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Table of Contents: Volume 11 Number 1 3 January, 2017

ARTICLES

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Full Length Research Paper

Comparative physicochemical, phytochemical and acute toxicity studies of two *Ocimum* species in Western Uganda

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***Ocimum gratissimum* and *Ocimum suave* are species belonging to the Lamiaceae family; they have been domesticated and are widely grown in gardens in Western Uganda for its claimed beneficial effect in ethnomedical practice. This study was aimed at comparative evaluation of the physicochemical, phytochemical and acute toxicity potentials of their leaves. Dried leaves of *O. gratissimum* and *O. suave* were subjected to physicochemical analysis, crude extraction in methanol and sequential extraction in n-hexane, chloroform, ethylacetate and methanol. Phytochemical constituents of the crude extracts were determined by both qualitative and quantitative methods, while acute toxicity potentials were studied in female rats. Total caffeic acid derivatives contents, total flavonoid contents and reducing power assay were evaluated in the sequential extracts. The physicochemical parameters of leaves of *O. gratissimum* and *O. suave* showed similar profiles. The extraction yield, phytochemical constituents and acute toxicity effects of the crude extracts were not significantly different. Hexane, chloroform and ethylacetate extract of *O. suave* showed significant ($p < 0.05$) higher total flavonoid contents than corresponding extracts of *O. gratissimum*. Similarly, *O. suave* extracts showed a stronger positive correlation of phenolic content to antiradical power than the corresponding extracts of *O. gratissimum.* Conclusively, the two species showed comparative physicochemical, phytochemical and acute toxicity profiles, and a positive correlation of phenolic contents to antiradical power.**

Key words: Lamiaceae, Uganda, phenolics, *Ocimum gratissimum*, *Ocimum suave*.

INTRODUCTION

Medicinal plants are of great importance to the health of individuals and communities. The therapeutic value of these medicinal plants lies in some active chemical substances that produce a definite physiological action on the human body (Edeoga et al., 2005). Nature has served as a rich repository of medicinal plants for

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thousands of years and an impressive number of modern drugs have been isolated from natural sources, notably of plant origin (Cowan, 1999). Medicinal plants were the main source for primary health care in the past centuries before the advent of conventional orthodox medicine. Herbal medicine, based on their traditional uses in the form of powders, liquids or mixtures, have been the basis of treatment for various ailments in African countries since ancient times. They provide diverse indications for which reason they are employed in the management of a wide range of conditions. Importantly, it is the most easily accessible and affordable health resource available to the local community (Mahomoodally, 2013).

The global surge in the use of medicinal plants as a result of the failures of synthetic drugs and the beliefs that herbal drugs provides safer, more reliable and cheaper phytomedicine, brings to the fore the need to improve on the quality of medicinal plants. One major problem associated with the traditional ethno-medical systems is that even though the medicinal plants seems to work very well and are readily available to the community, there are no sufficient data for quality assessment and standardization. Some of the problems associated with lack of quality include the multiple constituents of herbal drugs, absence of identified marker or active compounds in commercial quantity, and misidentification of chemo cultivars (Kunle et al., 2012). A key obstacle which has hindered the development of medicinal plant product in developing countries is the lack of documentation and stringent quality control. With this backdrop, it becomes extremely important to make an effort towards standardization of plant materials to be used as phytomedicine.

The genus *Ocimum* is ranked high among some of the astonishing herbs for having enormous medicinal potentials; they are widespread over Asia, Africa, Central and Southern America (Vani et al., 2009). All basils are member of the Lamiaceae family. Characterizations of each species in this genus (family Lamiaceae) are based on the leaves and habitat (Paton, 1992; Grayer et al., 2002). *Ocimum gratissimum* and *Ocimum suave* belong to the family Lamiaceae. Folkore medicine claims their use in many condition, they are widely grown in gardens in Uganda (Orwa et al., 2009). The leaves and flowers of *O. gratissimum* and *O. suave* are rich in essential oils, so it is used in preparation of teas and infusion. The plant materials are used throughout Uganda as home remedies (Tabuti et al., 2010). This research was conducted to compare the physicochemical, phytochemical and acute toxicity profile of two *Ocimum* species that are known locally to bear the same name (Omujaja) in Bushenyi district. This study provides scientific evidence to back the traditional use of these plants as a consequence of their chemical constituents. It will also provide documentation of phytochemical constituents and physicochemical parameters for monograph information about these two plants.

MATERIALS AND METHODS

Plants collections

The two plants were obtained from medicinal garden in Bushenyi District, Uganda, and taxonomically identified by Dr. Eunice Olet (Botanist) of the Biology Department, Faculty of Science, Mbarara University of Science and Technology and voucher specimens were retained with the voucher number LI 001 and 002 for *O. gratissimum* and *O. suave,* respectively. The plants collection was done in the early hours of the day between 9:00 a.m to 11:00 a.m. The leaves of *O. gratissimum* and *O. suave* were collected and dried under shade, then ground into powder using an electronic blender.

Determination of physicochemical parameters in powdered leaves of *O. gratissimum* and *O. suave*

Standard procedures as documented in the official literature were adopted in determining the dried leaf material moisture content, ash values and water and alcohol extractive values (World Health Organization, 2003; Kokate, 2000).

Moisture content

Four grams each of powdered leaves of *O. gratissimum* and *O. suave* were weighed into pre-weighed crucibles (in triplicate), and then dried in the oven at 100°C until constant weight. The crucibles were cooled in a dessicator, loss in weight was recorded as moisture content.

Determination of ash values

Four grams (in triplicate) of powdered leaves of *O. gratissimum* and *O. suave* were weighed into silica dish previously ignited, cooled and weighed. The material was ignited to ashes in an oven at 450°C, cooled in a dessicator for up to 24 h and weighed. Acid-insoluble ash content was analyzed by boiling the total ash for 5 min with hydrochloric acid (7%, v/v); the mixture in a crucible was ignited at 450°C to a constant weight. The total ash was dissolved in 200 ml of distilled water, stirred and the mixture was filtered using pre-weighed filter paper. The weight of the dried residue was determined as insoluble ash. The insoluble ash was subtracted from the total ash to obtain the amount of water soluble ash.

Determination of cold extractive values

Four grams of the powdered sample was weighed and soaked with 100 ml distilled water (or 70% ethanol) in a conical flask with a stopper. The materials were extracted for 24 h with intermittent shaking. The extracts were filtered rapidly through a dry filter; 20 ml of the filtrate was transferred to pre-weighed evaporating dish, concentrated to almost dryness on a water bath, and then dried at 105°C for 3 h. The percentage of water-soluble or ethanol soluble extractives were calculated with reference to the dried sample. Samples were prepared in triplicate.

Determination of hot extractive values

About 4.0 g of the powdered sample was accurately weighed and soaked with 100 ml distilled water (or ethanol) in a flask. The flask was attached to a reflux condenser and boiled gently for 1 h. The extracts were filtered rapidly through a dry filter; 20 ml of the filtrate

was transferred to pre-weighed evaporating dish, concentrated to almost dryness on a water bath, and then dried at 105°C for 3 h. The percentage of water-soluble or ethanol soluble extractives were calculated with reference to the dried sample. Samples were prepared in triplicate.

Crude and sequential extraction

Crude extraction was carried out with dried powdered leaves of *O. gratissimum* and *O. suave*. 100 g of powdered leaves was macerated in 500 ml of 80% methanol and shaken for 48 h on a laboratory rotator (Nuve SL 350 Quality System, Digisystem Laboratory inc, Taiwan). Sequential extraction was performed by successive maceration of dried powdered leaves (1:5 w/v) in solvent of increasing polarity (n-hexane, chloroform, ethylacetate and methanol) for 48 h each. All extractions were performed in triplicates, and were filtered using a Whatman® filter paper. The extracts were pooled together and concentrated in a Rotary evaporator (Buchi Rotavapor R-124) under reduced pressure and dried to a constant weight in oven at 40°C. All extracts were dried in separate containers in order to determine the percentage yield of extraction. The crude methanol extract of *O. gratissimum* and *O. sauve* were labeled as ME*Og* and ME*Os*, respectively. The successive extracts were labeled as *Og*HE and *Os*HE for hexane extracts; *Og*CE and *Os*CE for chloroform extracts; *Og*EAE and *Os*EAE for ethylacetate extracts; and *Og*ME and *Os*ME for methanol extracts. All the extracts were stored in a refrigerator (4°C±2); ME*Og* and ME*Os* were dissolved in 2% Tween 80 for acute toxicity test.

Qualitative phytochemical analysis

The standard methods described by Evans (2002), Harborne (1998) and Odebiyi and Sofowora (1982) were applied in the phytochemical screening of the individual constituents of the crude extracts. The presence of the compounds were rated as positive (+) or negative (-) in the two extracts.

Quantitative phytochemical estimation in the crude methanol extracts

Quantitative assessment for crude alkaloid, tannin, saponin and flavonoid were performed in crude methanol extracts of *O. gratissimum* and *O. suave* leaves. The alkaloid content of crude methanol extract was determined by the method of Harborne (1998). Tannin content was determined by the method described by Van Buren and Robinson (1969) as modified in Edeoga et al. (2005). Saponin content was determined by the method of Obadoni and Ochuko (2001). The flavonoid contents of the extracts were estimated by the method of Bohm and Koupai-Abyazan (1994).

Determination of total caffeic acid in sequential extracts of *O. gratissimum* and *O. suave* leaves

The Arnow's reagent method of spectrophotometric estimation of caffeic acid derivatives was used for the quantification method (Benedec et al., 2012). Briefly, 0.2 ml of various extracts (1 mg/ml) was added into test tubes in triplicates and hydrochloric acid (1 ml, 0.5 N), Arnow's reagent (1 ml) and sodium hydroxide solution (1 ml, 1 N) were added to the test tubes and allowed to stand for 5 min. The absorbance was read at 500 nm in a spectrophotometer (Spectronic 21D Milton Roy, USA). Caffeic acid was used as standard for the calibration curve.

Determination of total flavonoid content in sequential extracts of *O. gratissimum* and *O. suave* leaves

The total flavonoid contents (TFC) in the extracts were determined by the spectrophotometric aluminium chloride method (Sultana et al., 2009). Briefly, 1 ml of the extract (1 mg/ml) was added into test tubes. After that, 0.3 ml of 10% (w/v) $NaNO_2$ was added to the test tubes, and left to react for 5 min. Then, 0.3 ml of 10% (w/v) $AlCl_3$ was added and left for 1 min to react. Then, 2 ml of 1M NaOH was added and the mixtures shaken. Aliquots of the mixtures were transferred to a cuvette, and the absorbance values measured at 510 nm in a spectrophotometer (Spectronic 21D Milton Roy). Rutin was used as a standard for the calibration curve. All samples were prepared in triplicates.

Determination of reducing power of sequential extracts of *O. gratissimum* and *O. suave* leaves

The reducing power was determined according to the method of Oyaizu (1986) as described in Ferreira et al. (2007). It is based on the principle that antioxidant substances react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700 nm.

Various concentrations of the plants extracts or ascorbic acid (0.125, 0.25, 0.5, and 1.0 mg/ml) were mixed with 2.5 ml of phosphate buffer and 2.5 ml of 1% potassium ferricyanide, respectively. The mixtures were incubated in a 50°C water bath for 20 min. After cooling to room temperature (20 to 23°C), 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of freshly prepared 0.1% ferric chloride solution. The absorption was measured at 700 nm in a spectrophotometer (Spectronic 21D Milton Roy, USA). The inhibitory concentration providing 0.5 of absorbance (IC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration.

Comparative evaluation of acute toxicity effects of crude methanol extracts of *O. gratissimum* and *O. suave* leaves

Female Wistar rats were housed in the Animal Facility of the Department of Pharmacology and Toxicology, Kampala International University-Western Campus. The animals were kept in a cage lined with sawdust, at room temperature with adequate ventilation, under a naturally illuminated environment with 12 h of light and 12 h of darkness. They were fed with standard diet (Nuvita (R) Animal Feed Ltd, Jinja Uganda) and have access to clean drinking water *ad libitum*. The animal experiment was conducted according to the National Institute of Health Guide for the care and use of laboratory animals (NIH, 1996). The study was carried out after ethical consideration and approval by research committee of the Kampala International University – Western Campus.

Comparative toxicity screening was carried out in female Wistar rats (weighing 120 to 150 g) following the Organization for Economic Co-operation and Development (OECD) guidelines (423). The rats were assigned into groups (n=5) including a control group by the stratified random method according to their body weight. The route of administration was by oral gavage in accordance with the main route of intake of *O. gratissimum* and *O. suave* by humans for medicinal purposes. Single oral dose of 2000 and 5000 mg/kg per body weight of ME*Og* and ME*Os* was administered to overnight fasted animals. Animals were observed individually after dosing once during the first 30 min, periodically during the first twenty four hours, with special attention given during the first four hours and daily thereafter, for a total of 14 days. The effects of the extracts on

Table 1. Physicochemical parameters of leaves of *O. gratissimum* and *O. suave*.

Physicochemical parameter	*O. gratissimum*		*O. suave*	
	Mean ± SEM	**%**	**Mean ± SEM**	**%**
Moisture content	0.73 ± 0.07	18.3	0.50 ± 0.36	12.5
Ash values				
Total ash	0.73 ± 0.09	18.3	1.17 ± 0.09	32.5
Acid-insoluble ash	0.10 ± 0.05	2.5	0.18 ± 0.04	4.5
Water-Soluble ash	0.33 ± 0.18	8.3	0.75 ± 0.03	18.8
Extractive values				
Cold water soluble extractives	0.55 ± 0.25	13.8	0.45 ± 0.25	11.3
Cold Ethanol soluble extractives	0.37 ± 0.17	9.3	0.33 ± 0.09	8.3
Hot water soluble extractives	0.13 ± 0.03	3.3	0.27 ± 0.03	6.8
Hot ethanol soluble extractives	0.47 ± 0.07	11.8	0.33 ± 0.12	8.3

Values are Mean ± SEM (n=3). ^{ns}Values are not significantly different; $p > 0.05$ using unpaired T-test.

general behavior, feed intake and weight gain were observed in the animals for fourteen days post administration.

Statistical analysis

All data generated were presented as Mean ± Standard Error of the Mean (SEM). The reducing power activity was expressed in terms of IC_{50}, which was estimated from the non-linear regression curve using version 5 GraphPad prism®. The linear correlation coefficients between the total caffeic acid derivatives content and reducing power activity were calculated by Pearson test using the GraphPad Prism® software version 5.01 (GraphPad Software, Inc. La Jolla, CA 92037 USA). Comparative significant differences between two samples were analyzed using student's t test. All other analysis was by one-way ANOVA statistical model with Newman-Keuls Multiple Comparison post hoc test. Statistical significance was taken for $p<0.05$.

RESULTS

Physicochemical parameters of leaves of *O. gratissimum* and *O. suave*

The moisture content, ash values and extractive values of dried leaves of *O. gratissimum* and *O. sauve* were determined and are presented in Table 1. Percentage of moisture content in leaves of *O. gratissimum* (18.3%) was higher than what was obtained in *O. suave* (12.5%). The percentage total dry ash content for *O. suave* (32.5%) was greater than *O. gratissimum* (18.3%). Similarly, the acid-insoluble ash and water-soluble ash values for *O. suave* (4.5 and 18.8%) was greater than for *O. gratissimum* (2.5% & 8.3%). These differences were not significantly different ($p > 0.05$).

The cold water extractive yield was higher for *O. gratissimum* (13.8%) compared to *O. suave* (11.3%), while hot water showed higher values for *O. suave* (6.8%) than *O. gratissimum* (3.3%). Both cold ethanol and hot ethanol gave the same soluble extractive values for *O. suave* (8.3 %). Hot ethanol gave higher values for *O. gratissimum* (11.8%) than cold ethanol extractive (9.3%). Taken together, cold extraction gave the better yield for both samples compared to hot extraction.

Crude and sequential extraction yield

Crude extraction of powdered leaves of *O. gratissimum* and *O. suave* in aqueous methanol gave a percentage yield of 1.86 and 2.23%, respectively. Sequential extraction of MEO*g* and MEO*s* gave similar yields in all solvent extracts except in the methanol extract. The yield was as follows, *Os*HE (2%) > *Og*HE (1.7%); *Og*CE (1.49%) > *Os*CE (1.48%); *Og*EAE (1.22%) < *Os*EAE (1.23%) and *Og*ME (3.73) < *Os*ME (9.78%).

Phytochemical analysis

Qualitative phytochemical screening revealed the presence of the major phytochemicals in the methanol extracts of both *O. gratissimum* and *O. suave* leaves (Table 2). Quantitative phytochemical estimations in the methanol extracts revealed higher percentages of tannins and flavonoids in the MEOg (60 and 66%) than MEO*s* (40 and 56%). Similarly, the alkaloids composition in MEO*g* (24%) was higher than in MEO*s* (20%), saponin content was however lower in MEO*g* (10%) compared to MEO*s* (22%) (Table 3). These differences were not statistically significant ($p > 0.05$).

Total caffeic acid and total flavonoid contents in sequential extracts of *O. gratissimum* and *O. suave*

The level of phenolic compounds in the different solvent

Table 2. Qualitative screening results for phytochemicals found in methanol extracts of *O. gratissimum* (ME*Og*) and *O. suave* (ME*Os*) leaves.

Substance	Test/Reagent	ME*Og*	ME*Os*	Deduction
Saponins	Frothing test	+	++	Saponins detected in both species but more in *O. suave*
Tannins	Lead acetate	+	+	Both extracts contained tannin in the two methods used
	15% ferric chloride	++	+	
Terpenoids/Steroids	Salkowski test	+	+++	More terpenoids were found in *O. suave*
Alkaloids	Mayer test	+	+	Presence of alkaloids were detected in both extracts
	Wagners test	+	+	
Flavonoids	Shinoda assay	+	+	Presence of flavonoids were detected in methanol extracts of both plants
	2% lead acetate	++	+	
	Dilute ammonia solution	+	+	
Reducing sugars	Iodine	+	+	Reducing sugars present in both extracts
Cardiac glycosides	Ferric chloride + glacial acetic acid	++	+	There were more cardiac glycosides
Lactone coumarins	Balijet test	++	+	More coumarins in *O. gratissimum*

Table 3. Quantitative phytochemical composition of methanol extracts of *O. gratissimum* (ME*Og*) and *O. suave* (ME*Os*) leaves.

Phytochemicals	ME*Og*		ME*Os*	
	Mean ± SEM	% Content	Mean ± SEM	% Content
Alkaloids	0.12 ± 0.15^{ns}	24	0.10 ± 0.03	20
Flavonoids	0.30 ± 0.10^{ns}	60	0.20 ± 0.10	40
Tannins	0.33 ± 0.05^{ns}	66	0.26 ± 0.04	52
Saponins	0.05 ± 0.03^{ns}	10	0.11 ± 0.01	22

Values are Mean ± SEM (n=3). ^{ns}Values are not significantly different; $p > 0.05$ using unpaired T-test.

extracts (hexane, chloroform, ethylacetate and methanol) of the leaves of *O. gratissimum* and *O. suave* are shown in Figure 1A and B. Total caffeic acid derivatives expressed as mgCAE/g sample was as follows, *Os*HE (47.2) > *Og*HE (33.1); *Os*CE (42.3) > *Og*CE (34.9); *Og*EAE (76.7) > *Os*EAE (53.6); and *Os*ME (85.1) > *Og*ME (74.9). The findings of this study revealed a significantly ($p < 0.05$) higher caffeic acid contents in *Og*EAE than *Os*EAE.

Similar pattern was observed in total flavonoid contents, with *O. gratissimum* showing lower contents compared to *O. suave* extracts (Figure 1B). The total flavonoid content expressed as RE/g sample was as follows: *Os*HE (32.4) > *Og*HE (24.5); *Os*CE (39.1) > *Og*CE (32.2); *Os*EAE (45.7) > *Og*EAE (41); and *Os*ME (43.8) > *Og*ME (42.2). The results showed a significant ($p < 0.05$) higher flavonoid contents in hexane, chloroform and ethylacetate extracts of *O. suave* compared to *O. gratissimum*.

Reducing power of sequential extracts of *O. gratissimum* and *O. suave* leaves

The ability to act as antioxidant is based on the reducing potential of phenolics. To further compare the phenolic properties of the successive extracts, the reducing powers of the extracts were evaluated. A dose-dependent increase in reducing power of the extracts was observed (Figure 2). Ascorbic acid used as positive standard agent showed higher reducing power with an IC_{50} of 0.14 ± 0.08 compared to the extracts. The IC_{50} for the extracts is as presented in Table 4. All the extracts showed potent reducing potentials all within the range for the standard antioxidant (Ascorbic acid), but *Os*ME showed the best activity. Comparative anti-radical power

Table 4. Inhibitory concentration (IC50), antiradical power and Pearson correlation coefficients of successive extracts of *O. gratissimum* and *O. suave*.

Extract	Reducing power	Antiradical power	Correlation[a]
	IC_{50}	$1/IC_{50}$	TCADC[b]
*Og*HE	0.19 ± 0.07	5.26	0.9301*
*Os*HE	0.30 ± 0.08	3.33	0.9808**
*Og*CE	0.16 ± 0.05	6.25	0.8893*
*Os*CE	0.26 ± 0.05	3.84	0.9604**
*Og*EAE	0.18 ± 0.07	5.56	0.9188*
*Os*EAE	0.16 ± 0.05	6.25	0.8939*
*Og*ME	0.21 ± 0.08	4.76	0.9347*
*Os*ME	0.14 ± 0.04	7.14	0.8444^{ns}
Ascorbic acid	0.14 ± 0.08	7.14	-

[a]Pearson correlation coefficient (r). [b]Total caffeic acid derivative content. *$p < 0.05$, **$p < 0.01$ (two-tailed), ns: not significant.

Table 5. Effects of methanol extracts of *O. gratissimum* (ME*Og*) and *O. suave* (ME*Os*) leaves after single oral administration on body weight in Rats.

Treatment	No. of deaths	Body weight of experimental animals (kg) at different days[a]				
		0	4	7	10	14
ME*Og* (2 g/kg)	0/5	0.124 ± 0.005	0.131 ± 0.005	0.136 ± 0.005	0.139 ± 0.005	0.141 ± 0.005
ME*Og* (5 g/kg)	0/5	0.125 ± 0.006	0.129 ± 0.006	0.133 ± 0.006	0.136 ± 0.006	0.135 ± 0.006
ME*Os* (2 g/kg)	0/5	0.126 ± 0.006	0.131 ± 0.006	0.136 ± 0.006	0.141 ± 0.007	0.147 ± 0.005
ME*Os* (5 g/kg)	0/5	0.127 ± 0.006	0.134 ± 0.006	0.137 ± 0.006	0.140 ± 0.006	0.142 ± 0.007
Distilled water (10 ml/kg)	0/5	0.129 ± 0.007	0.136 ± 0.007	0.138 ± 0.007	0.141 ± 0.006	0.143 ± 0.006

[a]Values are Mean ± SEM (n=3). Values are not significantly different; $p > 0.05$ using one-way analysis of variance.

is as follows: *Og*HE > *Os*HE; *Og*CE > *Os*CE; *Og*EAE < *Os*EAE; and *Og*ME < *Os*ME. There were positive correlations between the reducing potential and the phenolic contents of all the extracts except *Os*ME.

Acute toxicity effects of crude methanol extracts of *O. gratissimum* and *O. suave* leaves

Comparative acute toxicity test showed no mortality in rats administered ME*Og* and ME*Os* at doses of 2 and 5 g/kg (Table 5). The two extracts did not produced significant changes in treated animals' behavioral pattern, such as locomotor activity, writhing response, fighting, convulsion, tremor, exophthalmos, ptosis, piloerection, tail elevation, traction, motor incoordination, catalepsy, pain response, pinna reflex, skin color, diarrhea, or impairment in food intake, or water consumption immediately after administration or during the 14 days observation period. There was no significant difference in body weights during the fourteen days post administration observations. However, there was a slight loss in weight of the animals administered with ME*Og* (5 g/kg) from days 10 to 14.

DISCUSSION

This study was conducted on the need to improve quality of local herbal medicines by systematic scientific studies and documentation of two *Ocimum* spp. bearing similar local names, in order to avoid the pitfall of misidentification. The physicochemical evaluation of herbal drugs is an important parameter in detecting adulteration or improper handling of drugs. These parameters are useful for identification of allied species as well as adulterants.

The low moisture content of *O. suave* (12.5%) as compared to *O. gratissimum* (18.3%) may discourage bacterial growth when the dried leaves are stored. Dried herbal materials with moisture content higher than 14% have been shown to support bacterial growth (African Pharmacopeia, 1986). The total ash is particularly important in the evaluation of purity of drugs, that is, the presence or absence of foreign inorganic matter such as metallic salts and/or silica (Musa et al., 2006). A lower acid-insoluble ash shows that a very small amount of the inorganic compounds is insoluble in acid. The implication is that adulteration of the raw materials with compounds such as silica or other metallic salts is very less likely to

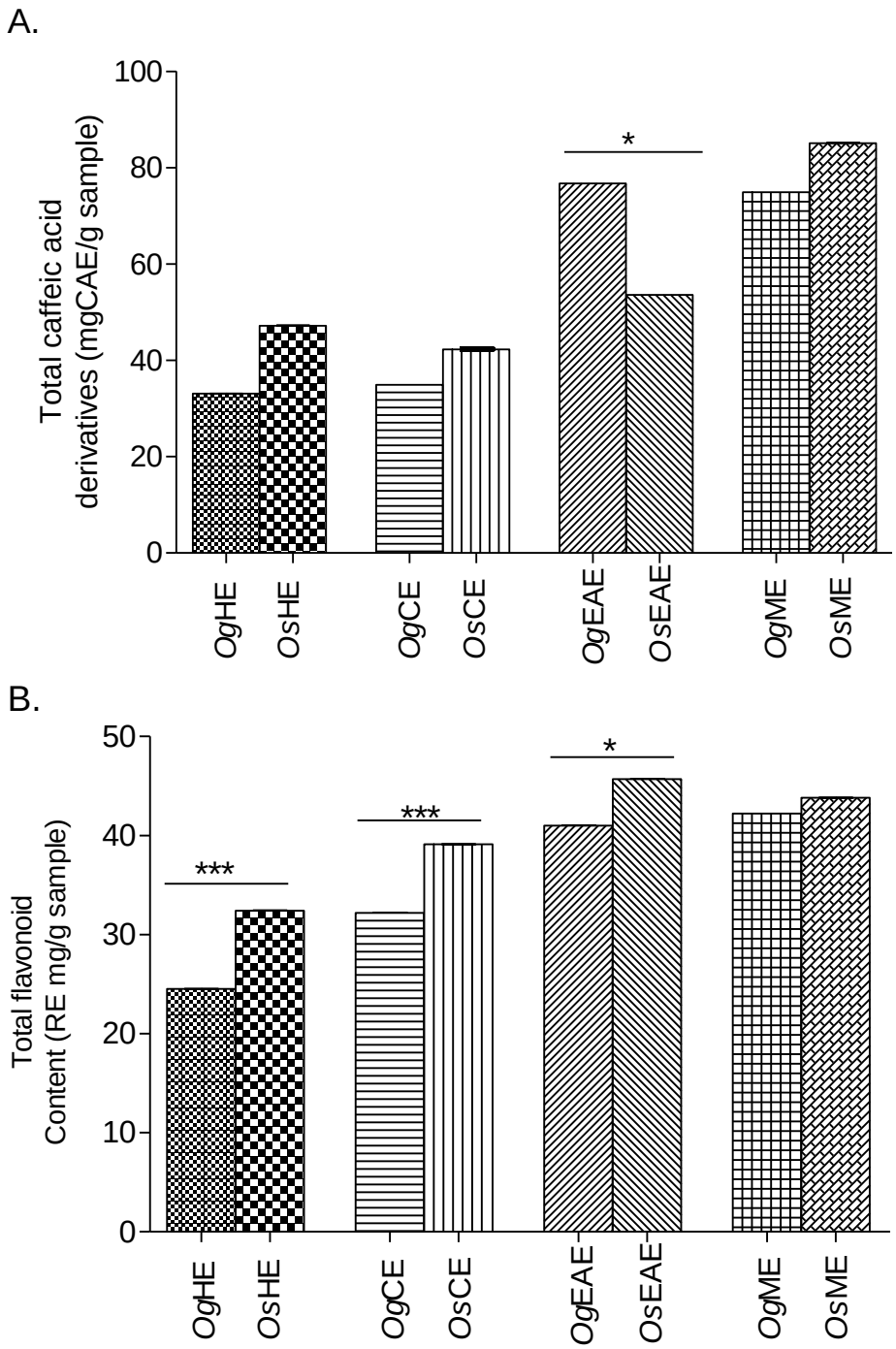

Figure 1. Phenolic contents of successive extracts of *O. gratissimum* and *O. suave* leaves. (A) Total caffeic acid derivatives content, and (B) Total Flavonoid content. *$p < 0.05$ using student t-test.

happen. The acid insoluble ash value may also mean improved oral absorption of the extracts of the leaves in the gastrointestinal tract.

The sequential extraction showed that *O. suave* phytochemicals showed relatively higher solubility than *O. gratissimum* in solvent of increasing polarity. The fact remains that plant extracts usually occur as a combination of various type of bioactive compounds or phytochemicals, which makes their separation a big challenge. Polarity based solvent extraction from medicinal plants is known to selectively extract components that are soluble in particular solvents.

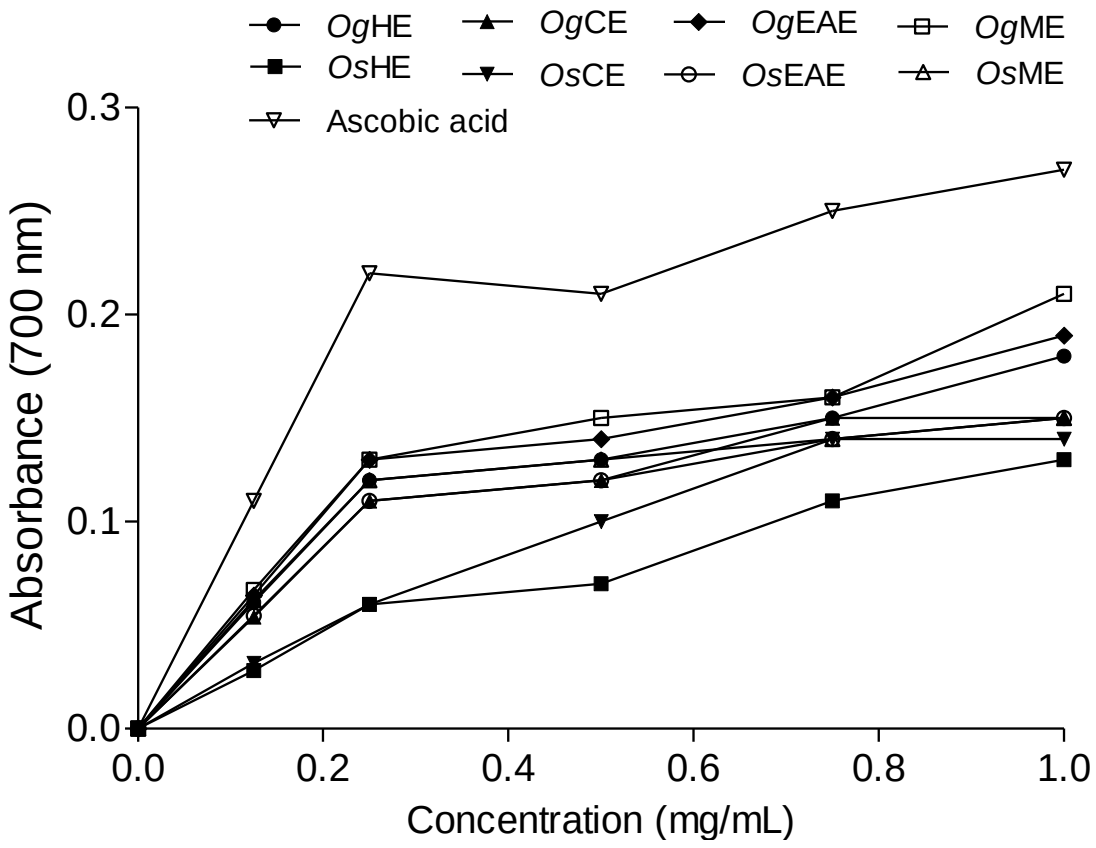

Figure 2. Reducing power assay of successive extracts of *O. gratissimum* and *O. suave* leaves.

Components vary from solvent extracts to whole extract and may be responsible for different biological effects.

Phenolic acids such as caffeic acid, gallic acid, ferulic and *p*-coumaric acid are distributed in nature in their free and bound forms. Caffeic acid and rutin were chosen, because these compounds were previously reported in the *Ocimum* spp. (Ola et al., 2009). It was observed that ethylacetate and methanol extracts have comparable amounts of the caffeic acids than hexane and chloroform. Caffeic acid (Phenolic acid) is found in many fruits, vegetables and medicinal plants existing as an esterified form of quinic acid. Caffeic acid exerts many pharmacological properties including antioxidant, anti-inflammatory and cytotoxic properties (Kelebek et al., 2015). The four major caffeic acid derivatives are caftaric acid, caffeic acid, chicoric acid and rosmarinic acid (Lee, 2010). Grayer et al. (2000) has shown the presence of rosmarinic acid in *O. gratissimum*. High performance liquid chromatographic characterization can help to differentiate and quantify the amount of the different caffeic acid derivatives in the two plants; this may serve as phytochemical markers or index for quality control and identification of the *Ocimum* spp. In a report by Hakim et al. (2004), comparative studies of phenolic content of *O. gratissimum* with seven other *Ocimum* spp., showed *O. gratissimum* to possess higher total phenolic content than the other species. It is noted that *O. suave* was absent among the *Ocimum* spp. in this report.

O. suave sequential extracts showed a more significantly positive correlation than their corresponding *O. gratissimum* extracts except in *Os*ME that was not significant. Though *Os*ME showed a higher total caffeic acid derivative contents and a comparable IC_{50} value to ascorbic acid, it however did not have a significant positive correlation; this implied that other compounds might be responsible for the reducing power other than the phenolic acids. Flavonoids and tannins are polyphenolic compounds that might be present in such extracts other than the phenolic acids. Reducing power of the extracts might be due to their hydrogen-donating ability, with a capacity to react with free radicals to stabilize and block chain reactions (Ferreira et al., 2007). Hakkim et al. (2004) demonstrated the reductive capacity of eight *Ocimum* spp., wherein *O. gratissimum* was the most effective at reducing iron (III).

Acute toxicity test provides preliminary information on the toxic nature of a material for which no other toxicological information is available. The acute toxicity study was aimed at identifying and characterizing adverse effects that can be produced in animals by a

single oral exposure to high dose of *O. gratissimum* or *O. suave* extracts. Acute toxicity results of methanol extracts of *O. gratissimum* and *O. suave* showed that none of the extracts caused death immediately after single oral administration or within fourteen days. This implied that the LD_{50} of both extracts is greater than 5 g/kg body weights of rats. There are many reasons why acute toxicity is performed, namely to serve as the basis for classification and labeling, to provide initial information on the mode of toxic action of a substance, to help arrive at a dose of a new compound and to help in dose determination in animal studies (Ukwuani et al., 2012; Balogun et al., 2014). Importantly, it also serves to validate the justification for safety and historical use of some medicinal plants. The high safety margin of the extracts of *O. gratissimum* and *O. suave* may partly explain the historical use of their leaves as infusions in the traditional management of several ailments.

Conclusion

Comparative physicochemical, phytochemical and acute toxicity analysis of two *Ocimum* spp. showed no major significant differences in the two plants. The information provided in this study will serve as an important contribution to knowledge as part of information required to prepare monographs, to promote the conservation of the two species, and in establishing quality parameters for standardization.

Conflict of Interests

The authors have not declared any conflict of interests.

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Chemical profiling and chemical standardization of *Vitex negundo* using 13C NMR

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Chemical profiling and standardization of the defatted methanol extract of the leaves of *Vitex negundo* L. were carried out using 13C nuclear magnetic resonance (NMR) analysis followed by chemometric analysis of the chemical shift data. Chemical profile was obtained using a k-means cluster profile and chemical standardization which was achieved using a multivariate control chart. The *V. negundo* samples were made up of four groups: the training set, submitted samples from production farms, commercial samples, such as tablets, capsules and teas, and experimental samples (samples which were allowed to degrade). Four groups were generated in k-means cluster, which generally corresponded to the four types of samples. The multivariate control chart identified samples whose quality exceeded the upper control limit, all of which were commercial samples and experimental samples. The samples were also analyzed by quantitative thin layer chromatography (qTLC) using agnuside as marker compound. Comparison of the qTLC results with the k-means cluster and the multivariate control chart showed poor correspondence. This means that a univariate analysis of a plant sample using a marker compound is useful only for quantification of the target compound. On the other hand, chemical profiling and standardization of medicinal plants should use a multivariate method.

Key words: *Vitex negundo*, 13C NMR, multi-variate cluster profile, multi-variate control chart.

INTRODUCTION

With the growing interest in medicinal plants today, numerous plants which are traditional home remedies are being developed for commercial production. This entails expansion of the supply chain from sourcing of validated planting material to farming and processing of the raw plant material, to manufacture of finished product. Because many herbal products are sold as dried plant material, such as tablets and teas, there is a need to develop effective methods of standardization and quality assurance. Medicinal plants are very complex mixtures of secondary metabolites which can vary significantly depending on the planting material, environment and farming conditions, age at harvest, storage, and processing.

Quality assurance of herbal products should meet the following needs: verification of plant identity; detection of

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adulteration or chemical deterioration; and quantification of active components, if known (Kumari and Kotecha, 2016). Quality assurance can be based on the targeted analysis of one or few compounds (univariate) or on the chemical profile of the plant extract (multivariate) (Ning et al., 2013). Chemical profiling of herbal products refers to the generation of a quantitative molecular description of the whole extract of plant secondary metabolites (MW < 1,000 Da) in order to establish plant identity and product quality (Yongyu et al., 2011) using chemical analytical methods such as chromatography, spectroscopy, or hyphenated chromatography-mass spectrometry.

Nuclear magnetic resonance (NMR) spectroscopy can yield considerable information in an untargeted analysis of a plant extract. NMR is robust, highly reproducible, and requires minimal sample preparation which minimizes experimental artifacts and bias. Because of the desire for highest sensitivity, 1H NMR is the most common technique used and is combined with chemometric methods to profile, fingerprint or discriminate among crude herbal samples (Bailey et al., 2002; Zulak et al., 2008; Lee et al., 2009; Kim et al., 2010; Mahmud et al., 2014), and for quality control (Wang et al., 2004, Rasmussen et al., 2006, van der Kooy et al., 2008). 1H NMR measurements of herbal medicines have been reported using magnetic fields from 300 to 800 MHz (Zulak et al., 2008; Kim et al., 2011). The limitation of 1H NMR, however, is that the spectra are magnetic field-dependent because the chemical shift in Hz is magnetic field-dependent while the 1H-1H J couplings are magnetic-field independent. This means that 1H NMR spectra taken at different magnetic fields will have different ratios $\Delta\upsilon/\Delta J$ and different spectral appearances. When the ratio between the difference in frequency ($\Delta\upsilon$) and coupling (ΔJ) is less than 20, the spectrum is second order and the appearance of the spectrum is sensitive to the magnetic field strength (Becker, 2000). Thus, data from 1H NMR spectra taken at different magnetic fields cannot be combined.

Compared to 1H NMR, 13C NMR is a more general chemical profiling technique because the fully 1H-decoupled 13C NMR signals are singlets and do not have second-order effects since 1H-13C coupling is zero. This means that 13C NMR data are amenable to spectral comparison across different magnetic field strengths. Unlike 1H NMR, 13C NMR does not require water suppression, which is another source of spectral variability since this is influenced by instrument and operator performance. 13C NMR however requires much longer acquisition times and this is its main disadvantage. To date, there are only a few examples of the use of 13C NMR for the profiling of biological extracts. 13C NMR was used to profile triacylglycerols from the seed oil of *Moringa oleifera* (Vlahov et al., 2002) and lipid extracts from Atlantic salmon (Aursand et al., 2009). 13C NMR was used to profile fractions from a crude extract of *Anogeissus leiocarpus* after which hierarchical clustering analysis (HCA) revealed correlations between 13C signals of the mixture with known compounds using a 13C NMR database (Hubert et al., 2014).

Chemometrics is a family of techniques that applies statistics to voluminous chemical data, such as spectroscopic signals from a collection of samples, with the objective of gaining insights into the characteristics of the samples through graphical representation or pattern-recognition (Wold, 1995). Chemometric analysis is an ideal tool for the classification of spectroscopic data from whole plant extracts to differentiate plants according to species, origin, processing treatment, age, and other quality parameters (Kim et al., 2010).

The overall objective of this paper is to explore the use of 13C NMR together with multivariate statistical methods for the chemical profiling and standardization of medicinal plants. This work will also compare the use of 13C NMR with 1H NMR. The results from the multi-variate control chart will be compared with a targeted univariate quantitative thin layer chromatography (qTLC) method using a marker compound.

MATERIALS AND METHODS

Study species

Vitex negundo, L. is an aromatic shrub which is found from tropical East Africa to South Asia, Southeast Asia, and Polynesia and from Japan southward to Malesia and is widely used in traditional medicine, especially in South and Southeast Asia (GRIN-Global, no date). *V. negundo* is grown all over the Philippines in commercial farms which supply the dried leaves to herbal pharmaceutical companies. The iridoid agnuside is a major constituent in the dried leaves of *V. negundo* (Dayrit and Lagurin, 1994). A validated method has been reported for the analysis of the leaves by qTLC using agnuside as a marker compound (Roy et al., 2015).

Samples

There was a total of 64 samples, which were made up of four sets: training set (n=15), submitted samples (n=17), commercial samples (n=13), and experimental set (n=19). The training set was made up of *V. negundo* leaf samples that we collected from 5 locations around the Philippines. The training set samples were immediately washed and dried at ≤ 60°C to < 5% moisture. The submitted set was made up of dried or powdered leaves that were submitted by 5 commercial farms from various parts of the country. Commercial products (n = 13) were tablets, capsules, and tea products that were purchased from supermarkets and drug stores. Experimental samples (n = 19) comprises a heterogeneous set which include; old samples (> 4 years), flowers, plant tops, and samples that deliberately allowed to degrade (fresh samples were allowed to stand for 3 days before drying).

Sample preparation

To determine the reproducibility of the procedure (extraction and 13C NMR and qTLC analyses), each of the 64 plant samples was extracted and analyzed in duplicate. The results of each duplicate run were not averaged but were considered as a separate sample.

Therefore, the number of NMR and qTLC runs is twice the number of samples.

All samples were milled and sieved (30 to 100 mesh). Five grams of plant material were defatted using n-hexane in a Soxhlet apparatus for 4 h. Two grams of the hexane defatted material were extracted with methanol in a Soxhlet apparatus for 4 h at 90°C. The same defatted sample was used for NMR and qTLC.

NMR analysis

To prepare the NMR sample 0.1 g of the defatted methanolic plant extract was dissolved in 0.7 ml of methanol-D_4 (with added TMS, Cambridge Lab., USA) in a 5 mm NMR tube. A measured amount of DMSO was added as internal standard.

1H NMR spectra were acquired on a 400 MHz on a JEOL Lambda 400 NMR spectrometer (9.4 Tesla) and on a 500 MHz Varian (11.75 Tesla). The same spectral parameters were used for both instruments: pulse angle: 45°; number of scans: 4; number of points: 32k. The following spectral parameters were adjusted according to the magnetic field: at 400 MHz: spectral width: 7,993 Hz; at 500 MHz: spectral width: 10,000 Hz. FIDs were processed using exponential multiplication with auto-processing to avoid operator bias. Line broadening was set at 2.4 Hz for 400 MHz spectra and 3.0 Hz for 500 MHz spectra.

13C NMR spectra were acquired at the corresponding frequencies: 100 MHz (9.4 Tesla) and 125 MHz (11.75 Tesla). The same spectral parameters were used for both instruments: pulse angle: 45°; broad-band 1H decoupling; number of scans: 2,200; number of points: 32k. The following spectral parameters were adjusted according to the magnetic field: at 100 MHz: spectral width: 27,100 Hz; at 125 MHz: spectral width: 33,875 Hz. FIDs were processed using exponential multiplication with auto-processing to avoid operator bias. Line broadening was set at 1.20 Hz for 100 MHz spectra and 1.5 Hz for 125 MHz spectra.

Data processing and statistical analysis

For the 100 MHz 13C NMR spectrum, a bin size of 4 Hz was used across the spectral range of 27,100 Hz. For 125 MHz spectrum, a bin size of 5 Hz was used across the spectral range of 32,768 Hz. Sixty of the tallest peaks in each 13C NMR spectrum were selected. The duplicate extracts were treated as separate samples. The peaks were aligned and normalized using the signal of the DMSO internal standard.

The tallest 60 peaks in each 13C NMR spectrum were selected, normalized against the DMSO internal standard and then aligned. NMR peaks which were, absent in greater than 90% of the samples were removed. This yielded 108 chemical shifts. These were loaded as a table in JMP for chemometric analysis. Chemometrics analysis was performed using JMP version 11 (SAS).

Quantitative thin-layer chromatography (qTLC)

qTLC analysis was performed on silica gel-60 F254 aluminum backed plates (Merck 5554), using the solvent system: EtOAc:HOAc:H_2O (16:2:1). Agnuside was purified from *V. negundo* leaves and used as TLC marker compound. The 1H and 13C NMR and melting point agreed with literature (Dayrit and Lagurin, 1994) and gave a single spot by TLC.

Weighed volumes of each sample were spotted on the TLC plate in 5 mm bands using an automated TLC applicator (CAMAG Linomat 5, Switzerland). Each plate contained 5 calibration bands of the marker compound and six extracts spotted in duplicate. The plates were recorded using a digital camera under UV-254 nm light and processed using QuantiScan v3.0 software (Biosoft, UK).

The correlation coefficient, R^2, for the marker compound in all TLC plates was > 0.99.

RESULTS

NMR profiles at different magnetic field strengths

The 1H NMR 400 MHz and 500 MHz spectra and 13C 100 MHz and 125 MHz spectra of the same *V. negundo* extract are shown in Figures 1 and 2, respectively. The 1H NMR spectra taken at 400 MHz and 500 MHz show significant differences in peak heights and peak patterns which are expected from theory. We did not subject the 1H NMR spectra to further analysis. On the other hand the 13C NMR spectra taken at 100 MHz and 125 MHz show very similar profiles.

Principal components analysis (PCA) cluster plot

PCA is the most common method used to reduce the number of dimensions in a large data set by creating linear combinations of the data that can be used to represent the entire sample using fewer dimensions. PCA has been utilized to discriminate among commercial feverfew samples (Bailey et al., 2002), for quality control and authentication of chamomile (Wang et al., 2004), differentiation of *Artemisia* species (van der Kooy et al., 2008), and metabolite fingerprinting of ginseng (Lee et al., 2009).

Initially, we used PCA to generate the sample clusters. The result was that PC1 and PC2 could account only for about 41% of the variability which meant that this was not a sufficiently good model for the 128 samples (Figure 3). The data required up to PC9 to reach 80% explained variability but there is no simple way to show the resulting clusters.

K-Means cluster plot

An alternative to PCA is k-means clustering, which can be used to classify a given data set starting from an a priori number of clusters. K-means cluster analysis was used to classify different chemotypes of *Chamerion angustifolium* L., a medicinal plant used in food supplements, according to their geographic origin (Kaškonienė et al., 2015). The k-means cluster was generated directly from the 13C NMR chemical shifts. The procedure for k-means involves obtaining the differences ($y_i - \bar{y}$), where y_i is the intensity of a chemical shift y of run i; $\bar{y}$ is the average intensity of the chemical shift y for all runs, i = 1 to n. In this work, i = 128 runs and y = 108 chemical shifts. The magnitude of these differences ($y_i - \bar{y}$), equivalently $(y_i - \bar{y})^2$ to remove the effect of the sign, determines the k-means clustering of the samples (Johnson and Wichern, 2007).

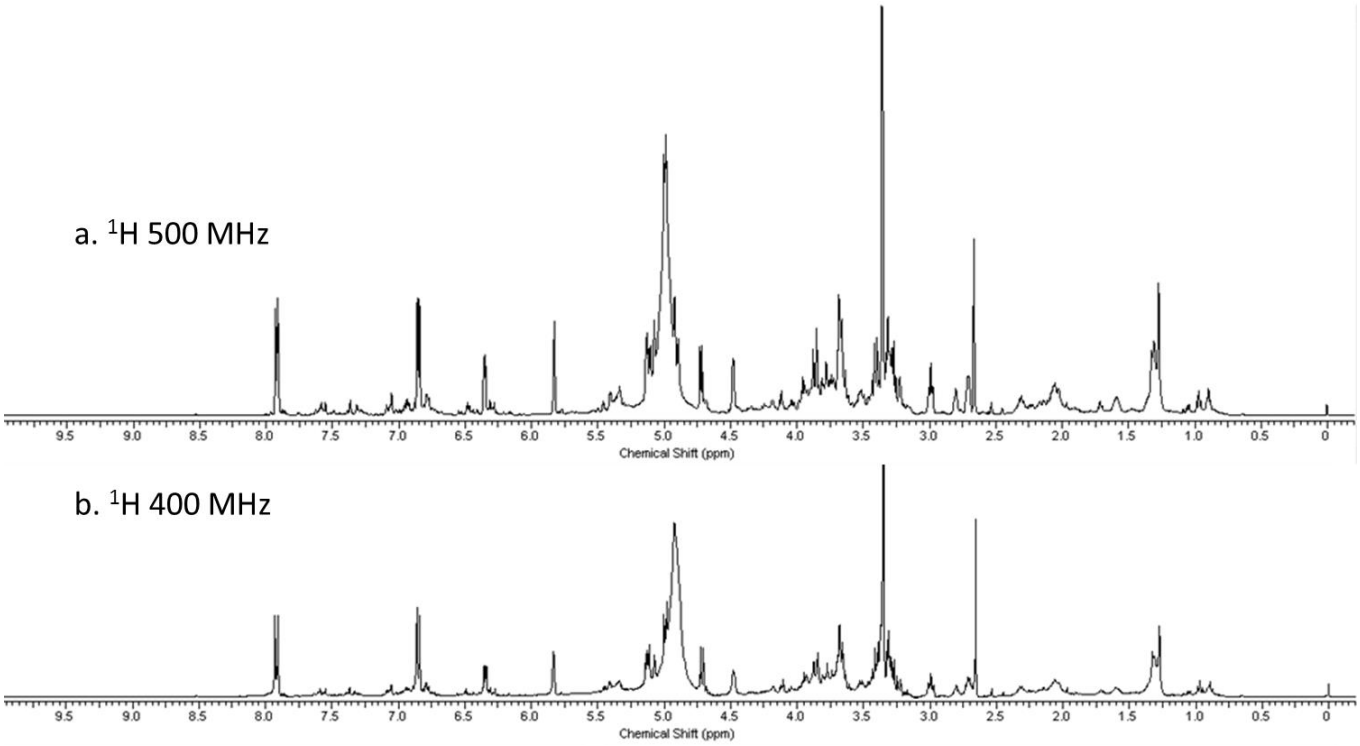

Figure 1. 1H NMR of the defatted MeOH extract of *V. negundo*. (a) 500 MHz (11.75 tesla); (b) 400 MHz (9.4 tesla). The NMR solvent used was methanol-D_4 and the internal standard was DMSO.

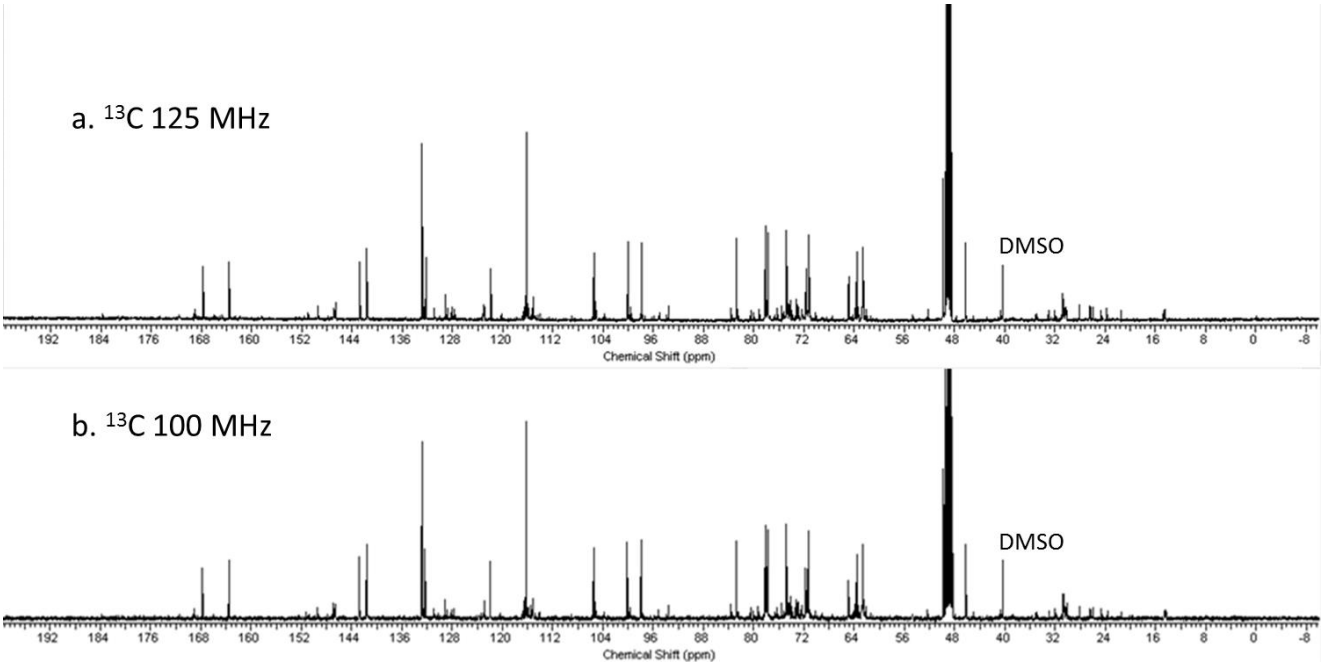

Figure 2. 13C NMR of the defatted MeOH extract of *V. negundo*. (a) 125 MHz (11.75 Tesla); (b) 100 MHz (9.4 Tesla). The NMR solvent used was methanol-D_4 and the internal standard was DMSO. The peaks were normalized to the DMSO peak.

The k-means cluster obtained for 128 runs is shown in Figure 4 and the membership of each cluster is summarized in Table 1. Four clusters were defined a priori and the groupings obtained were consistent with

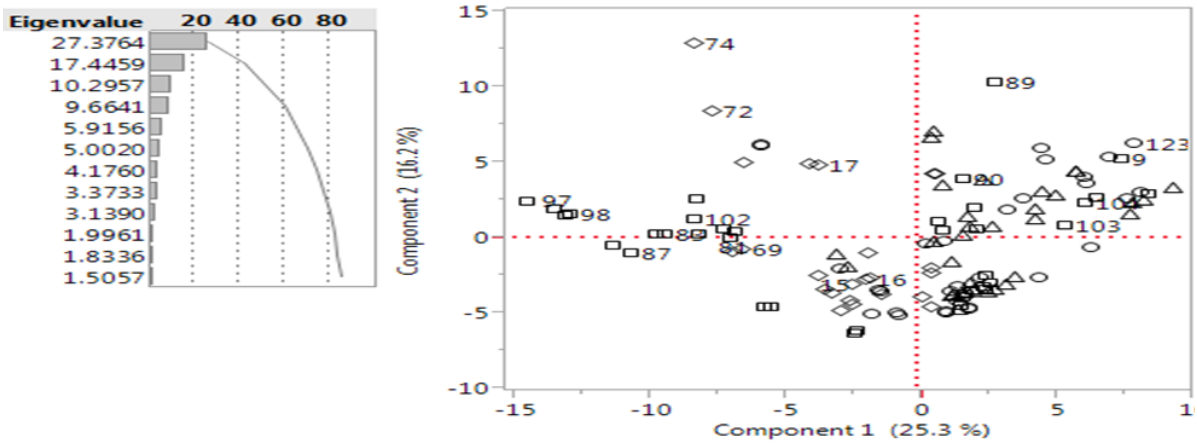

Figure 3. PCA of 128 runs. PC1 and PC2 explains only 41.5% of the variability (25.3% + 16.2%). The skree plot indicates that 9 PCs are needed to reach 80% explained variability. The numbers refer to the sample number which are given in Table 2.

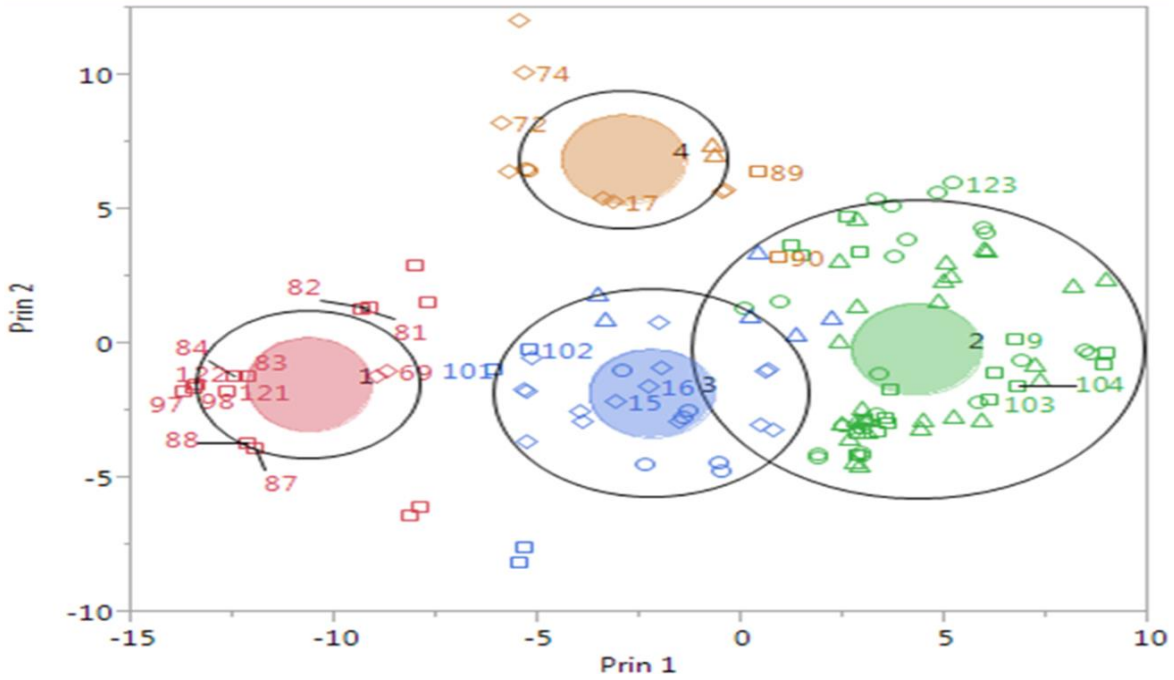

Figure 4. Multi-variate cluster profile by k-means. Cluster 1 is composed mainly of experimental samples that were deliberately allowed to degrade; cluster 2 is composed mainly of training set and submitted samples; clusters 3 and 4 are composed mainly of commercial products. Legend: o - training set; Δ - submitted samples; ◊ – commercial products; □ - experimental set. The numbers refer to the run numbers which are given in Table 2.

the type of sample. Cluster 1 consists mainly of the experimental set which refers to samples that were intentionally allowed to degrade. Cluster 2 consists mainly of the training set and set of submitted samples. This indicates that commercial farms generally prepared their samples using a good drying protocol. Clusters 3 and 4 are the commercial products. The experimental samples, however, were distributed in both clusters 1 and 2 since their characteristics varied widely depending on the sample treatment.

Comparison 13C NMR at 100 MHz and 125 MHz

In this experiment, we sought to compare the results of the 13C NMR spectra taken at100 and 125 MHz. The

Table 1. Distribution of the different samples among the four clusters. (n = number of runs. % is calculated as = n/128).

Sample Type	Cluster									
	1		2		3		4		Total	
	n	%	n	%	n	%	n	%	n	%
Training	0	0.0	22	17.2	6	4.7	2	1.6	30	23.4
Submitted	0	0.0	26	20.3	6	4.7	2	1.6	34	26.6
Commercial	2	1.5	0	0.0	16	12.5	8	6.3	26	20.3
Experimental	14	10.9	18	14.1	4	3.1	2	1.6	38	29.7
Total	16	12.4	66	51.6	32	25.0	14	11.1	128	100.0

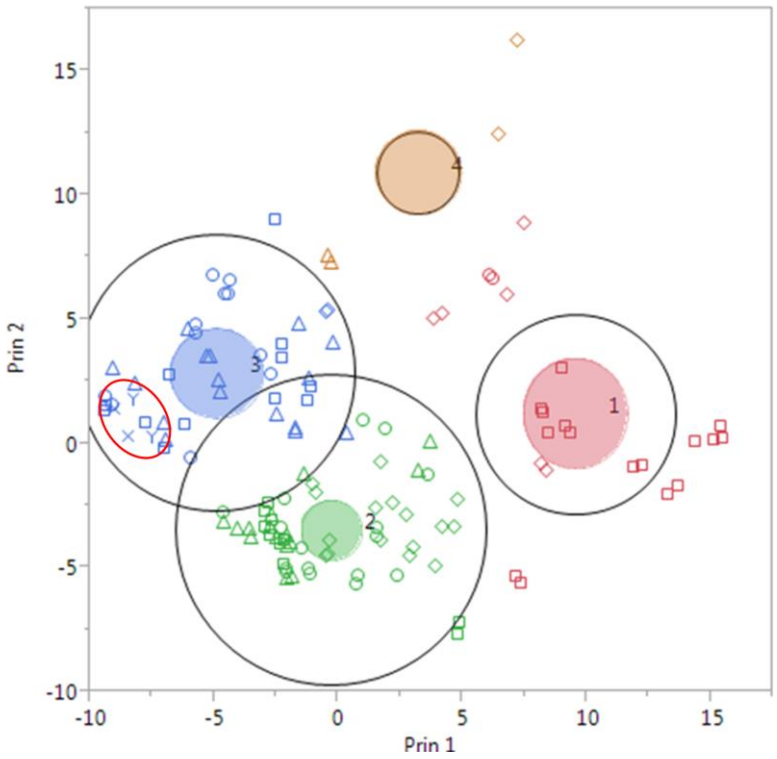

Figure 5. Multi-variate cluster profile by k-means with four additional data points from two training set samples, each of which was analyzed at 125 MHz (Y) and 100MHz (X). The four new samples appeared close to each other in cluster 3 (encircled).

13C NMR of two samples was run at 100 MHz and 125 MHz and the data from these runs were added to the k-means cluster. Figure 5 shows the resulting k-means profile, where the new data at 100 MHz and 125 MHz are indicated. The new data points clustered very closely. This indicates that the 13C NMR spectra taken at 100 MHz and 125 MHz give very similar results.

Multivariate control chart from 13C NMR data

Nine PCs were used to generate the Hotelling's T^2 multivariate control chart (Figure 6). The upper control limit (UCL) was set to the training set sample with the highest T^2 value (in this case, this was run 123). This means that the runs that exceeded the UCL were considered rejected based on their 13C NMR profile.

qTLC analysis

The 64 *V. negundo* samples were analyzed twice by qTLC to measure the agnuside content giving 128 runs (Table 2). This is a univariate analysis using agnuside as

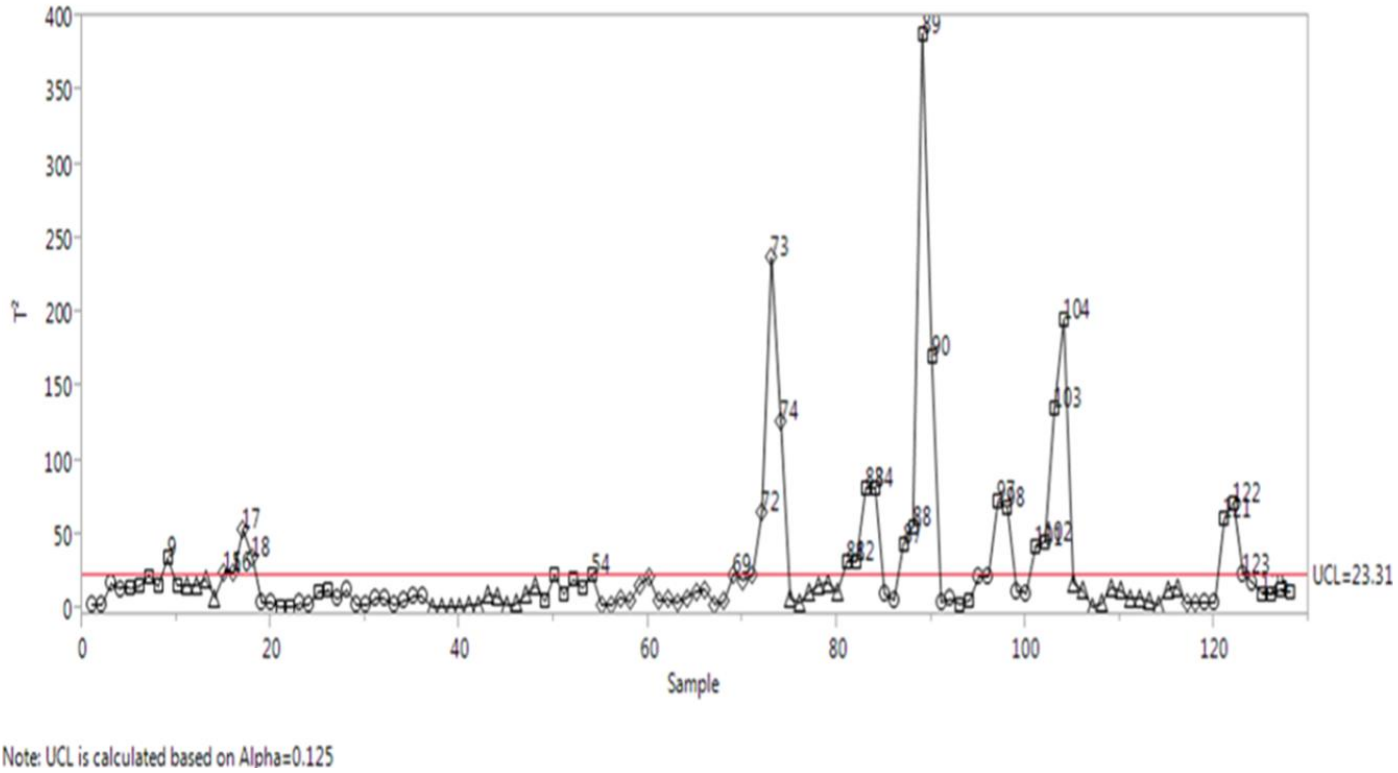

Figure 6. Multi-variate control chart showing the T^2 for 128 runs. Alpha = 0.115 is set so that the line of the upper control limit (UCL) crosses the training sample with highest value (run 123). Runs which are above the UCL of T2 = 23.31 are rejected. Legend: o - training set; Δ - submitted samples; ◊ - commercial products; □ - experimental set. The numbers refer to the run number as indicated in Table 2.

quantitative marker compound.

There was no clear relationship between %agnuside content as measured by qTLC, its cluster grouping (Figure 4), and its T^2 value in the control chart (Figure 5). For example, runs 75 to 78 (submitted samples) had low %agnuside content of 2.3, 2.2, 0.4, and 0.4%, respectively, but were below the UCL, while runs 103 and 104 (experimental samples) had relatively high agnuside content (4.9 and 4.8%, respectively) but were rejected based on their T^2 value. Some runs such as 69 and 70, had 0% agnuside, but were still within the UCL line.

DISCUSSION

The official pharmacopoeia method for the validation of herbal medicines relies on the use of thin layer chromatography (TLC), gas chromatography (GC), or high performance liquid chromatography (HPLC) for the analysis of chemical markers or pharmacologically-active components (EDQM, 2007; WHO, 2011). However, these methods which are based on the targeted analysis of one or two compounds cannot give an adequate assessment of the quality of an herbal sample which contains hundreds of compounds.

The objective of this work was to determine whether the profile of all carbon atoms generated by 13C NMR is able to provide an accurate multivariate profile of a complex mixture, such as extracts of a medicinal plant. To do this, four types of samples were obtained: a training set, submitted samples, commercial samples, and experimental samples. The results from the k-means cluster, closely agreed with the type of samples that were analyzed. This gives good confidence that the use 13C NMR with subsequent multivariate analysis using k-means cluster is able to accurately generate a chemical profile of the extract. Further, a multivariate control chart was generated from which an upper control limit (UCL) of the multivariate profiles of the samples could be set.

Comparison of the results of the multivariate control chart and the univariate qTLC analysis using agnuside as marker compound showed poor correspondence. The results showed that a sample can have a high content of agnuside but be above the UCL of the multi-variate control chart. This highlights the difference between a targeted analysis of a single compound and a multivariate chemical profile: a single compound cannot represent the quality of a complex mixture.

To obtain reliable statistical results, a large training set is needed and the method of extraction and spectroscopic measurement must be optimized and standardized to avoid bias, maximize reproducibility and minimize

Table 2. Summary of results of qTLC analysis using agnuside as marker compound, runs with T2 value above 23.31 are rejected. cluster grouping, and Hoteling T^2 value. Selected runs are indicated in the cluster plot (Figure 4) and control chart (Figure 5).

Run No.	Sample Type	% Agnuside	Cluster No.	T^2 Value	Run No.	Sample Type	% Agnuside	Cluster No.	T^2 Value
1	Training	6.2	2	3.7	65	Commercial	1.9	3	12.33
2	Training	6.1	2	2.9	66	Commercial	1.8	3	13.13
3	Training	2.8	2	17.43	67	Commercial	4.3	3	3.95
4	Training	2.8	2	14.19	68	Commercial	4.4	3	5.82
5	Experimental	0.6	1	14.29	69	Commercial	0.0	1	24.29
6	Experimental	0.7	1	15.94	70	Commercial	0.0	1	20.00
7	Experimental	0.9	1	22.92	71	Commercial	1.7	4	23.25
8	Experimental	0.9	1	15.79	72	Commercial	1.7	4	65.93
9	Experimental	7.4	2	34.89	73	Commercial	1.6	4	237.38
10	Experimental	7.3	2	16.59	74	Commercial	1.6	4	126.98
11	Submitted	6.4	2	16.18	75	Submitted	2.3	2	7.64
12	Submitted	6.8	2	16.17	76	Submitted	2.2	2	4.75
13	Submitted	5.4	3	20.73	77	Submitted	0.4	3	12.06
14	Submitted	5.4	3	8.38	78	Submitted	0.4	3	16.72
15	Commercial	1.2	3	24.52	79	Submitted	4.4	2	18.27
16	Commercial	1.1	3	24.82	80	Submitted	3.9	2	12.14
17	Commercial	2.8	4	54.16	81	Experimental	0.0	1	32.29
18	Commercial	2.9	4	35.28	82	Experimental	0.0	1	32.62
19	Training	3.7	3	4.45	83	Experimental	0.0	1	81.93
20	Training	3.5	3	4.59	84	Experimental	0.0	1	82.27
21	Experimental	5.0	2	1.54	85	Training	2.4	2	10.47
22	Experimental	5.3	2	1.80	86	Training	2.5	2	6.74
23	Training	5.8	2	4.11	87	Experimental	0.0	1	44.84
24	Training	5.4	2	3.97	88	Experimental	0.0	1	55.63
25	Experimental	0.3	3	11.80	89	Experimental	2.1	4	388.86
26	Experimental	0.4	3	12.80	90	Experimental	2.0	4	170.53
27	Training	2.4	3	7.31	91	Training	2.2	2	5.37
28	Training	2.4	3	13.16	92	Training	2.3	2	7.10
29	Training	4.9	2	3.33	93	Experimental	1.4	2	3.89
30	Training	4.9	2	2.87	94	Experimental	1.7	2	6.33
31	Training	5.0	2	7.12	95	Training	2.0	4	22.62
32	Training	5.0	2	7.27	96	Training	1.8	4	22.12
33	Training	7.0	2	3.41	97	Experimental	0.0	1	72.77
34	Training	6.8	2	5.85	98	Experimental	0.0	1	69.64
35	Training	3.8	3	8.56	99	Training	7.0	2	12.53

Table 2. Contd.

36	Training	3.7	3	8.58	100	Training	6.6	2	11.16
37	Submitted	4.6	2	2.47	101	Experimental	0.3	3	42.75
38	Submitted	4.4	2	1.60	102	Experimental	0.2	3	46.10
39	Submitted	5.4	2	1.71	103	Experimental	4.9	2	136.11
40	Submitted	5.6	2	1.78	104	Experimental	4.8	2	195.96
41	Submitted	4.8	2	3.30	105	Submitted	1.3	3	18.19
42	Submitted	4.5	2	3.64	106	Submitted	1.4	3	12.95
43	Submitted	3.9	2	10.55	107	Submitted	2.5	2	2.01
44	Submitted	4.3	2	8.79	108	Submitted	2.4	2	4.36
45	Submitted	4.4	2	2.83	109	Submitted	3.7	2	15.10
46	Submitted	4.9	2	4.22	110	Submitted	3.1	2	12.93
47	Submitted	4.5	2	10.85	111	Submitted	2.5	2	7.24
48	Submitted	4.1	2	16.55	112	Submitted	2.2	2	8.26
49	Experimental	4.4	2	5.82	113	Submitted	2.7	2	5.81
50	Experimental	4.1	2	23.28	114	Submitted	2.5	2	3.46
51	Experimental	5.2	2	10.45	115	Submitted	1.5	4	13.95
52	Experimental	5.1	2	20.39	116	Submitted	1.5	4	15.39
53	Experimental	5.6	2	14.88	117	Commercial	1.5	4	4.12
54	Experimental	5.5	2	23.48	118	Commercial	1.5	4	4.75
55	Commercial	2.6	3	2.94	119	Training	1.7	2	5.06
56	Commercial	2.5	3	2.92	120	Training	1.7	2	4.66
57	Commercial	4.3	3	8.34	121	Experimental	0.0	1	60.98
58	Commercial	4.6	3	5.49	122	Experimental	0.0	1	72.50
59	Commercial	0.9	3	17.00	123	Training	3.1	2	23.93
60	Commercial	0.9	3	22.19	124	Training	3.0	2	18.34
61	Commercial	2.1	3	6.11	125	Experimental	0.5	2	11.23
62	Commercial	2.4	3	7.06	126	Experimental	0.5	2	10.33
63	Commercial	3.5	3	4.95	127	Experimental	7.7	2	13.73
64	Commercial	3.4	3	8.33	128	Experimental	7.7	2	12.29

variation. In this procedure, the 60 highest 13C NMR peaks in each spectrum were selected. The use of fewer peaks makes the statistics easier to calculate but may decrease the chemical reliability. On the other hand, the use of a large number of peaks (>60) will require more training set samples, which will make the procedure more time-consuming.

Comparison of the 13C NMR profile generated at 100 and 125 MHz showed that, comparable profiles are generated. On the other hand, the 1H NMR spectra obtained at 400 and 500 MHz were clearly different. This means that 1H NMR profiles are comparable only at the same magnetic field strength while 13C NMR spectra from different magnetic field strengths may still be compared. However, further comparisons of 13C NMR spectra using bigger differences in magnetic field

should be done to determine how general this is.

Finally, it is worth noting that NMR is one of several methods that can be used for a multivariate or fingerprint analysis of plant extracts. For example, fingerprint analysis of *V. negundo* seed samples from different regions in China was done using high-performance liquid chromatography (HPLC) with diode array detection, with hierarchical cluster analysis (HCA) (Shu et al., 2016); mass spectrometry together with HCA were used for the identification and quantitative analysis of phenolic compounds in *V. negundo* in other to identify possible chemical markers (Huang et al., 2015).

Conclusions

13C NMR spectra of extracts of medicinal plants can be used to generate a k-means cluster, which accurately represents the chemical profile of the samples. The 13C NMR data can also be used to generate a multivariate control chart which sets the upper control limit based on the 13C NMR profile. Comparison of the multivariate control chart with qTLC results showed poor correspondence. This indicates that a univariate analysis of a plant sample is useful only for quantification of the target compound but cannot be used for chemical profiling and standardization of medicinal plants.

Conflict of Interests

The authors have not declared any conflict of interests.

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