
Caffeine (mg/g extract)	ND	ND	ND
Nicotine (mg/g extract)	ND	1.23 ± 0.11	1.54 ± 0.14

Data presents the mean ± SD. EGC = (-)-Epigallocatechin, EC = Epicatechin, C = Catechin, EGCG = epigallocatechin gallate, ECG = Epicatechin gallate, ND = non-detectable (n = 5). # $p < 0.01$ when compared to other extracts.

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4.2 Activities on catecholamine, oxidative stress and toxicity in chromosome

4.2.1 Activities on catecholamine in rats

Table 4.3 and **Figure 4.4** present the results of catecholamine neurotransmitters in plasma as dopamine (**Figure 4.4.A**), noradrenaline (**Figure 4.4.B**), and adrenaline (**Figure 4.4.C**) levels in all of wistar rats. Results from statistically different evaluation between the groups showed significant levels of dopamine, noradrenaline and adrenaline after treatment with nicotine at 0.6 mg/kg BW, when compared to normal saline treatment in the control group ($p < 0.01$). After treatment with bupropion, the results showed a significantly lower dopamine level and higher noradrenaline and adrenaline levels, when compared to the nicotine-treated or control group ($p < 0.01$). After extract had been administered orally for 20 days, the dopamine levels reduced significantly in the stem, flower, and leaf treated groups, respectively as same as in the bupropion treated group ($p < 0.01$), when compared to the nicotine-treated group. Additionally, the dopamine levels reduced significantly in the stem treated group, when compared to the bupropion treated group ($p < 0.01$). Whereas the noradrenaline and adrenaline levels significantly increased in the leaf treated group ($p < 0.01$) when compared to the nicotine-treated group or compared to the flower or stem treated groups, but no significant difference from the bupropion treated group ($p > 0.01$). Furthermore, the flower and stem extracts did not increase the noradrenaline or adrenaline levels, with significant difference from the bupropion treated group ($p < 0.01$).

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Table 4.3 Catecholamine parameters (dopamine, noradrenaline and adrenaline) in non nicotine- or nicotine-treated rats with each extracts

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 ± 0.008	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 of Levels of dopamine, nor adrenaline and adrenaline (ng/mL).

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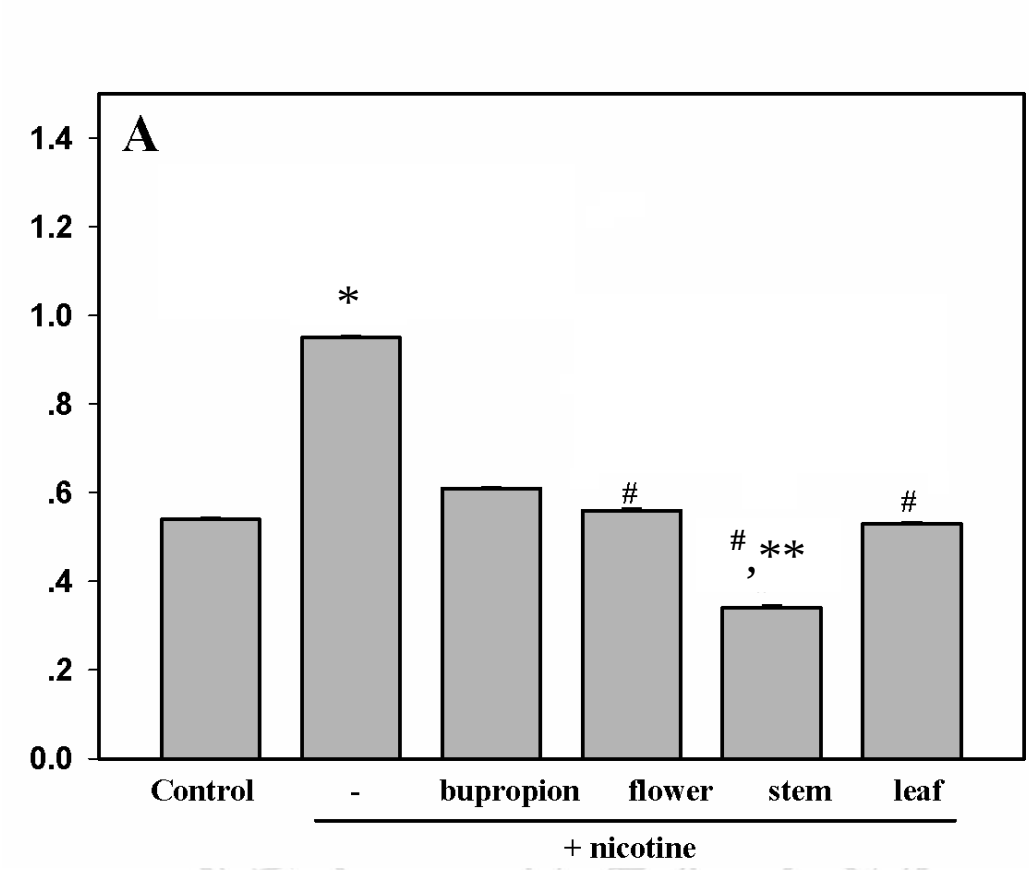

Figure 4.4 Catecholamine levels; dopamine (A), noradrenaline (B) and adrenaline (C) in plasma after treatment with VC extracts (flower, stem and leaf), control with normal saline solution and bupropion in nicotine-treated rats (n = 10). * $p < 0.01$ when compared to control and bupropion-treated groups. ** $p < 0.01$ when compared to flower, leaf, bupropion and nicotine-treated groups. # $p < 0.01$ when compared to nicotine-treated group.

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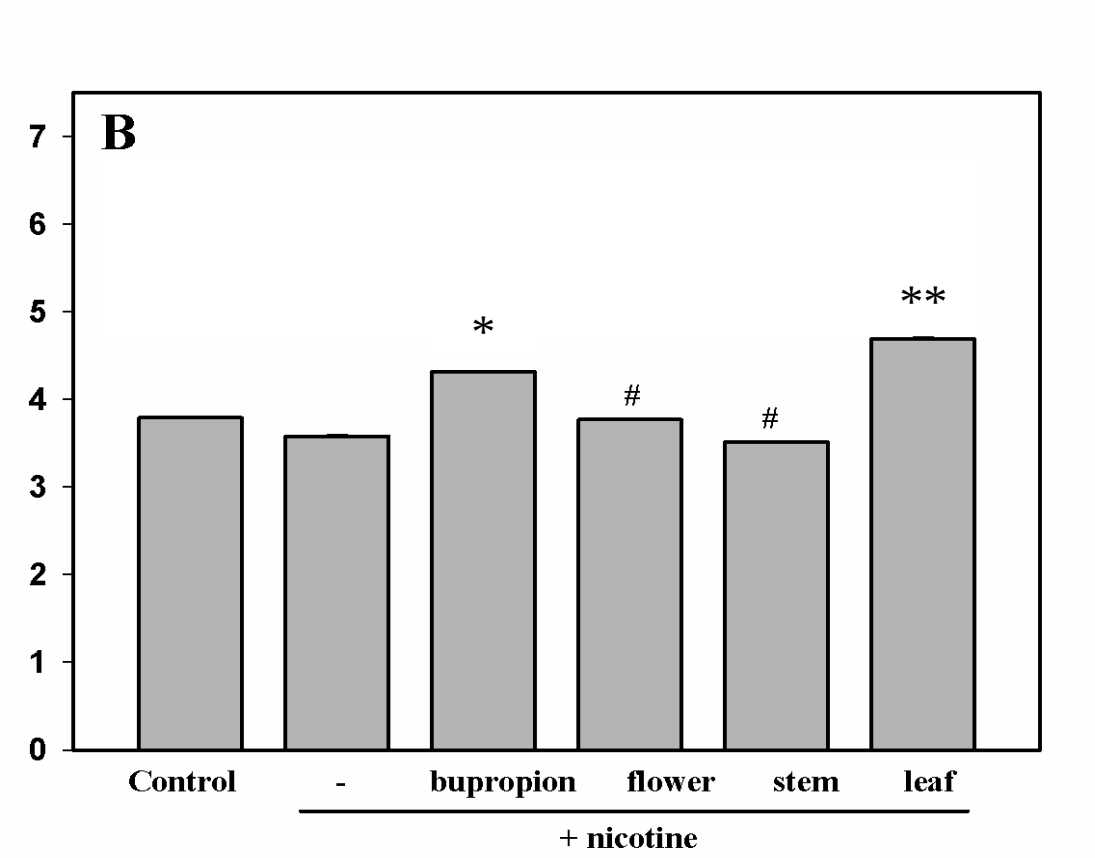

Figure 4.4 (**Cont.**) Catecholamine levels; dopamine (A), noradrenaline (B) and adrenaline (C) in plasma after treatment with VC extracts (flower, stem and leaf), control with normal saline solution and bupropion in nicotine-treated rats (n = 10). * $p < 0.05$ when compared to control and nicotine-treated groups. ** $p < 0.01$ when compared to stem and flower-treated groups. # $p < 0.01$ when compared to stem and bupropion-treated groups.

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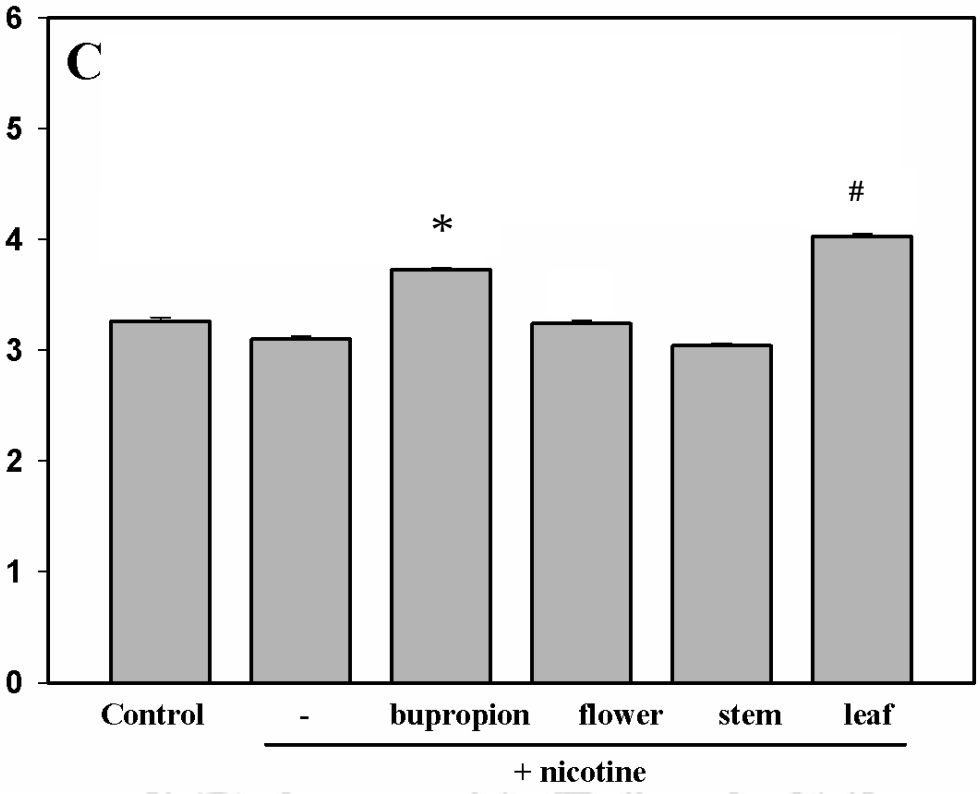

Figure 4.4 (**Cont.**) Catecholamine levels; dopamine (A), noradrenaline (B) and adrenaline (C) in plasma after treatment with VC extracts (flower, stem and leaf), control with normal saline solution and bupropion in nicotine-treated rats (n = 10). * $p < 0.05$ when compared to control and nicotine-treated groups. # $p < 0.01$ when compared to stem and flower-treated groups.

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4.2.2 Oxidative stress in rats.

Results of the TAC levels in plasma in **Table 4.4** and **Figure 4.5** showed that there did not significant difference between the control and nicotine-treated group ($p > 0.05$). After treated with bupropion, the TAC level non-significantly increased when compared to either the control or nicotine group ($p > 0.05$). Furthermore, TAC levels showed did not significant increase after the administration of stem, flower and stem extracts, respectively with no statistical difference when compared to the nicotine administered group ($p > 0.05$).

MDA level in **Table 4.4** and **Figure 4.6** showed a non-significant increases after treated with nicotine ($p > 0.05$), but MDA level decreased significantly after treatment with bupropion ($p < 0.01$). The MDA levels showed tendency decrease when treated with flower, stem and leaf extract, respectively. In among of extract administered groups, MDA level decreased significantly in a leaf treated group, when compared to flower and stem treated groups that had similar activity as the bupropion treatment.

Table 4.4 Oxidative stress parameters; total antioxidant capacity (TAC) and malondialdehyde (MDA) in all groups

	TAC (mmol/L)	**MDA (µmol/L)**
Control	12.04 ± 0.29	24.77 ± 0.79
Nicotine	11.74 ± 0.06	29.17 ± 1.68
Nicotine + bupropion	13.15 ± 0.38	15.14 ± 0.60$^{\#}$
Nicotine + flower extract	12.03 ± 0.42	28.67 ± 2.14
Nicotine + stem extract	11.83 ± 0.21	23.45 ± 2.03
Nicotine + leaf extract	12.34 ± 0.15	18.99 ± 0.89$^{\#}$

Data are Mean ± SEM. $^{\#}$ $p < 0.01$ when compared to control and nicotine-treated groups. # $p < 0.01$ when compared to the flower treated-group (n = 6; 3 male and 3 female).

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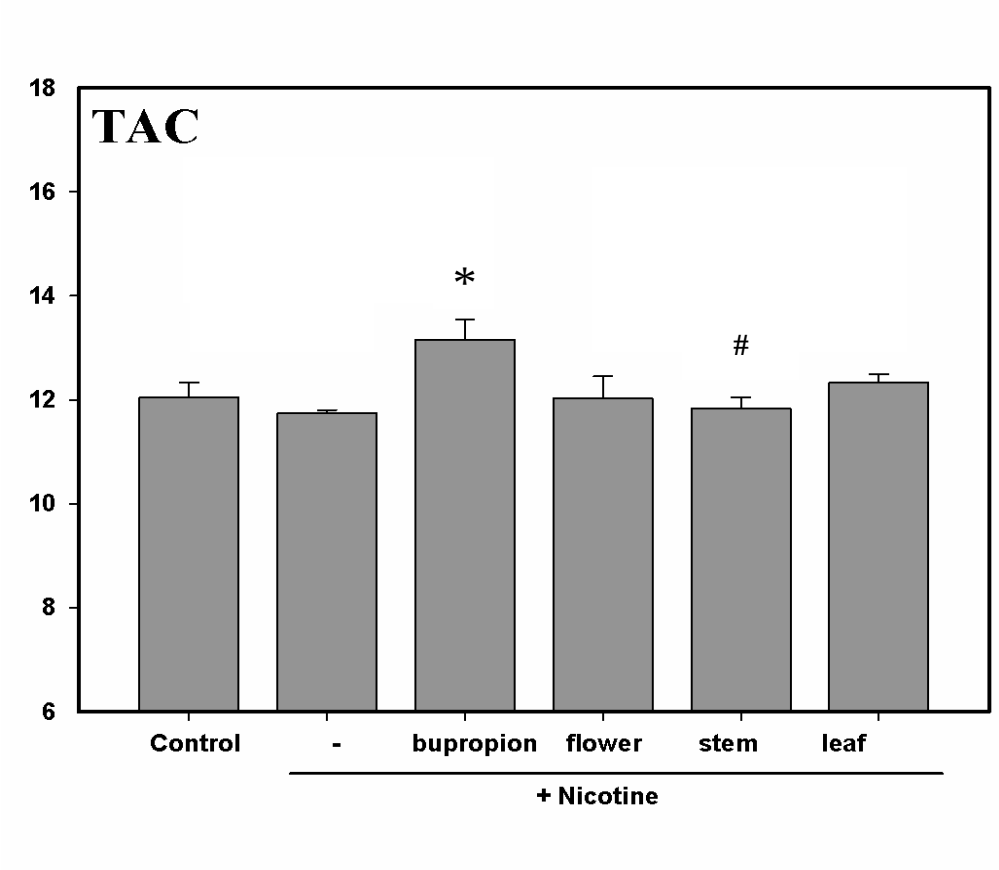

Figure 4.5 Total antioxidant capacity (TAC) levels in plasma after treatment with VC extracts (stem, flower and leaf), control with normal saline solution and bupropion in nicotine-treated rats (n = 6; 3 males and 3 females). * $p < 0.01$ when compared to nicotine-treated group. # $p < 0.05$ when compared to bupropion-treated group.

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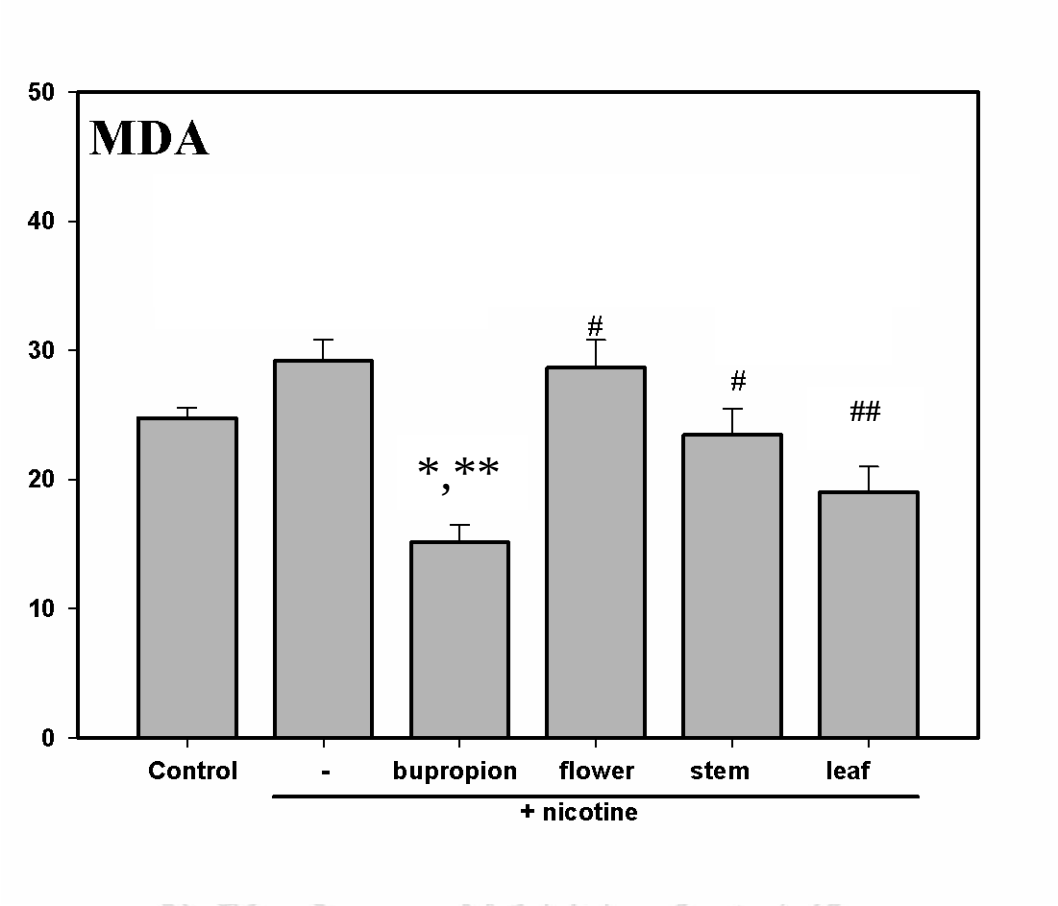

Figure 4.6 Malondialdehyde (MDA) levels in plasma after treatment with VC extracts (stem, flower and leaf), control with normal saline solution and bupropion in nicotine-treated rats (n = 6; 3 males and 3 females). * $p < 0.01$ when compared to nicotine-treated group. ** $p < 0.01$ when compared to control group. # $p < 0.05$ when compared to bupropion-treated group. ## $p < 0.01$ when compared to flower-treated group.

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4.2.3 Toxicity on chromosome in rats

The results of chromosomal analysis on bone marrow cells at the metaphase stage in nicotine-treated and positive control (PC) male Wistar rats have been summarized in **Table 4.5**.

After VC stem, flower, or leaf extract had been administered orally at a single dose of 2 g per kg body weight, comparing to the positive control with CP intraperitoneally injected at 50 mg/kg body weight. The results of the percentages of mitotic index (MI) in both male and female groups were presented. There was no statistical difference in any VC extract treated groups (flower = 6.34 ± 0.13%, stem = 6.30 ± 0.22% and leaf = 6.30 ± 0.20%), when compared to the distilled water treated male group (6.28 ± 0.16%). Whereas, a significant decrease in % MI was significantly seen after treatment with cyclophosphamide (3.96 ± 0.23%) ($p < 0.01$). Additionally, CP also induced chromosomal damage (5.80 ± 1.59 per cell). Moreover, the chromosome aberration also presented with break (6.80 ± 2.76%), exchange (1.00 ± 0.77%) and multiple aberration (1.2 ± 0.49%) pattern significantly when compared to distill water or any extract treated groups. In the female group, the %MI results was similar to the male group, with no significant difference in any of the extracts (flower = 6.28 ± 0.11%, stem = 6.02 ± 0.19% and leaf = 6.12 ± 0.19%), when compared to the control group (5.80 ± 0.33%). Whereas, the %MI also decreased significantly after treatment with CP (4.10 ± 0.14%) ($p < 0.01$), which induced chromosomal damage (9.20 ± 0.97 per cell) and significantly different types of chromosomal aberration (21.20 ± 0.14% break, 2.00 ± 0.84% exchange and 1.40 ± 0.51% multiple aberration), when compared to other groups.

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Figure 4.7 shows the characteristic of chromosome damage from a single dose administration comparing the positive agent as cyclophosphamide (CP). From the chromosome slides after extract treatment did not present any changes both in male and female rat groups. Whereas the chromosome slide in cyclophosphamide treated group demonstrated the various type of chromosome damage such as exchange and breaking pattern.

Table 4.5 Mitotic index (MI); types of chromosomal aberration and damage in various extract-treated rats

Male	M.I. (%)	Types of Chromosomal aberration (%)			Damage per Cell
		Break	Exchange	Multiple Ab.	
Distilled water	6.28 ± 0.16	0	0	0	0
Stem extract	6.30 ± 0.22	0	0	0	0
Leaf extract	6.30 ± 0.20	0	0	0	0
Flower extract	6.34 ± 0.13	0	0	0	0
Cyclophosphamide	3.96 ± 0.23*	6.80 ± 2.76*	1.00 ± 0.77*	1.20 ± 0.49*	5.80 ± 1.59*
Female					
Distilled water	5.80 ± 0.33	0	0	0	0
Stem extract	6.02 ± 0.19	0	0	0	0
Leaf extract	6.12 ± 0.19	0	0	0	0
Flower extract	6.28 ± 0.11	0	0	0	0
Cyclophosphamide	4.10 ± 0.14*	21.20 ± 1.62*	2.00 ± 0.84	.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 (n = 10). * Percentage of Mitotic Index (% MI). * $p < 0.01$ compared to distilled water and VC extract groups.

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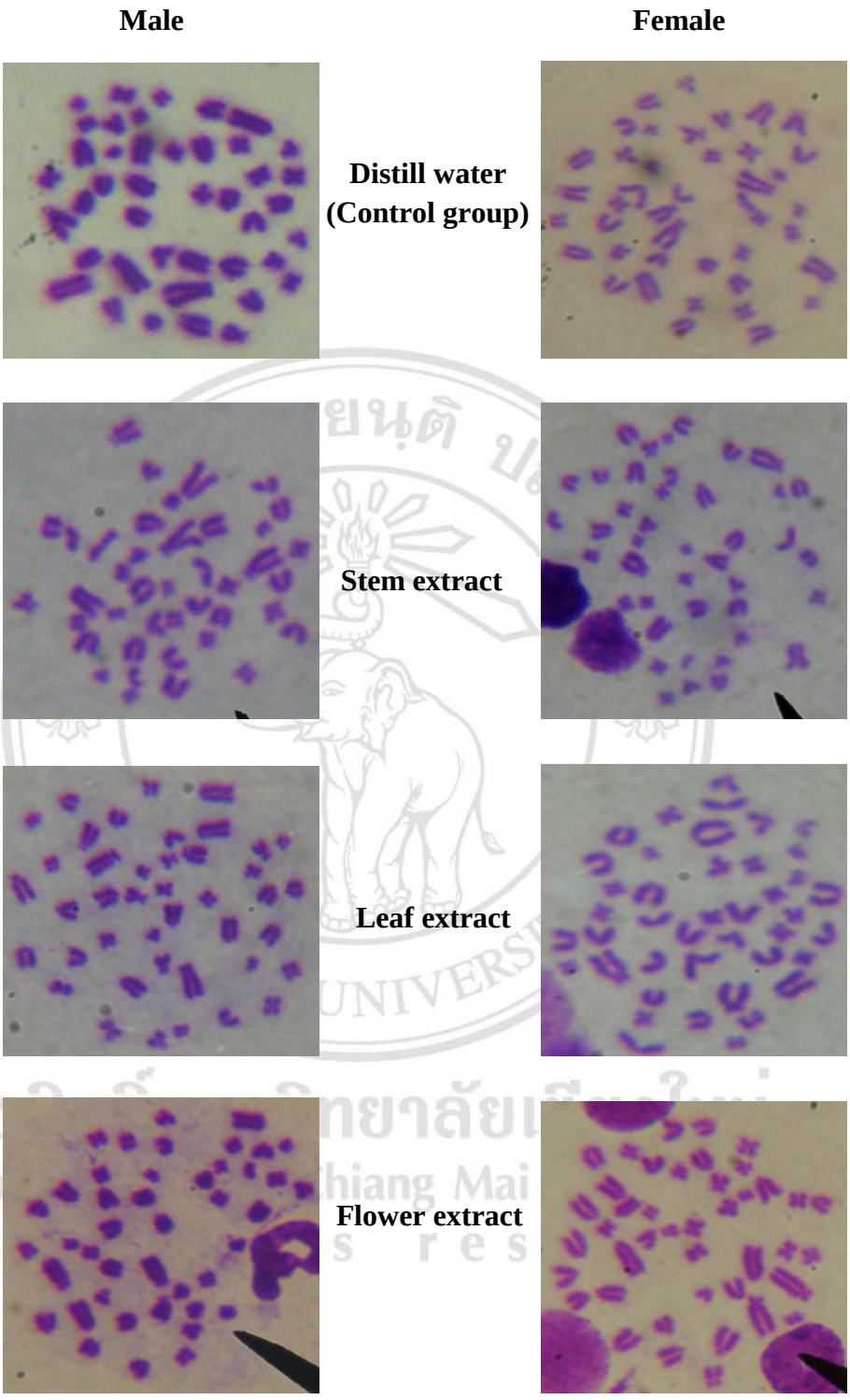

Figure 4.7 Characteristics of chromosomal aberrations from the bone marrow of male and female Wistar rats after treatment with distilled water, and VC extracts from the stem, leaf and flower, compared to cyclophosphamide (Giemsa stain, 1000x).

Cyclophosphamide (CP) treatment

Male **Female**

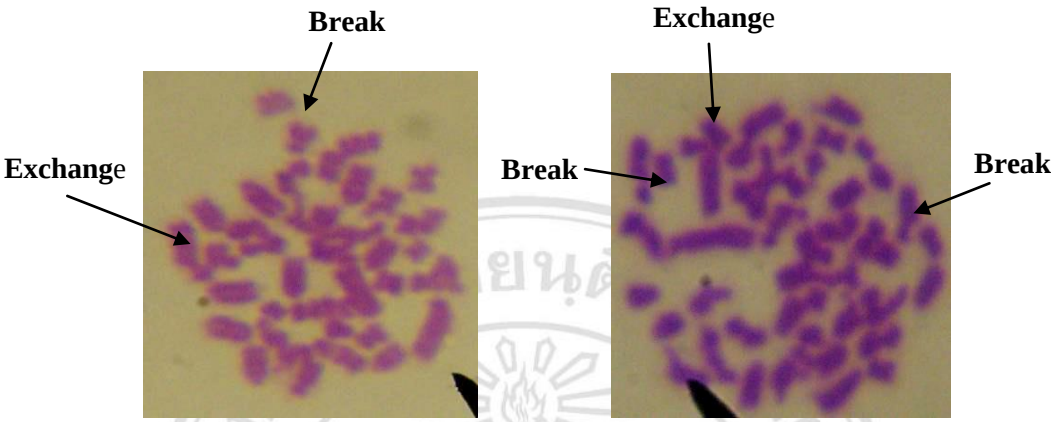

Figure 4.7 (**Cont.**) Characteristics of chromosomal aberrations from the bone marrow of male and female Wistar rats after treatment with distilled water and VC extracts from the stem, leaf and flower, compared to cyclophosphamide (Giemsa stain, 1000x).

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