CHAPTER 5

DISCUSSION AND CONCLUSION

From the overall results of *Vernonia cinerea* Less. (VC) extracts in this study is possibly support to the previous study on the efficiency on smoking cessation in clinics, especially antioxidant activity, active compounds, including neurotransmitters and acute toxicity on chromosome. From the previous study about the VC either in a tea bag that could help to stop smoking in clinic (**Wongwiwatthananukit, 2003**) or prepared by boiling in water and evaporation system also can stop smoking and increases on antioxidant status in active smokers (**Leelarungrayub *et al.*, 2010**).

In this study, different parts of the VC showed varied antioxidant activities such as total antioxidant capacity (TAC). Also, leaf extract presented the highest total antioxidant capacity (TAC), when compared to flower and stem extracts. Moreover, The stem extract had the significant highest scavenging activity on nitric oxide (NO) (0.91 ± 0.23 mg/mL), when compared to the flower extract (1.08 ± 0.11 mg/mL) and leaf extract (2.77 ± 0.75 mg/mL) ($p < 0.01$). The stem and flower extracts presented 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 in the flower extract (1.68 ± 0.23 mg/mL) when compared to that from the stem extract (3.03 ± 0.12 mg/mL) and leaf extract (3.90 ± 0.13 mg/mL), respectively ($p < 0.01$) (**Table 4.1**).

From the results showed the dominant active compound, especially, the total phenolic in leaf extract was higher than in flower and stem extracts, as same as the total tannin in flower and leaf extracts (**Table 4.2**). From the results showed the interested active compounds and its antioxidant activity that is possibly to support the previous study on the blood antioxidant activity in smokers after supplemented the VC condense

juice (**Leelarungrayub *et al.*, 2010)**. Although the extract protocol in this study with evaporation in hot water and freeze dry technique, the interested results of catechins (ECG, EC and C), isoflavone (diadzin and fenistin), and flavonoid (myricetin and quercetin) (**Figure 4.2**) in leaf extract were higher than in flower and stem extracts signficantly. Therefore, these results are similar to the previous results in VC from methanolic extraction (**Misra *et al.*, 1984**; **Azevedo *et al.*, 2013**) that showed the active compounds as flavonoid (**Misra *et al.*, 1993**), phenols and tannins (**Latha *et al.*, 1998**). However, there are still other active compounds such as N-hexadecanoic acid, 1, 2-benzene-dicarboxylic acid, squalence, caryophyllene oxide, guaiol, octadecanoic acid and 9, 12-octadecanoic (**Prasopthum *et al.*, 2015**) that did not evaluated in this study. Therefore, the prepared protocol either from methanol or water also presents the similar results. Interested results of another compounds; nitrate and nitrite that found in stem and leaf extracts, non in flower part. Whereas the caffeine could not identified in this study. Surprising results in this study that represented the low concentration of nicotine in leaf and flower extracts that did not identified before and possible relates to the benefits on withdrawing on smoking. Therefore, the possible mechanism on help to stop smoking is possible from the nicotine replacement. Although, in the previous study of Wongwiwatthananukit and co-worker with mixed crude VC in tea bag in clinic showed the adverse events such as upper abdominal pain (21.9%), nausea (28.1%), headache (40.6%), palpitation (15.6%), drowsiness (59.4%) and craving reduction (59.4%) (**Wongwiwatthananukit *et al.*, 2009**), whereas the nausea (10.5%) and headache (5.2%) were presented only initial week of supplementation with VC condensed juice in the Leelarungrayub's study (**Leelarungrayub *et al.*, 2010**). Different preparation in both studies between a tea bag and condensed juice, the nicotine concentration possibly involves these adverse effects. Unfortunately, there was no reported on the nicotine level in a mixed VC tea bag in the last study (**Wongwiwatthananukit *et al.*, 2009)**, but the lower level of nicotine in the a mixed VC tea bag may be possibly explained relating to the more adverse symptoms than in the condensed VC juice containing more nicotine.

From the previous evidences about active compounds (**Misra *et al.*, 1993; Latha *et al.*, 1998),** and anti-inflammatory activity in animal study (**Mazumder *et al.*, 2003**).

Moreover, how the antioxidant status was improved after supplement with condensed VC juice in active smokers (**Leelarungrayub *et al.,* 2010**). Therefore, scavenging activity by flavonols or catechins on radicals; NO, $O_2^{\bullet-}$, and $OH^{\bullet}$ had also confirmed. Previous evidences has been strongly confirmed that flavonoids and other phenolic compounds could scavenge the organic radicals as DPPH (**Seyoum *et al.,* 2006**; **Zhang *et al.,* 2012).** But the controversial results in this study showed that the dominant activity on radial scavenging is presented in stem extract, whereas the active compounds especially catechins, isoflavone and flavonoids were lower when compared to the leaf extract. In basic theory of those active compounds should be more activity. Possibly, although all active compounds can be identified by HPLC, but non-purity compounds or complex structure may non-active in radical scavenging protocols. Moreover, there also have many active compounds in each VC parts that did not identified on which possibly effects to the results in this study.

From, the previous studies when applied this VC plant in all active smokers had been reported on an interested adverse effect with the tongue numbness and dislike for the taste or smell of cigarette smoke at 46.9% (**Wongwiwatthananukit *et al.,* 2009**) and 100% (**Leelarungrayub *et al.,* 2010**). From unpublished information has been proposed that nitrite and nitrate in VC is possible a direct substance to the tongue numbness.

In the second experimental study, the effects of extracts on catecholamine neurotransmitter had been evaluated in rats. In basic knowledge of catecholamine neurotransmitters is replied on three important neurotransmitters; dapamin, noradrenaline and adrenaline (**Kopin, 1964**). Dopamine is found in many tissues, especially localized in relatively high concentration in the brain, e.g. basal ganglia, and is conversed to noradrenaline and adrenaline in the adrenal medulla respectively that generally accepted as the major transmitter substance for sympathetic system. When the nicotine is preserved in the blood circulation, it can bind to a nicotine receptor and directly effects on the dopamine level by inhibition the dopamine catabolism to noradrenaline and adrenaline. Previous data confirmed that nicotine induces dopamine release in the brain (**Benowitz *et al.,* 2002**), thus, the noradrenaline and adrenaline may be increased after the dopamine is metabolized by beta-hydroxylase (DBH) and

phenylethanolamine N-methyltransferase (PNMT) activities, respectively (**Kapoor and Jones, 2005; Rossi *et al.*, 2005**). The high nicotine also affects the release of other substances such as acetylcholine, serotonin, nor-epinephrine, glutamate, vasopressin, beta-endorphin and gamma-aminobutyric (GABA) (**Cryer *et al.*, 1976**). Nicotine is able to activate the sympathetic nervous system, which increases heart rate (HR) and blood pressure (BP) (**Irving and Yamamoto, 1963**), stroke volume (SV) and cardiac output (CO) (**Bargeron *et al.*, 1957**) and coronary blood flow (**Vezina *et al.*, 2007**). In experimental design, a previous evidence showed 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 could be a lethal dosage in adult humans (**Okamoto *et al.*, 1994**). Therefore, the dose of nicotine supplement in rats was 0.6 mg/kg that is sufficient dose and safely in rats. Moreover, the route of nicotine intake for fast bioavailability and maximum venous blood concentration is depend on the type of nicotine product, such as cigarette (1-2 mg), nicotine gum (1 or 2 mg), nicotine inhaler (2 mg/cartridge) or nicotine patch (15-22 mg), with the time of maximum concentration possibly varies from 10 to 30 minutes (**Stratton *et al.*, 2001**). Furthermore, a previous study showed that daily subcutaneous nicotine injection at 0.4 mg/kg body weight in rats was able to induce nicotine tolerance, and affected on locomoter activity from nicotine addiction in only 12 days (**Cohen *et al.*, 1991**). Thus, 20 days of nicotine injections and co-feeding with different VC extracts was designed and compared to the bupropion (standard nicotine antagonist agent) (**Slemmer *et al.*, 2000**). Bupropion 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). Therefore, this interaction can help to reduce withdrawal symptoms from nicotine cessation or maintains the dopamine in the blood (**Slemmer *et al.*, 2000**).

Table 4.3 shows the results of this study. After injection the nicotine, the level of dopamine increased significantly when compared to non-treated group, whiling the noradrenaline and adrenaline level slightly reduced. This result is also supported by the previous evidence of dopamine releasing by nicotine in the mesolimbic system relating to nAChRs (**Koob and Yolkow, 2010; Slemmer *et al.*, 2000**). When co-treated with the bupropion, dopamine level decreased and noradrenaline or adrenaline improved

significantly. This can be explained by antagonism with nicotine receptor of bupropion, and blocking the uptake of synaptic dopamine (**Prasopthum *et al.*, 2015; Paterson, 2009**). In medical treatment in among of smokers, it also has other potentially therapeutic actions, such as blockage of nicotinic acetylcholine receptor and norepinephrine uptake (**Paterson, 2009**). Interestingly, treatment with stem, flower or leaf extracts showed dominantly on lower dopamine levels as same as in bupropion-treatment. But, the levels of noradrenaline or adrenaline were lower after treated with flower and stem extracts. Only in a leaf treated group, that presented the similar results of all neurotransmitters as the bupropion function. Higher levels of noradrenaline and adrenaline in the body has been proposed to many advantages such as increased blood pressure, respiration, and peripheral nervous alert, whereas adrenaline effects on the central nervous system by enhancing respiration and increasing muscle activity (**Prasopthum, 2015**). In this study, the mechanism of flower and stem extracts on inhibition the dopamine is still unclear, especially the leaf extract can increase the noradrenaline and adrenaline levels. But in previous research proved that phenolic monoterpenoid, as a carvacrol in many plants, could able to bind to AChRs at a binding site distinct from nicotine (**Tong *et al.*, 2013**) and possibly stimulated the function of mitochondrial monoamine oxidases (MAOs) (**Fowler *et al.*, 2003**), which increased dopamine degradation to noradrenaline and adrenaline (**Prasopthum, 2015; Slemmer *et al.*, 2000**). This hypothesis can support the result of total phenolic content in this study that presented the highest in the leaf extract and significant increased of noradrenaline and adrenaline levels after treatment compared to the flower or stem extract treatment. Moreover, the results of noradrenaline and adrenaline levels after leaf treatment were similar to those in the bupropion treated group. Thus, the hypothesized pathway on inhibition of AChRs may be possibly, but it still needs to be confirmed in the future.

Part of the results from oxidative stress on TAC and MDA levels. The reason for the non-significant increase on MDA level in the nicotine-treated group can be explained by condition of its oxidative stress, which supported in a previous results (**Crowley-Weber *et al.*, 2003**). Nicotine is possible an up-regulator or trigger the oxidative stress under NF-κB and apopotosis pathway, with decreased glutathione

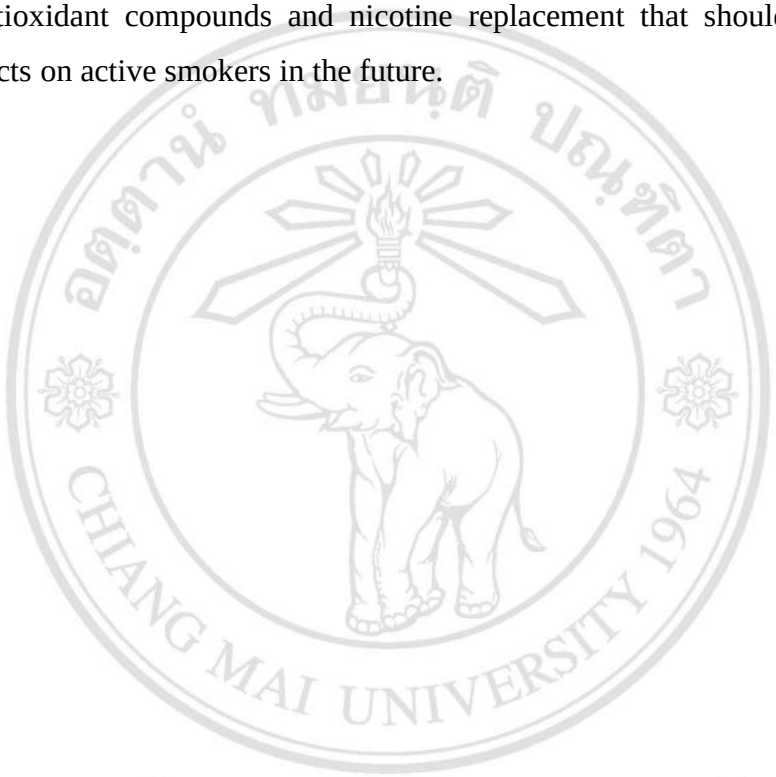
(GSH) and increased MDA (**Yildiz *et al.*, 1999**). A slightly increases on TAC and significantly lower on MDA levels after treatment with bupropion, when compared to the control group, is unclear and controversial. Although, the update evidence proves that bupropion is not an antioxidant agent, but has the distinguished function of inhibiting dopamine and noradrenaline reuptake or the post-synaptic acetylcholine nicotine receptor (**Slemmer *et al.*, 2000**). But recent evidence has been proposed and reported that bupropion can modulate NO synthesis and nitrosative stress-signaling pathway in rats (**Dhir and Kulkarni, 2007**), that possibly supported the mechanism of bupropion on NO pathway in this study. Interesting in the results after co-treated with different VC extracts, especially in leaf extract with the lowest MDA level and highest TAC level when compared to flower or stem extracts and also non-significantly different to the bupropion treated group. Furthermore, dominant results in a leaf treated group on MDA and TAC status still need be confirmed.

Finally, the results of mutagenic effect from a single high dose treatment of all extracts in chromosome from bone marrow in both male and female rats are presented in **Table 4.5.** Cyclophosphamide (CP) is used as a positive toxicity inducer in the system. It is an alkylating agent and the most commonly used anticancer, chemotherapeutic drug, which damages chromosomes through generating free radicals and alkylating on DNA (**Povirk and Shuker, 1994**). It is often used as a positive control even in acute genotoxic test in any animal models, presenting chromosomal aberrations, chromosome break, chromatid break, chromatid exchange, chromosomal exchange and ring chromosome.

After oral administration of VC extracts from the stem, leaf and flower at 2 g per kg body weight, and cyclophosphamide injected intraperitoneally, the toxicity was evaluated within 24 hr. This dose of VC extracts was designed according to the standard protocol for testing by the Organization for Economic Cooperation and Development (**OECD, 2000**). The results in **Table 4.5** show no mutagenic effect in all groups after treated with different VC extracts, as in the control group. Whereas, in the cyclophosphamide treated group, a significant reduction of % MI and chromosomal aberration or damage have been presented in **Table 4.5** and **Figure 4.7**. Therefore, the result possibly indicates the safety condition from administrated any VC extracts under

dose of 2 g per kg body weight that can possibly apply in human. However, the side effects of long term administration is still not evaluated and studied in the future.

From overall in this study between in *vitro* and in animal model that showed different results, especially relating catecholamines and oxidative stress with many active compounds. Especially, non-selective part of VC materials in order to help on stop smoking is proposed better than using with only stem, flower or leaf part because of various antioxidant compounds and nicotine replacement that should have more beneficial effects on active smokers in the future.



Conclusion

The results in both *in vitro* and *in vivo* studies show interested antioxidant compounds and activity of *Vernonia cinerea* Less. (VC); for example, tannin, catechine, flavonoid and some compounds as nitrate, nitrite and nicotine. Especially, the leaf VC extract has the dominant active compounds and activity when compared to the stem and flower extracts, including involves the catecholamine neurotransmitters by reduction on dopamine and enhances noradrenaline or adrenaline releasing. Finally, a high dose of all VC extracts does not any mutagenic effects on chromosomes. Therefore, clinical application of *Vernonia cinerea* Less. (VC) that is a natural plant that shows the possibility for apply to stop smoking or a part of smoking cessation program combined with psychological consulting or behavior adjustment. However, the many controversial issues on its mechanisms need to be investigated and proven, especially relating to other neurotransmitter receptors and gene regulators that may answer how VC can help to stop smoking.