International Journal of Agriculture and Environmental Research

ISSN: 2455-6939

Volume:02, Issue:04

PHYTOCHEMICALS AND CHEMICAL MARKERS OF *Orthosiphon stamineus* IN RESPONSE TO ORGANIC FERTILIZERS

Running title : Biochemical properties of *Orthosiphon stamineus*

Esther Yap Shiau Ping[1], Siti Hajar Ahmad[1*], Rosenani Abu Bakar[2], Khozirah Shaari[3], Surisa Phornvillay[1], Nur Indah Abdul Shukor[1], Tan Xue Yi[1]

[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

[2]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

[3]Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

ABSTRACT

Objective: To determine the effect of different types and rates of organic fertilizers on the phytochemicals and chemical markers of *O. stamineus.*

Methods: Animal- and plant-based fertilizers with equivalent rates of 0, 100, 200, 300 and 400 kg N/ha were applied. Plants were then harvested at 8 weeks after transplanting for extraction using 70% ethanol. Total phenolic contents (TPC) were determined by using the Folin-Ciocalteu method, total flavonoid contents (TFC) by using complexation with aluminum chloride (AlCl3) and antioxidant activity by DPPH radical scavenging assay. Sinensetin (SEN) and rosmarinic acid (RA) were both quantified by high performance liquid chromatography (HPLC).

Results: TPC were not significantly affected by fertilizer types and rates. TFC showed a quadratic regression trend for plant-based fertilizer. Both animal- and plant-based fertilizer showed a linear decrease in DPPH as fertilizer rates increased. RA showed a linear decrease for plant-based fertilizer as rates increased whereas a quadratic trend was seen for animal-based fertilizer. SEN showed a quadratic trend only for animal-based fertilizer but no relationship for plant-based fertilizer.

ISSN: 2455-6939

Volume:02, Issue:04

Conclusions: Fertilizer type and rates influenced the amount of phytochemicals and chemical markers that the plants produced. The suitable fertilizer type and rate to produce optimum phytochemicals and chemical markers is plant-based fertilizer at 300 kg N/ha.

Keywords: Rosmarinic acid, Sinensetin, *Orthosiphon stamineus,* Fertilizer types and rates

1. INTRODUCTION

Traditional medicine usages have been on the rise since the 1990s in many developed and developing countries [1]. The reasons for this rise are due to lower costs of herbs as compared to synthetic drugs, the need for alternative treatments for drug-resistant pathogens and increasing popularity for products that are natural and environmental friendly [2]. Organic production is the most likely answer to cultivating herbs for medicinal purposes. It is a production system that takes into consideration the health of soils, ecosystems and people and discourages use of inputs with adverse effects but relies on ecological processes, biodiversity and cycles adapted to local conditions [3]. Organic farming of tea plantation was reported to help in increasing leaf growth by 23% and also major polyphenols by 38% [4]. Use of organic inputs such as organic and bio-organic fertilizers in production of sweet fennel increased fresh yield (20%), total phenolic contents (45%) and total flavonoid contents (51%) [5]. Different levels of organic nitrogen fertilizers also help in the improvement of biomass yield and nutrient absorption in herbs such as Stevia rebaudiana and Clinacanthus nutans [6,7].

Orthosiphon stamineus has been identified as a potential high value herbal medicinal product. The plant has high antioxidant compounds such as flavones, polyphenols, bioactive proteins and glycosidesin which give the plant its diuretic, hepatoprotective, antifungal, antimicrobial and antidiabetic roles in human health [8,9,10,11,12]. The two main chemical markers which make O. stamineus a valuable medicinal herb are rosmarinic acid (RA) and sinensetin (SEN). RA, a phenolic acid present in plants as a secondary metabolite, is a water-soluble ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid. These compounds can be found naturally in many plants, especially in the species of Boraginaceae and Lamiaceae [13,14]. RA has been reported to be an antioxidant, possessing anti-inflammatory, antiapoptotic, antifibrotic and antimicrobial activities as well as neuroprotective and neurorescue effects [15,16,17]. SEN, a flavonoid, is a rare polymethoxylated flavone (PMF) and is mostly found in citrus plants with 5-methoxy groups on the basic benzo-γ-pyrone skeleton with a carbonyl group at the C4 position. SEN has been reported to show anti-inflammatory activity, antioxidant potential and antiproliferative activity [18,19,20].

The common practices by farmers to produce O. stamineus are by using a combination of both chemical and organic fertilizers. For example, 10 t/ha chicken manure combined with 1 t/ha

ISSN: 2455-6939

Volume:02, Issue:04

inorganic fertilizer NPK Green (10% N) are recommended for BRIS soils and half the rates are needed if the plant is planted on alluvial soil [21]. Organic fertilizers such as chicken dung as a source of fertilizer was also reported to give a higher dry matter yield of O. stamineus (135 kg/ha) when applied at the rate of 0.9 kg/plant as compared to oil palm empty fruit bunch and cow dung [22]. Organic fertilizers are mixed into the soil one week before planting to ensure that the organic fertilizers are able to decompose and react with the soil to allow ease of absorption by the roots [23].

Organic fertilizer refers to soil amendments, from natural sources such as plant and animal by-products, with minimum amount of nitrogen, phosphate, and potash [24]. Organic fertilizers help to increase or maintain the organic matter in the soil, act as a slow release nutrient for the plants, thus increase or maintain yield of plants. Combinations of oil seed extract and liquid fish produced the same quality and yield of Calibrachoa as compared to using chemical fertilizers [25]. An increase in onion marketable yield fertilized by organic fertilizer by 1.9 t/ha as compared to chemical fertilizer was also reported [26]. The benefit of organic fertilizer in crop improvement was further strengthen in a study whereby broiler litter based organic fertilizer has shown to increased yield of containerised marigold production in glasshouse by 30% as compared to control [27].

However, the amount of chemical compounds produced by herbs is not always consistent and insufficient, thus the compounds are often altered structurally to produce drugs that are more active than the original compounds. It is expected that the use of organic fertilizer could increase the phytochemical and chemical marker contents in plants. Thus, the objective of this study was to determine the effect of different types and rates of organic fertilizers on the phytochemicals and chemical markers of *O. stamineus.*

2. MATERIALS AND METHODS

2.1 Media preparation and seedling transplanting

The media for transplanting *O. stamineus* seedlings comprised soil of Bungor series with 63% clay, obtained from Field 16, Universiti Putra Malaysia (UPM), Serdang (2.9917˚ N, 101.7163˚ E), Selangor, Malaysia. The soil was sieved using a 1-cm mesh and 8 kg of the soil was filled into a 35.6 cm x 35.6 cm black polybag. Soil amendments at rates of 7.8 g chicken manure and 13.3 g rice husk biochar were added into each polybag. Then, the polybags were arranged at a distance of 45 cm between rows x 35 cm between plants under semi-controlled glasshouse condition. A 4-week old rooted cuttings of *O. stamineus* was transplanted into each polybag a week after application of the soil amendments. The plants were treated with two types of organic fertilizers, an animal-based (50% chicken manure) and a plant-based (20% chicken manure), at

ISSN: 2455-6939

Volume:02, Issue:04

equivalent rates of 0, 100, 200, 300 and 400 kg N/ha. The fertilizers were applied during transplanting of cuttings. Watering of the plants was done manually once a day while hand weeding was done at least once a week.

2.2 Harvesting and drying

The plants were harvested at 8 weeks after transplanting by cutting the basal stem at 15 cm from the soil level, leaving behind 3 to 4 buds for the next ratoon growth, using a sharp pair secateurs. The harvested plants were packed in labeled baskets and transported to the Postharvest Laboratory, Department of Crop Science, Universiti Putra Malaysia. The plant samples were washed to remove dirt and soil, air dried to remove excessive water, cut to a length of 10 cm and packed into labeled 40 cm × 15 cm paper bags with 70 g of sample in each bag. The plants were harvested at 8 weeks after transplanting. Dry weight biomass yield was calculated based on the spacing of plants at 45 cm × 35 cm in a 9 m × 5 m plot.

2.3 Extraction of plant samples

Metabolites from dried *O. stamineus* plants were extracted with some modifications [28]. Dried ground samples of 0.2 g were weighed and extracted with 5 ml of 70% ethanol for an hour at 40 ˚C using a sonicator (Fisherbrand FB15055, United Kingdom). The extracts were then filtered using a Whatman No. 5 filter paper and the filtrate was used for the quantification of antioxidant compounds and activities. The whole process was repeated three times using a fresh solvent at each extraction.

2.4 Total phenolic contents determination

The total phenolic content (TPC) in the extracts were determined by using the Folin-Ciocalteu colorimetric method some modifications [29]. A total of 200 µl of extract was mixed with 1.5 ml Folin-Ciocalteu reagent and 1.5 ml sodium carbonate (Na_2CO_3). A blank was also prepared by replacing 200 µl of extract with 70% ethanol. The mixture was then incubated in the dark for 2 hours at ambient temperature before measuring the absorbance against the blank using a spectrophotometer (Fisher Thermo Scientific, Multiskan Go, United Kingdom) at wavelength of 725 nm. The data were compared with gallic acid (Scharlau, Spain) external calibration curve to give the concentration of TPC as gallic acid equivalent and was calculated using the following equation: ((GAE equivalent × V × D × 10^{-6} × (100/W))/100) × 1000 (V = total volume of sample in ml; D = dilution factor; W = weight of sample in gram).

2.5 Total flavonoid contents determination

ISSN: 2455-6939

Volume:02, Issue:04

Total flavonoids content (TFC) of the extract was determined with some modifications [28]. A total of 1 ml extract was mixed with 0.3 ml sodium nitrate ($NaNO_3$), 0.3 ml aluminium chloride ($AlCl_3$) and 2 ml sodium hydroxide (NaOH). A blank was also prepared by replacing 1 ml of extract with 70% ethanol. The mixture was then measured against the blank using the same spectrophotometer as the above at wavelength of 510 nm. The data were compared with quercetin (Sigma-Aldrich, Germany) external calibration curve to give the concentration of TFC as quercetin equivalent and calculated using the following equation: ((QE equivalent × V × D × 10^{-6} × (100/W))/100) × 1000 (V = total volume of sample in ml; D = dilution factor; W = weight of sample in gram).

2.6 2,2-diphenyl-1-picrylhdrazyl (DPPH) free radical scavenging activity

The DPPH radical scavenging capacity of the extract was determined with some modifications [30]. A total of 1 ml of extract was mixed with 2 ml ethanolic DPPH (Sigma-Aldrich, Germany) and stored in the dark for 30 minutes. A blank was also prepared by replacing 1 ml of extract with 70% ethanol. Subsequently, the extract and blank were measured using the above spectrophotometer at a wavelength of 517 nm. The percentage of DPPH radical scavenging capacity was calculated using the following equation: ((Ab – Ae)/Ae) x 100% (Ab = absorbance of blank at 517 nm; Ae = absorbance of extract at 517 nm).

2.7 HPLC analysis of chemical markers, sinensetin and rosmarinic acid

A total of 5 ml extract was filtered through a 0.45 µm nylon membrane filter prior to HPLC analysis. An Agilent HPLC (1200 series), utilized to perform the analysis, was equipped with an autosampler, column, oven and UV/VIS detector. A C18 HPLC column with a specification of 5 microns at 250 mm long and 4.6 mm wide was used. The samples were analyzed using a gradient mobile phase 0.1 % formic acid: deionized water. Each sample was analyzed at the gradient mobile phase flow rate of 1 ml/min, detector wavelength of 320 nm at 40 ˚C for 25 minutes by gradient flow (Table 1). For qualification and quantification purposes, a calibration curve for each compound was made with the standard marker compounds, SEN and RA.

ISSN: 2455-6939

Volume:02, Issue:04

Table 1. Gradient elution program used in separation of sinensetin and rosmarinic acid.

Time	Solvent ratio	
	A (0.1% Formic Acid)	B (Acetonitrile)
0	90	10
3	90	10
5	85	15
8	85	15
18	35	65
21	85	15
22	90	10
25	90	10

2.8 Experimental design and statistical analysis

The experiment was conducted using a randomized complete block design in two factorial arrangements of treatments, with four replications and three plant subsamples per replication. The treatments were two types of organic fertilizers (plant- and animal-based) at five rates of each organic fertilizer (0, 100, 200, 300, 400 kg/ha N). The results were statistically analyzed using analysis of variance (ANOVA) of SAS version 9.3 (SAS Institute, Cary, NC) and means were compared by Tukey test at P = 0.05. Regression analysis was conducted to study the relationship between the independent and dependent variables when interactions between treatments existed.

3. RESULTS

3.1 Total phenolic content

The TPC of the plants in the current study were not significantly affected by the interaction between fertilizer type × fertilizer rates. Application of either animal- or plant-based fertilizers did not affect the TPC (Table 2). Increasing the fertilizer rates from 0 to 400 kg/ha N resulted in no significant linear or quadratic effects on the TPC of the plants.

ISSN: 2455-6939

Volume:02, Issue:04

Table 2. Main and interaction effects of two fertilizer types and five fertilizer rates on total phenolic contents, total flavonoid contents and 2,2-diphenyl-1-picrylhdrazyl (DPPH) free radical scavenging activity of *Orthosiphon stamineus*.

Factor	Total phenolic contents (mg GAE/g DW)	Total flavonoid contents (mg QE/g DW)	DPPH free radical scavenging activity (% inhibition)
Fertilizer type (FT)			
Animal-based	15.94	28.50	72.13
Plant-based	19.37	29.59	72.76
Significance	NS	**	**
Fertilizer rates (FR) (kg/ha N)			
0	17.61	30.62	74.43
100	20.68	23.85	73.20
200	15.25	29.35	71.96
300	14.86	27.99	71.60
400	19.87	25.93	71.04
Significance			
Linear	NS	NS	**
Quadratic	NS	NS	*
FT × FR	NS	**	*

*,**,NS = significant and nonsignificant at P = 0.05 or 0.01, respectively, (n = 12).

Mean comparison of fertilizer types by F test at P = 0.05.

ISSN: 2455-6939

Volume:02, Issue:04

3.2 Total flavonoid content

In the present experiment, there was a significant interaction effect between fertilizer type × fertilizer rates. The relationship of simple effects of fertilizer rates on TFC was quadratic for plant-based fertilizer, while the effect of animal-based fertilizer did not fit any tested regression model (Figure 1). For the plant-based fertilizer, TFC decreased with increasing fertilizer rates, then followed by a gradual decrease. Thereafter, TFC increased in response to fertilizer rate but lower by 17% at 400 kg/ha N as compared to the control.

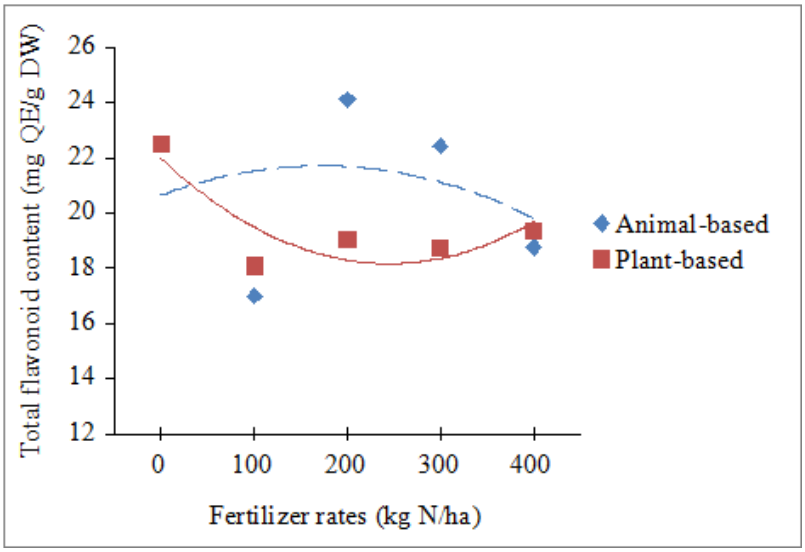

Figure 1. Relationship between total flavonoid content of *Orthosiphon stamineus* and fertilizer rates. The solid line indicates a significant quadratic regression trend at P=0.05, (n = 12). For plant-based fertilizer, $y = 29.84 – 0.042x + 0.000086x^2$, $R^2 = 0.48$.

3.3 2,2-diphenyl-1-picrylhdrazyl (DPPH) free radical scavenging activity

DPPH free radical scavenging activity was significantly affected by fertilizer type × fertilizer rates interaction. Animal-based fertilizer applied to plants at a rate 200 kg/ha N caused a linear decrease (R^2=0.71) of 3% in the DPPH radical scavenging activity as compared to the control (Figure 2). The plant-based fertilizer also followed the same decreasing trend as animal-based fertilizer; however, the antioxidant activity was higher than animal-based fertilizer at all rates of nitrogen (Figure 2).

ISSN: 2455-6939

Volume:02, Issue:04

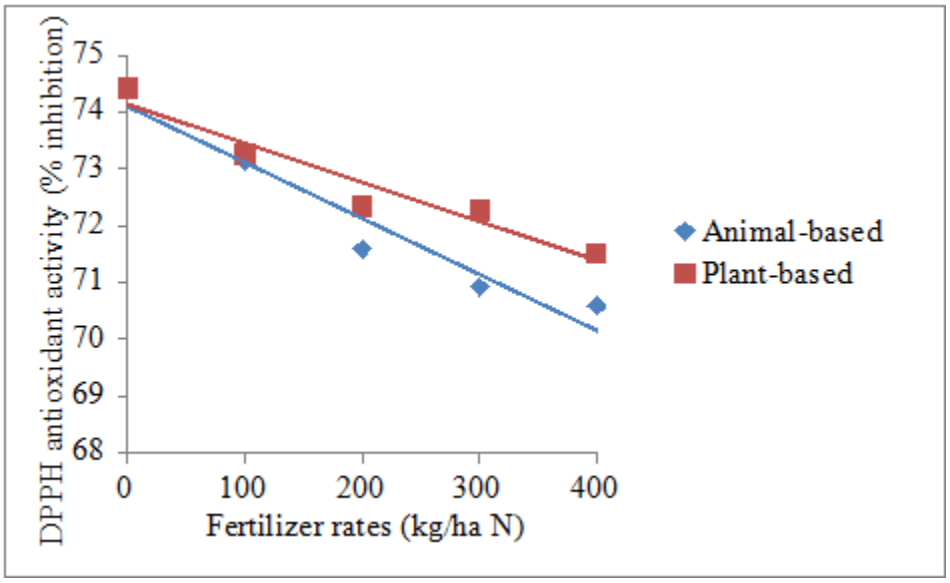

Figure 2. Relationships between 2,2-diphenyl-1-picrylhdrazyl (DPPH) free radical scavenging activity of *Orthosiphon stamineus* and fertilizer rates. Animal-based fertilizer, y = 74.11 – 0.0099x, R^2 = 0.71; plant-based fertilizer, y = 74.13 - 0.0068x, R^2 = 0.56.

3.4 Chemical markers

In this current study, the interaction effect between fertilizer type × fertilizer rates was significant for RA. The simple effects of fertilizer rates on RA was a significant and negative linear relationship for plant-based fertilizer and a significant and negative quadratic relationship for animal-based fertilizer (Figure 4). For the plant-based fertilizer, RA decreased with increasing fertilizer rate by 2% at 400 kg/ha N as compared to control. As for animal-based fertilizer, RA decreased with increasing fertilizer rate followed by a gradual decrease. Thereafter, RA increased in response to fertilizer rate but lower by 2.14% w/w at 400 kg/ha N as compared to the control. Animal-based fertilizer gave optimum RA of 1.70% w/w at fertilizer rate of 250 kg/ha N. Based on Figure 4, plant-based fertilizer at 300 kg/ha N was selected since the RA was higher than at all rates of nitrogen from animal-based fertilizer. A difference of 1.3% w/w of RA at 300 kg/ha N was obtained as compared to the control.

ISSN: 2455-6939

Volume:02, Issue:04

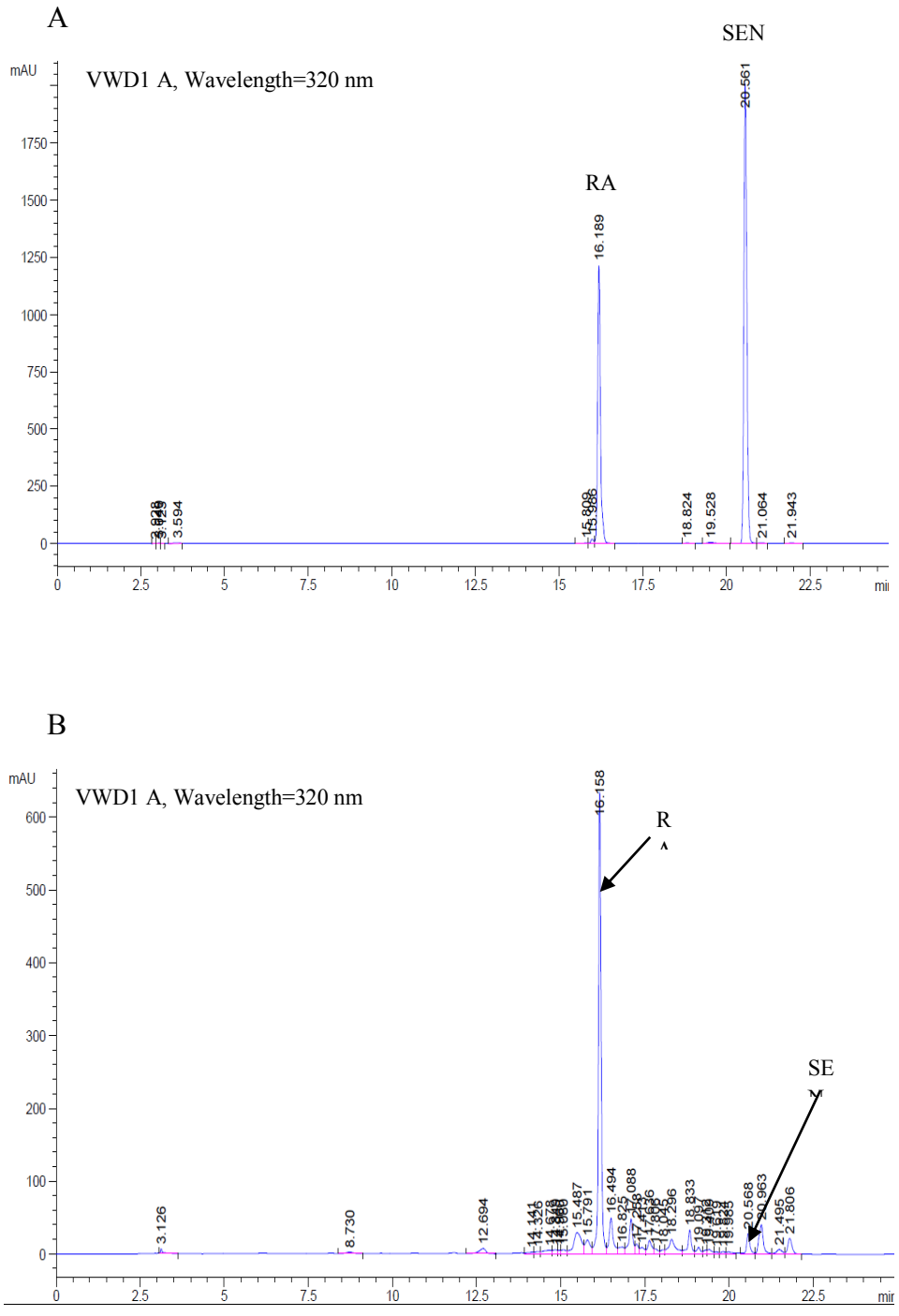

Figure 3. HPLC chromatogram of the *Orthosiphon stamineus* plant extracts (A) reference markers; (B) Aqueous ethanol (70%) extract.

ISSN: 2455-6939

Volume:02, Issue:04

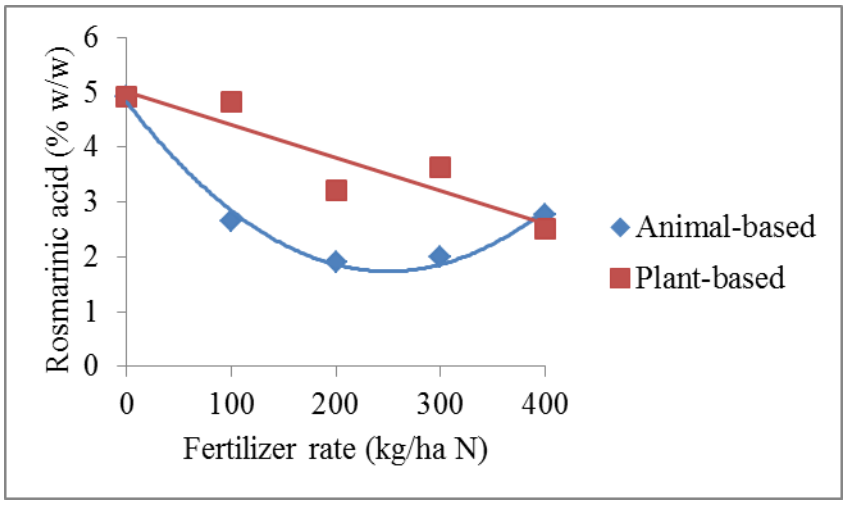

Figure 4. Relationships between rosmarinic acid of *Orthosiphon stamineus* and fertilizer rates of animal- and plant-based fertilizers. Solid line indicates a significant regression trend at P=0.05, (n = 12). Animal-based fertilizer, $y = 4.82 – 0.025x + 0.000050x^2$, $R^2 = 0.72$; Plant-based fertilizer, $y = 5.02 – 0.006x$, $R^2 = 0.42$.

The interaction effect between fertilizer type × fertilizer rates was significant for SEN. The relationship of simple effects of fertilizer rates on total flavonoid contents was quadratic for animal-based fertilizer, whereas that of plant-based fertilizer did not fit any tested regression models (Figure 5). For the animal-based fertilizer, SEN decreased with increasing fertilizer rates followed by a gradual decrease. Thereafter, SEN increased in response to fertilizer rates but higher by 0.03% w/w at 400 kg/ha N as compared to the control.

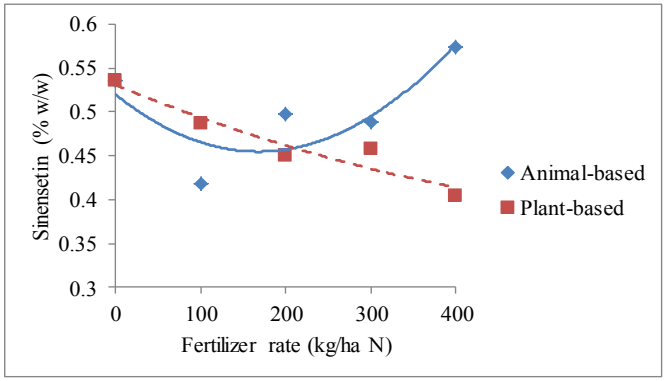

Figure 5. Relationships between sinensetin of *Orthosiphon stamineus* and fertilizer rates of animal- and plant-based fertilizers. The solid line indicate a significant regression trend at P=0.05, (n = 12). Animal-based fertilizer, $y = 0.52 - 0.00077x + 0.00000229x^2$, $R^2 = 0.50$.

ISSN: 2455-6939

Volume:02, Issue:04

3.5 Correlation coefficients between contents of total phenolic, total flavonoid, rosmarinic acid and sinensetin and 2,2-diphenyl-1-picrylhdrazyl (DPPH) free radical scavenging capacity.

Main effects of treatments on TPC, TFC, DPPH free radical scavenging activity, RA and SEN were pooled by fertilizer rates and correlation analysis was conducted in order to determine the relationships between the measured variables (Table 3). TPC was positively correlated to RA and it is classified under the phenolic group. As more phenolic content was found in the plant, RA would also increase. Similarly, TFC was correlated with SEN, whereby SEN increased with increased TFC. This is rather expected since SEN is classified under the flavonoid group. However, the DPPH free radical scavenging activity was positively correlated only to RA, which indicated that RA contributed to the antioxidant properties of *O. stamineus*.

Table 3. Correlation coefficients (R) between total phenolic contents (TPC), total flavonoid contents (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, rosmarinic acid (RA) and sinensetin (SEN).

	TFC	DPPH activity	RA	SEN
TPC	-0.01^{NS}	-0.18^{NS}	0.50**	0.08^{NS}
TFC	-	-0.06^{NS}	0.06^{NS}	0.40*
DPPH activity		-	0.47**	0.13^{NS}
RA			-	0.27^{NS}

^{*,**,NS} = significant and nonsignificant at P = 0.05 or 0.01, respectively. (n = 40).

ISSN: 2455-6939

Volume:02, Issue:04

4. DISCUSSION

Plant phenolic compounds are secondary metabolites that are the most common and widespread groups of substances in plants. It is found throughout the plant kingdom but the type of compound present depends on the phylum or family. Secondary metabolites are chemicals produced by plants for protective value and can be divided into three main groups, terpenes, phenolics and nitrogen-containing compounds. Out of the three main groups, phenolic compounds (made from simple sugars, containing benzene rings, hydrogen and oxygen) are the most common and widespread in plants whereby it plays a role in pigmentation, growth, reproduction and resistance to pathogen [31]. The antioxidant properties of phenolic compounds are due to their ability to donate electron or hydrogen atoms [32].

A study on Labisia pumila showed that the total phenolic decreased as the nitrogen rates increased from 0 to 270 kg N/ha [28,33]. An inverse relationship between nitrogen rates and TPC was also seen in Brassica oleracea [34]. These studies showed that nitrogen rates negatively influence the TPC in herbs and vegetables. However, a study showed an increase in TPC of winter wheat grain as the nitrogen rates increased from 0 to 300 kg N/ha [35]. These studies showed that different crops react differently to increasing levels of nitrogen fertilization. It is known that secondary metabolites would only be synthesized when plants are under stress such as exposures to drought, differences in temperature, nutrient stress and other environmental stresses. In the current study, however, nitrogen rates did not significantly affect the TPC. The soil pH in the current study was in the range of 4.0 to 5.0, which indicated an acidic soil. Thus, nitrogen availability was reduced which induced nutrient stress to the plant. When nitrogen availability is limited for plant uptake, a reduction in ribulose bisphosphate carboxylase or oxygenase (Rubisco) activity occurred, thus, reducing amount of secondary metabolites produced by plants.

Flavonoids are the most common and widely distributed group of plant phenolics whereby it is present in almost all fruits and vegetables. Flavonoids are antioxidants due to the hydrogen donating properties of their phenolic hydroxyl groups, which are attached to the ring structures [36]. There are seven major flavonoid classes, anthocyanidins, chalcones, flavanols, flavanones, flavones, flavonol and isoflavones.

The decreasing trend in TFC after application of 200 kg/ha N fertilizer is in line with reports, whereby TFC decreased in the leaves of Labisia pumila with increasing fertilization rate until 270 kg N/ha due to the decrease in carbon to nitrogen ratio [37,38]. Plants tend to direct nitrogen to increase their sink size as compared to producing secondary metabolites when the availability of nitrogen in the soil is higher. However, inconsistent effects of nitrogen on TFC can be seen whereby total flavonoid contents values in Rubia tinctorum increased by 14% as compared to

ISSN: 2455-6939

Volume:02, Issue:04

control when nitrogen rates increased [39]. There is no clear cut environmental and physical factors that could contribute to all of the flavonoid expression within a plant tissue, but it is a combination of different factors, such as light intensity and temperature. The total flavonoid concentration of Anoectochilus formosanus, an orchid plant, also increased by 9% (60 µmol·m-2·s-2) when compared to control (10 µmol·m-2·s-2) [40]. As for temperature, a positive and significant correlation between phenols and growing degree days of broccoli florets and kale leaf tissues were reported [41]. The reason was due to the induction of different enzymes involved in the biosynthesis of flavonoids when the plant is being exposed to certain stress conditions, such as changes in temperature, irradiation, water stress and pest attacks.

Antioxidant properties of ethanol plant extract of Orthosiphon stamineus at different rates and types of nitrogen fertilizer were evaluated to find the optimum fertilizer to be applied. DPPH free radical scavenging assay is popularly used in natural product antioxidant studies as this method is simple and sensitive. The DPPH assay is based on the theory that a hydrogen donor is an antioxidant, which would then change colour from purple to yellow upon absorption of hydrogen from an antioxidant [42]. The antioxidant effect can be measured easily by using a spectrophotometer, at an absorbance of 517 nm.

It was also found that cow-dung treated soil showed a higher antioxidant activity at IC50 432.80 µg/ml, followed by compost and vermicompost treated soil in Andrographis paniculata leaves [43]. In contrast, there were no significant differences in the antioxidant activity of Passiflora incarnata when different organic fertilizers were used [44]. The low levels of plant nitrogen with high carbon to nitrogen ratio increased the production of secondary metabolites and antioxidant activities [38]. This could be attributed to the condition of the soil that immobilize nitrogen in their particles. However, a reduction of antioxidant activities in animal-based fertilizers showed that nitrogen is easily available for plant uptake. Another study also reported that nitrogen fertilizer at 3.0 g/plant increased oil composition of clary sage but reduced oil composition by 12% when nitrogen fertilization increased up to 6.0 g/plant [45]. Reduction of antioxidant for animal-based fertilizer could also be due to the availability of nitrogen for plant uptake as compared to plant-based fertilizer. Also, animal-based fertilizer contains lignin which is more difficult to break down, thus, causing a slow-release characteristic for nitrogen.

Chemical markers, also known as bioactive compounds are essential and non-essential compounds that occur in nature as a part of the food chain and shown to have an effect on human health [46]. The selection of chemical markers in plants is important for the quality control of herbal medicines, authentication of genuine species, harvesting if best quality raw materials, evaluation of postharvest handling, assessment of intermediates and finished products, and detection of harmful or toxic ingredients [47, 48]. The main chemical markers for O. stamineus

ISSN: 2455-6939

Volume:02, Issue:04

are rosmarinic acid (RA) and sinensetin (SEN). RA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid which is commonly found in the species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae. It is one of the phenolic compounds which has beneficial effects on human health such as protection again diabetes, antidepressant, antibacterial, anti-inflammatory, antimutagen and antiocidative [49, 50, 51, 52, 53].

SEN is one of the flavonoids that can be found in O. stamineus. SEN is a rare polymethoxylated flavone (PMF) is mostly found in citrus plants with 5 methoxy groups on the basic benzo-γ-pyrone skeleton with a carbonyl group at the C4 position. SEN has the capability to inhibit α-glucosidase and α-amylase activity which controls the glucose absorption in type 2 diabetes [54]. SEN also has anti-inflammatory activity whereby inflammatory mediators were inhibited [55]. SEN in combination with other chemical markers found in herbs also contributed to being an antitumour as, whereby 50% ethanolic extract of O. stamineus helped to reduce colorectal cancer [56]. The reduction might be due to the promotion of secondary metabolites by low nutrient concentration. The effects of fertilization on chemical markers also depend on the chemical structure of the chemical markers and if N molecules are needed for the buildup.

The content of RA and SEN content in O. stamineus from different parts of Malaysia ranged from 5.1% to 29.90% and 0.22% to 1.76% of total dry leaf weight, respectively [57]. It is also reported that an increase in RA content and yield in Satureja hortensis by nitrogen fertilization, which is explained by the presence of nitrogen in the structure of amino acids and enzymes that increases the biosynthesis of RA precursors and catalyzer enzymes [58]. In another study, canavanine content in Sutherlandia frutescens microshoots was reported to increase as the concentration of sodium chloride given increased although there were no significant differences between the concentrations [59]. The same decreasing trend for RA concentration as nitrogen levels increased in all cultivar of basil was also reported [60]. In addition, it was reported that a reduction of aloins content in two Aloe species was found as fertilizer rates increased [61].

In conclusion, both fertilizer rates and type influenced the amount of phytochemicals and chemical markers that were produced by the plants. Thus, in order to achieve optimum phytochemicals and chemical markers, the suitable fertilizer type and rate is plant-based fertilizer at 300 kg N/ha. However, more research needs to be done to further investigate the effects of the treatment in the field.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

ISSN: 2455-6939

Volume:02, Issue:04

ACKNOWLEDGMENTS

The authors would like to express their gratitude for grants and financial support from NKEA Research Grant Scheme (NRGS), Ministry of Agriculture, Malaysia and Universiti Putra Malaysia for providing the equipment and facilities to carry out the experiment.

REFERENCES

[1] Traditional medicine. 2015 [Online] Available from: http://www.who.int/tropics/traditional_medicine/en/ [Accessed on 30 Aug 2015]

[2] Bandaranayake M, Mumivand H, Hadian J, Tabatabaei SMF. Effects of nitrogen and calcium carbonate on growth, rosmarinic acid content and yield of *Satureja hortensis* L. J of Agricultural Science 2010; 2(3):92-98.

[3] Definition of organic agriculture. 2008 [Online] Available from: http://www.ifoam.bio/en/organic-landmarks/definition-organic-agriculture [Accessed on 18 November 2015]

[4] Chin FS, Ho TY, Chong KP, Mohamadu BJ, Nyet KW. Organic versus conventional farming of tea plantation. Borneo Science 2010; 26:19-26.

[5] Salama ZA, El Baz FK, Gaafar AA, Zaki MF. Antioxidant activities of phenolics, flavonoids and vitamin C in two cultivars of fennel (*Foeniculum vulgare* Mill.) in responses to organic and bio-organic fertilizers. J of the Saudi Society of Agricultural Sciences 2015; 14(1):91-99.

[6] Jennifer B, Charles LC, Tessema A, Valtcho DZ, Archana G, Carmen S. Organic versus conventional fertilization effects on sweet basil (*Ocimum basilicum* L.) growth in a greenhouse system. Industrial Crops and Products 2015; 74:249-254.

[7] Marcio C, Maira U, Daniel VM, Giuseppina L, Lin CM. Effect of organic fertilization on biomass production and bioactive compounds in *Passiflora incarnata* L. International Journal of Phytocosmetics and Natural Ingredients 2015; 2:11.

[8] Hossain MA, Ismail Z, Rahman A, Sun CK. Chemical xomposition and anti-fungal properties of the essential oils and crude extracts of *Orthosiphon stamineus* Benth. Industrial Crops and Products 2007; 27:328-334.

ISSN: 2455-6939

Volume:02, Issue:04

[9] Adam Y, Somchit MN, Sulaiman MR, Nasaruddin AA, Zuraini A, Bustamam A, et al. Diuretic properties of *Orthosiphon stamineus* Benth. J of Ethnopharmacology 2009; 124:154-158.

[10] Alshawsh MA, Mahmood AA, Ismail S, Amin ZA. Hepatoprotective effects of *Orthosiphon stamineus* extract on thioacetamide-induced liver cirrhosis in rats. Evidence-Based Complementary and Alternative Medicine 2011; 1-6.

[11] Alshawsh MA, Abdulla MA, Ismail S, Amin ZA, Qader SW, Hadi HA, et al. Free radical scavenging, antimicrobial and immunodulatory activities of *Orthosiphon stamineus*. Molecules 2012; 17:5385-5395.

[12] Elsnoussi AHM, Mohammad JAS, Lee FA, Amirin S, Sue HC, Soo C, et al. Potent α-glucosidase and α-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from *Orthosiphon stamineus* Benth as anti-diabetic mechanism. BCM Complementary and Alternative Medicine 2012; 12:176.

[13] Petersen M, Simmonds MSJ. Molecules of interest: rosmarinic acid. Phytochemistry 2003; 12:121-125.

[14] Petersen M. Rosmarinic acid: new aspects. Phytochemistry Reviews 2013; 12:207-227.

[15] Jordon MJ, Lax V, Rota MC, Loran S, Sotomayor JA. Relevance of carnosic acid, carnosol and rosmarinic acid concentrations in the in vitro antioxidant and antimicrobial activities of *Rosmarinic officinalis* (L.) methanolic extracts. Journal of Agricultural and Food Chemistry 2012; 60:9603-9608.

[16] Wang J, Xu H, Jiang H, Du X, Sun P, Xie J. Neurorescue effect of rosmarinic acid on 6-hydroxydopamine-lesioned nigral dopamine neurons in rat model of parkinson's disease. Journal of Molecular Neuroscience 2012; 47:113-119.

[17] Braidy N, Matin A, Rossi F, Chinain M, Laurent D, Guillemin GJ. Neutoprotective effects of rosmarinic acid on ciguatoxin in primary human neurons. Neurotoxicity Research 2014; 25:226-234.

[18] Du Q, Chen H. The methoxyflavones in *Citrus reticulate Blanco cv. Ponkan* and their antiproliferative activity against cancer cells. Food Chemistry 2010; 119:567-572.

[19] Jiang Y, Wong JH, Fu M, Ng TB, Liu ZK, Wang CR, et al. Isolation of adenosine, iso-sinensetin and dimethylguanosine with antioxidant and HIV-1 protease inhibiting activities from fruiting bodies of *Cordyceps militaris*. Phytomedicine 2011; 18:189-193.

ISSN: 2455-6939

Volume:02, Issue:04

[20] Shin HS, Kang SI, Yoon SA, Ko HC, Kim SJ. Sinensetin attenuates LPS-induced inflammation by regulating the protein level of IκB-α. Bioscience, Biotechnology and Biochemistry 2012; 76:847-849.

[21] *Orthosiphon stamineus*. 2015 [Online] Available from: http://www.globinmed.com/index.php?option=com_content&view=article&id=79215:orthosiphon-stamineus&Itemid=139 [Accessed on 30 Aug 2015]

[22] Affendy H, Aminuddin M, Azmy M, Amizi MA, Assis K, Tamer AT. Effect of organic fertilizers application to the growth of *Orthosiphon stamineus* Benth. intercropped with *Hevea brasiliensis* Willd. and *Durio zibenthus* Murr. International Journal of Agricultural Research 2011; 6:180-187.

[23] Affendy H. Misai kucing: panduan lengkap jana pendapatan. Alaf 21 Sdn Bhd; 2010.

[24] Organic fertilizers. 2015 [Online] Available from: http://www.cmg.colostate.edu/index.shtml [Accessed on 30 Aug 2015]

[25] Eaton TE, Cox D, Barker AV. Sustainable production of marigold and calibrachoa with organic fertilizers. HortScience 2013; 48(5):637-644.

[26] Lee J. Effect of application methods of organic fertilizer on growth, soil chemical properties and microbial densities in organic bulb production. Scientia Horticulturae 2010; 124:299-305.

[27] Bi G, Evans WB, Spiers JM, Witcher AL. Effects of organic and inorganic fertilizers on marigold growth and flowering. HortScience 2010; 45(9):1373-1377.

[28] Ibrahim MH, Jaafar HZE. The relationship of nitrogen and C/N ratio with secondary metabolites levels and antioxidant activities in three varieties of Malaysian kacip Fatimah (*Labisia pumila* Blume). Molecules 2011; 16:5514-5526.

[29] Farhan M, Abdul Razak S, Pin KY, Chuah AL. Antioxidant activity and phenolic content of different parts of *Orthosiphon stamineus* grown under different light intensities. Journal of Tropical Forest Science 2012; 24(2):173-177.

[30] Akowuah GA, Zhari I. Effect of extraction temperature on stability of major polyphenols and antioxidant activity of *Orthosiphon stamineus* leaf. Journal of Herbs, Spices and Medicinal Plant 2010; 16:160-166.

ISSN: 2455-6939

Volume:02, Issue:04

[31] Lattanzio V, Lattanzio VM, Cardinali A. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Research Signpost 2006; 37/661(2):24-67.

[32] Jothy SL, Zuraini Z, Sasidharan S. Phytochemicals screening, DPPH free radical scavenging and xanthine oxidase inhibitory activities of *Cassia fistula* seeds extract. Journal of Medicinal Plants Research 2011; 5(10):1941-1947.

[33] Ibrahim MH, Jaafar HZE, Karimi E, Ghasemzadeh A. Impact of organic and inorganic fertilizers application on the phytochemical and antioxidant activity of kacip Fatimah (*Labisia pumila* Benth). Molecules 2012; 18:10973-10988.

[34] Effect of organic nitrogen fertility on yield and nutritional content of basil and collard greens. 2014 [Online] Available from: https://getd.libs.uga.edu/pdfs/saperstein_raleigh_l_201405_ms.pdf [Accessed on 5 Jan 2016]

[35] Ma D, Sun D, Li Y, Wang C, Xie Y, et al. Effect of nitrogen fertilisation and irrigation on phenolic content, phenolic acid composition, and antioxidant activity of winter wheat grain. J Sci Food Agric. 2015; 95(5):1039-1046.

[36] Salter A, Wiseman H, Tucker G. Phytonutrients. Wiley-Blackwell; 2012.

[37] Ibrahim MH, Jaafar HZE. The relationship of nitrogen and C/N ratio with secondary metabolites levels and antioxidant activities in three varieties of Malaysian kacip Fatimah (*Labisia pumila* Blume). Molecules. 2011; 16:5514-5526.

[38] Ibrahim MH, Jaafar HZE, Karimi E, Ghasemzadeh A. Impact of organic and inorganic fertilizers application on the phytochemical and antioxidant activity of kacip fatimah (*Labisia pumila* Blume). Molecules. 2013; 18:10973-10988.

[39] Zamani Z, Zeinali H, Sinaki JM, Madani H. Effect of nitrogen and phosphorous fertilizers on the yield and secondary metabolites of medicinal plant *Rubia tinctorum* L. under salinity conditions. Iranian Journal of Plant Physiology. 2014; 4(2):949-955.

[40] Ma Z, Li S, Zhang M. Light intensity affects growth, photosynthetic capability, and total flavonoid accumulation of *Anoectochilus* plants. HortScience. 2010; 45(6):863-867.

[41] Kang MK, Juvik JA. Environmental stress and methyl jasmonate-mediated changes in flavonoid concentrations and antioxidant activity in broccoli florets and kale leaf tissues. HortScience. 2013; 48(8):996-1002.

[42] Lewis MJ. Protocol 15.3b Natural product screening: antioxidant screen for extracts; 2012.

ISSN: 2455-6939

Volume:02, Issue:04

[43] Upadhyaya S, Mahanta JJ, Saikia LR. Antioxidant activity, phenol and flavonoid content of a medicinal herb *Andrographis paniculata* (Burm. F.) Nees grown using different organic manures. Journal of Pharmacy Research. 2011; 4(3):614-616.

[44] Campos M, Uliana M, Montero DV, Lima G, Lin CM. Effect of organic fertilization on biomass on production and bioactive compounds in *Passiflora incarnata* L. International Journal of Phytocosmetics and Natural Ingredients. 2015; 2:11.

[45] Sharma S, Kumar R. Effect of nitrogen on growth, biomass and oil composition of clary sage (*Salvia sclarea* Linn.) under mid hills of North Western Himalayas. Journal of Natural Products and Resources. 2012; 3(1):79-83.

[46] Biesalski K, Dragsted LO, Elmadfa I, Grossklaus R, Muller M, Schrenk D, et al. Bioactive compounds: definition and assessment activity. Nutrition. 2009; 1-4.

[47] Li S, Han Q, Qiao C, Song J, Cheng CL, Xu H. Chemical markers for the quality control of herbal medicines: an overview. Chinese Medicine. 2008; 3:7.

[48] Rasheed NMA, Nagaiah K, Goud PR, Sharma VUM. Chemical marker compounds and their essential role in quality control of herbal medicines. Annals of Phytomedicine. 2012: 1(1):1-8.

[49] Patrick PM, Kaliclas S. Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase in vitro. Asia Pacific Journal Clin. Nutr. 2004; 13(1):101-106.

[50] Teresa I, Daniele DF, Giuseppe E, Alessandra D, Angelo AI. The spice sage and it's active ingredient rosmarinic acid protect PC12 cells from amyloid-β peptide-induced neurocity. JPET. 2006; 317(3):1143-1149.

[51] Renzulli C, Galvano F, Pierdomenico L, Speroni E, Guerra MC. Effects of rosmarinic acid against aflatoxin B1 and ochratoxin-A-induced cell damage in a human hepatoma cell line (HepG2). Journal of Applied Toxicology. 2004; 24(4):289-296.

[52] Luan H, Kan Z, Xu Y, Lv C, Jiang W. Rosmarinic acid protects against experimental diabetes with cerebral ischemia: relation to inflammation response. Journal of Neuroinflammation. 2013; 10:28.

[53] Konda S, El Omri A, Han J, Isoda H. Antidepressant-like effects of rosmarinic acid through nitrogen-activated protein kinase phosphatase-1 and brain-derived neurotrophic factor modulation. Journal of Functional Foods. 2015; 14:758-766.

ISSN: 2455-6939

Volume:02, Issue:04

[54] Mohamed EAH, Mohammad JAS, Lee FA, Amirin S, Sue HC, Soo CT, et al. Potent α-glucosidase and α-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from *Orthosiphon stamineus* Benth as anti-diabetic mechanism. BMC Complementary and Alternative Medicine. 2012; 12:176.

[55] Shin HS, Kang SI, Yoon SA, Ko HC, Kim SJ. Sinensetin attenuates LPS-induced inflammation by regulating the protein level of IκB-α. Bioscience, Biotechnology and Biochemistry. 2012; 76: 847-849.

[56] Foaud SRA, Elham F, Mohamed BKA, Ismail Z, Aman SAM, Amin MSAM. Marked antitumor activity of cat's whiskers tea (*Orthosiphon stamineus*) extract in orthotopic model of human colon tumor in nude mice. Journal of Biochemical Technology. 2012; 3:170-176.

[57] Akowuah GA, Zhari I, Norhayati I, Sadikun A, Khamsah SM. Sinensetin, eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and rosmarinic acid contents and antioxidative effect of *Orthosiphon stamineus* from Malaysia. Food Chemistry. 2004; 87:559-566.

[58] Babalar M, Mumivand H, Hadian J, Tabatabaei SMF. Effects of nitrogen and calcium carbonate on growth, rosmarinic acid content and yield of *Satureja hortensis* L. Journal of Agricultural Science. 2010; 2(3):92-98.

[59] Colling J, Stander MA, Makunga NP. Nitrogen supply and abiotic stress influence canavanine synthesis and the productivity of in vitro regenerated *Sutherlandia frutescens* microshoots. Journal of Plant Physiology. 2010; 167:1521-1524.

[60] Nguyen PM, Niemeyer ED. Effects of nitrogen fertilization on the phenolic composition and antioxidant properties of basil (*Ocimum basilicum* L.). Journal of Agricultural and Food Chemistry. 2008; 56:8685-8691.

[61] Cardarelli M, Rouphael Y, Rea E, Lucini L, Pellizzoni M, Colla G. Effects of fertilization, arbuscular mycorrhiza, and salinity on growth, yield, and bioactive compounds of two *Aloe* species. HortScience. 2013; 48(5):568-575.

