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Effect of solvent systems using ultrasonic assisted extraction on the extractability of water soluble vitamins and application to their determination in *Elephantorrhiza burkei* and *Euphorbia tirucalli* plants from Botswana

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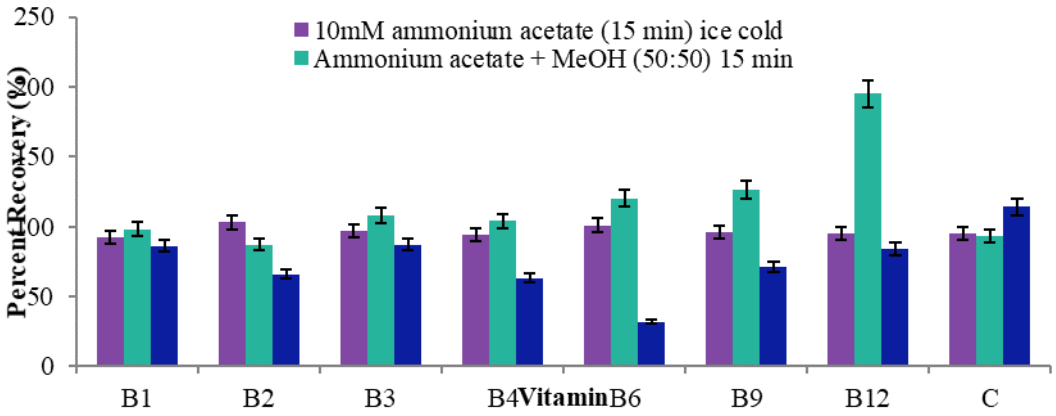
Abstract: A study to evaluate the water-soluble vitamin contents of two medicinal plants *Elephantorrhiza burkei* and *Euphorbia tirucalli* root extracts i.e. from plants that have a rich history of use in folklore human medicine from Botswana was conducted. Three solvent extraction strategies were compared and the best i.e. ultrasonic assisted extraction (UAE) employing 10 mM ammonium acetate was used. Ice-cold UAE was used to extract water-soluble vitamins from the plants and clean-up was accomplished using silica-based C-18 solid phase extraction (SPE). Vitamins were separated and quantified by reversed phase HPLC using a Synergi silica-based C18 column. Quantitative analysis of vitamins in the *Elephantorrhiza burkei* yielded the following: vitamin B_1 (3.5); vitamin B_2 (1.0); vitamin B_4 (27.5); vitamin B_6 (1.9), vitamin B_9 (not detected) and vitamin C (32.0) and from *Euphorbia tirucalli* root extracts yielded vitamin B_1 (1.8); vitamin B_2 (0.06); vitamin B_4 (12.7); vitamin B6 (0.14); vitamin B_9 (0.008) and vitamin C (13.6) all expressed in mg/100g, dry weight (DW) as averages of three extractions i.e. n = 3, respectively. These vitamin contents could contributors to the medicinal properties of the two plants and support their popular use in herbal medicine in the South-Western and the South-Eastern parts of Botswana. The high concentrations obtained for vitamin B_1, B_2, B4, B6 and C in the *Elephantorrhiza burkei* root is a blue print for its use as a pharmaceutically active ingredient or food supplement compared to the *Euphorbia tirucalli* root.



Keywords: **Medicinal plants, Botswana, Water-soluble vitamins, UAE, SPE, Reversed phase HPLC-DAD**

1. Introduction

Vitamins are a low molecular weight group of organic compounds required in small quantities to perform various chemical and physiological functions for normal growth, and maintenance of human and animal bodies. They are derived from every plant part and some parts of animal sources. Early nutritional studies identified thirteen (13) vitamins that are generally classified into two groups according to their solubility, water-soluble vitamins and fat-soluble vitamins (Ball, 2006). The water-soluble vitamins excluding vitamin C (ascorbic acid) are known as the vitamin B-complex group: thiamin (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3),

pantothenic acid (vitamin B5), vitamin B6 (pyridoxine), folate (folic acid) (vitamin B9), Cyanocobalamin (vitamin B12), and biotin (vitamin B7) are mainly essential for the physiological functions of human body which have being claimed as an antioxidant supplement compounds (Aja *et al.,* 2013). The most outstanding and attractive properties of these compounds is that they have wide ranges of pharmacological activities. All water-soluble vitamins circulate in the blood and are stored in limited amounts than fat-soluble vitamins. They serve as biological catalysts for certain metabolic reactions in the body and are only required in very small amounts. Lack of vitamins can cause serious human health diseases and sometimes, very small concentrations are required for maintenance of good human health (Hussian *et al*., 2006). For example, hundredths of a grams would be enough for a body to function normally (Combs, 2008). It has been reported that plants rich in anti-oxidant vitamins play a protective role in health and against incident mortality from diabetes, cancer, heart disease, fever, flu, hypertension and stroke (Adewale *et al*., 2013).

Traditional herbal medicines have for years been used to treat infectious diseases as an alternative method of medications as well as for the development of pharmaceutical drugs, nutraceuticals and health products in various parts of the world (Cowan 1999; WHO, 2002; Adebayo and Krettli, 2011, Laandrault *et al*., 2001). In recent times, vitamins from natural sources are gaining considerable attention, since these bioactive substances are often used in the preparation of dietary supplements, functional food ingredients, nutraceuticals, food additives, pharmaceuticals and cosmetic products to improve the health of individuals as well as communities. Several studies have reported that some water-soluble vitamins are responsible for various specific and vital functions in many metabolic process, and their deficiency or excess availability can cause specific diseases (Berger, 1985; McCormick, 1996; FAO/WHO, 1974).

Over the years, human beings recognized the curative properties of plants in their surrounding environment and developed different preparations for their healing purposes. These experiences form what is commonly referred to as ethno-medicine, and the practices were then documented through the centuries to build traditional medicine. Botswana is a developing country in the Southern Africa which has abundant traditional herbs used to relieve and treat many ailments. In rural areas, medicinal herbs are cheap sources used almost to cure most of their common ailments. In a review by Chauke *et al,* 2015, it was reported that about 51% of the medicines prepared for treatment of a variety of diseases were derived from roots of plants with leaves being the least preferred sources. Generally, most common medicines, food nutrients and supplements have a group of compounds extracted from plants as their primary bioactive ingredients and many have also provided blueprints for synthesis or partial synthesis of pharmaceutical products.

This study evaluates and compares the content of water-soluble vitamins in the extracts of two medicinal plants *Elephantorrhiza burkei* (*E. burkei*) and *Euphorbia tirucalli* (*E. trucalli*) roots that are native to Botswana and Southern Africa in general. These two plants are used extensively in the management and treatment of a number of ailments. *E. burkei* belonging to the Mimosaceae species is also commonly called "elephant's root. This indigenous plant is locally known as Mositsane or Mosidi in Botswana and occurs naturally throughout the dry parts of Southern Africa. They flower from September to November and pollinated mainly by African honeybee. In a paper reviewed by Alfred Maroyi, (2017), it was revealed that the species leaf, root, stem and stem bark decoctions are used in Southern Africa as traditional medicine (muthi) for a wide range of human diseases and ailments including abdominal pains, backache, high blood pressure, sores of the penis and vulva, dermatological diseases, gastrointestinal system disorders, sexual dysfunction, sexually transmitted infections, and wounds (Danley Kristen, 2006; Hedberg and Staugard, 1989; Mander *et al*., 2005; Hedberg and Staugard, 1989).

Cultivated *E. tirucalli* belongs to the plant family *Euphorbiaceae*, which is commonly known as Barki-thohar (Gupta *et al*., 2013). *E. tirucalli* has been used widely to treat many ailments including snakebites, warts, syphilis, sexual impotence and in skin parasites decoction extraction in Africa (Kokwaro, 1993; Wal *et al*., 2013; Mwine *et al*., 2011).

Numerous analytical methods have been developed for variety of complex matrices to quantify water soluble vitamin compounds. To establish analytical figure of merits of extraction and chromatographic separation are the most challenging. The objectives of this study were to evaluate the influence of extracting solvents on a simple and low cost ultrasonic assisted extraction (UAE) procedure for water-soluble vitamins using *Moringa oleifera* species as a model sample and to determine and compare quantitatively the number of water-soluble vitamins and the contents in two medicinal plants *E. burkei* and *E. tirucalli* roots from Botswana. Analysis would follow using reversed phase high performance liquid chromatography (RP-HPLC).

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2. Results and discussion

2.1. Peak identification

Chromatographically, eight (8) water-soluble vitamins were resolved within a time span of 12 minutes. Figure 1 shows the HPLC chromatogram of a water-soluble vitamin standards mixture in a single run.

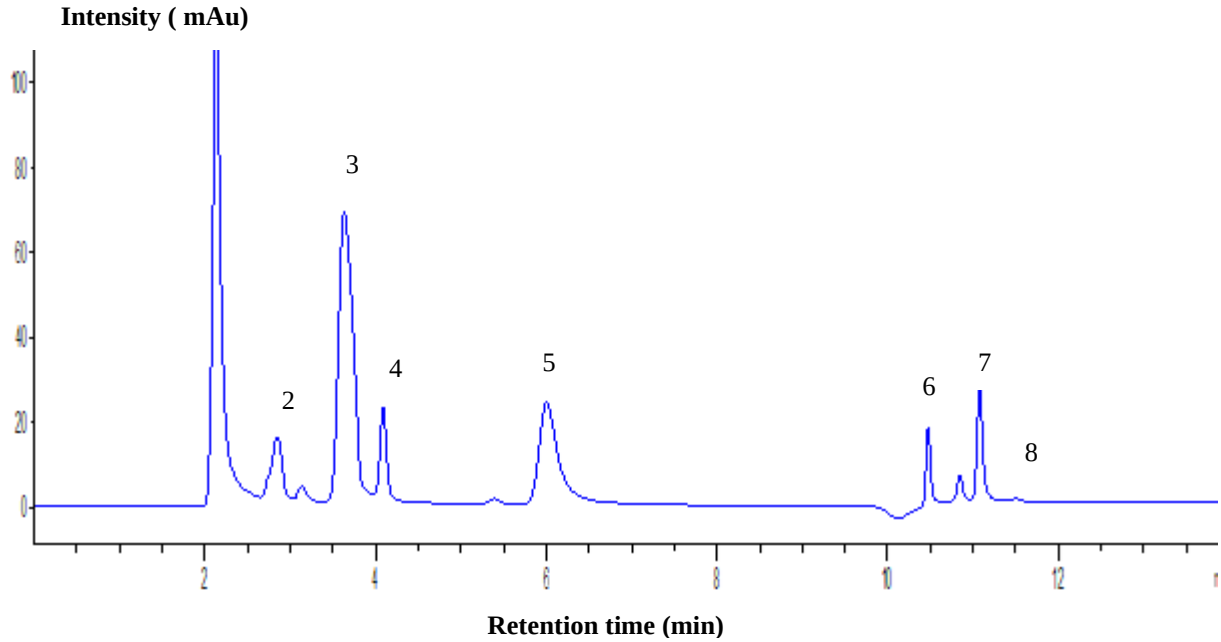

Fig. 1. Chromatogram of a mixture of eight water-soluble vitamin standards at 266 nm
1 = B1; 2 = B6; 3 = B4; 4 = C; 5 = B3, 6 = B12; 7 = B9, 8 = B2

Figure 2 shows the chromatogram of a real E. burkei root sample extract using 10 mM ammonium acetate as solvent for the ultrasonic assisted extraction (UAE) procedure.

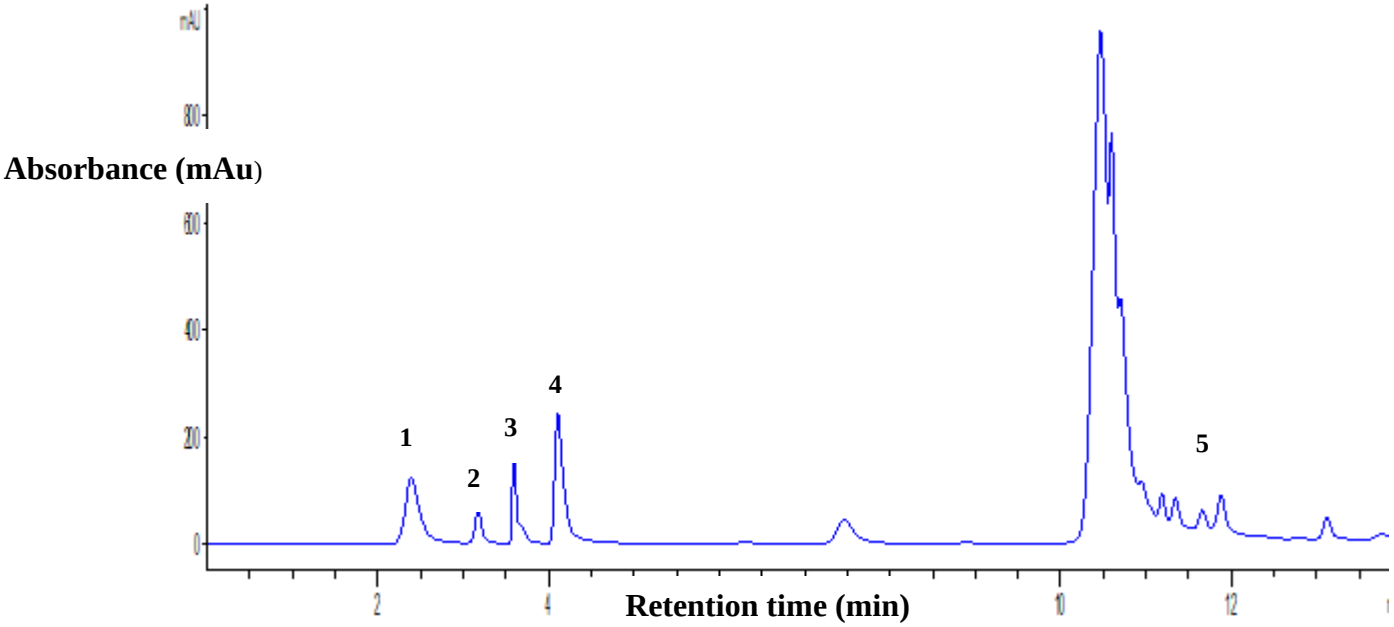

Fig. 2. Chromatogram of *E. burkei* root extract at the same chromatographic conditions. 1= B_1; 2 = B_6; 3 = B_4; 4 = C; 5 = B_2

2.2. Extraction efficiencies of the three solvents compared for UAE extraction

To ascertain the influence of solvent polarity as a factor and to what extent it influenced the efficiency of extractions, one-way analysis of variance (ANOVA) was carried out. The null hypothesis was that the variances that arose were from random effects for a significance level of 0.05. Using MINITAB Version 19, the calculated *F* value was 0.53 with a *p* value of 0.799. As a result, the null hypothesis was rejected implying that the polarity of the solvent played a significant role. Figure 3 shows the percentage recoveries of the vitamins from *Moringa oleifera* used as a model sample using three solvents and UAE. The results showed the solvent dependency of the UAE procedure.

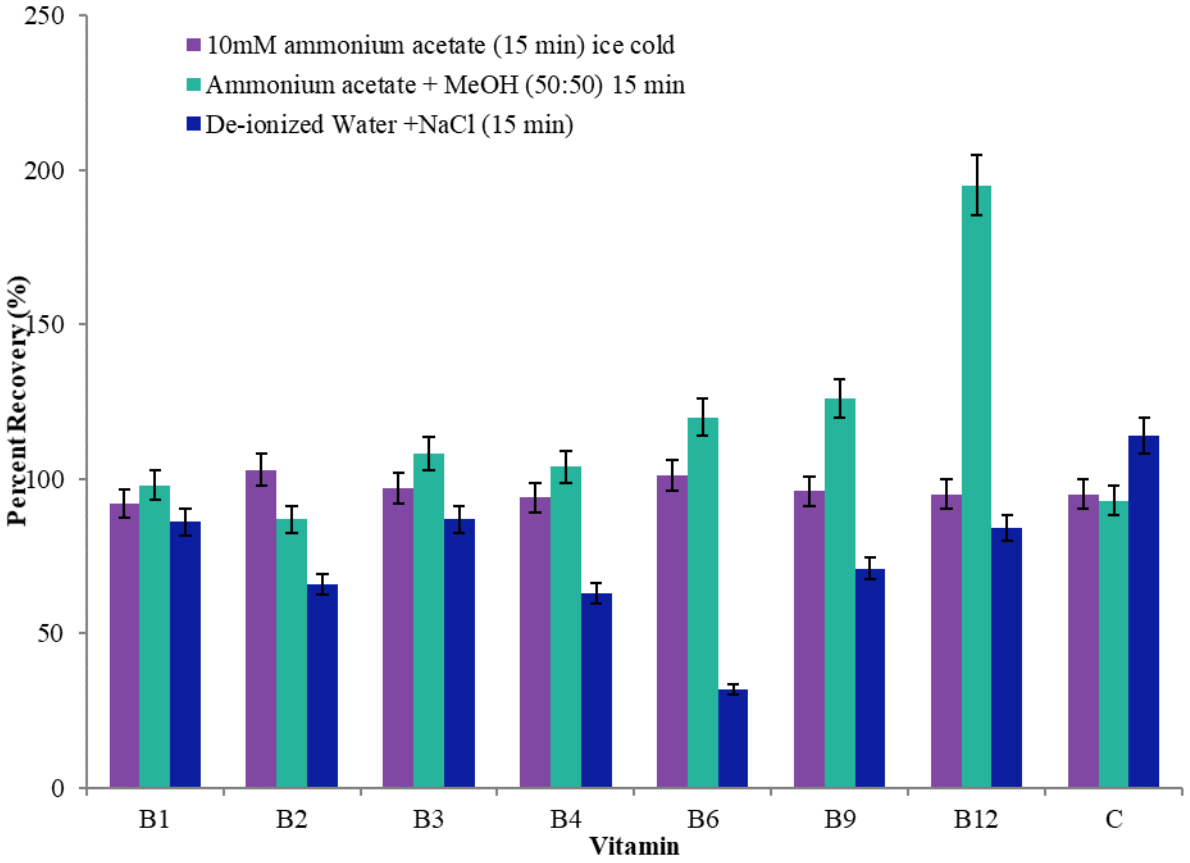

Fig. 3. Recoveries of free water-soluble vitamins (%) from *Moringa oleifera* as a model sample under UAE

By visual inspection, the extraction procedure employing ammonium acetate : methanol in a 50:50 ratio gave the best vitamin yields i.e. extracted the highest average percent yield as seen in Figure 3. However, a caveat was discovered when an exaggerated extraction efficiency of 195 % for vitamin B_{12} was observed. It was speculated that this procedure yielded a positive error whose source was not investigated but which coincidentally was within the error margin for the extraction procedure employing 10 mM ammonium acetate from the overlap of the ±5 % vertical error bars in Figure 3. This led to the selection of the 10 mM ammonium acetate method for use in subsequent experiments. Figure 4 is an interval plot of the three extraction procedures showing the 95% confidence interval (CI) for the mean percent recoveries. The pooled standard deviation was used to calculate the intervals. Using the same ultrasound conditions, the 10 Mm ammonium acetate procedure yielded percent recoveries with a 95 % confidence interval (CI) of 78.8-114.4 % compared to ammonium acetate plus methanol (50:50) where the 95 % CI was 98.6-134.2 %. Using a solution of sodium chloride in de-ionized water, the percent recoveries 95 % Ci ranged between 57.6 – 93.2 %. The differences in the extraction

efficiencies of the analytes were speculated to be caused by the differences in the polarities of the solvents as has been reported in the literature previously (Melecchi *et al*., 2006) and demonstrated using ANOVA.

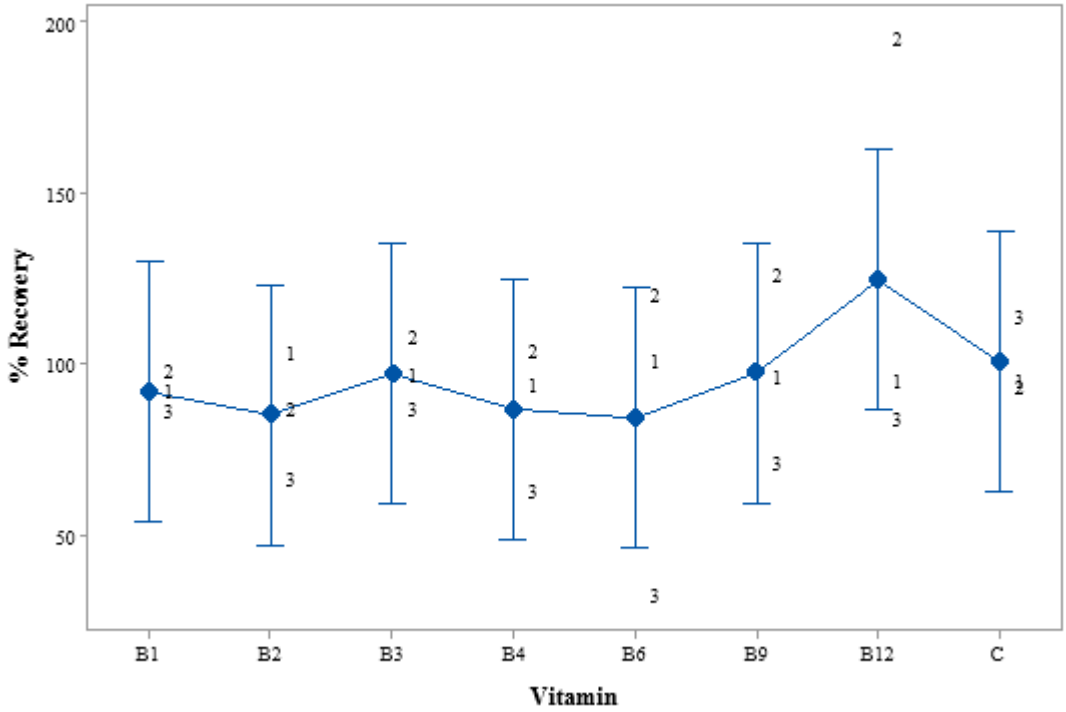

Fig. 4. Interval plot of the three extraction procedures showing the 95% confidence interval (CI) for the mean percent recoveries for all the extracted vitamins
1 = 10 mM ammonium acetate; 2 = ammonium acetate + methanol (50:50); 3 = de-ionized water + sodium chloride

2.3. Concentrations of water soluble vitamins in E. burkei and E. Tiucalli root extracts

The free water-soluble vitamins contents in *E. burkei* and *E. tirucalli* root extracts were detected in significant quantities as seen in Table 1. Vitamin B_1 (thiamine), B_2 (riboflavin), B_4 (adenine), B_6 (pyridoxine) and C (ascorbic acid) were quantified in *E. burkei* while in *E. tirucalli* roots, six vitamins i.e. vitamin B_1 (thiamine), B_2 (riboflavin), B_4 (adenine), B_6 (pyridoxine), B_9 (folic acid) and C (ascorbic acid) were identified and quantified. Vitamin B_3, and B_{12} were not detected in both plant root extract samples as seen in Table 2. *E. burkei* contained the highest amount of vitamin C at 32.0 ± 1.00 mg/100g DW compared to *E. tirucalli*, which contained 13.57 ± 0.01 mg/100g DW. Other vitamin B_1, B_2, B_6, B_9 and adenine (B_4) were detected in both types of plant species roots, while vitamin B_9 was not found in *E. burkei.*

Table 1. Concentration of free water-soluble vitamins in *E. burkei* and *E. tirucalli* roots and recommended daily allowances by the National Academy of Sciences Food and Nutrition (1989)

Vitamin	Concentration of vitamins (mg/100g) DW		Recommended daily dose (mg/day)
	E. burkei	E. tirucalli	
B_1	3.5 ± 0.20	1.83 ± 0.02	1.0-1.5
B_2	1.0 ± 0.10	0.064 ± 0.01	1.3-1.8
B_3	ND	ND	15-20
B_4	27.5 ± 0.10	12.72 ± 0.02	Not found
B_6	1.9 ± 0.10	0.14 ± 0.01	2
B_9	ND	0.0075±0.0004	0.4
B_{12}	ND	ND	2.4
C	32.0 ± 1.00	13.57 ± 0.01	30-180

ND = not detected, Means of n = 3, 95% confidence interval

2.2. Correlation of the results with recommended daily vitamin intake allowances by the National Academy of Sciences Food and Nutrition

Using MINITAB Version 19, correlation analysis using the Pearson correlation coefficients was fitted as shown in Figure 5. The correlation between the vitamin levels in *E. burkei* and the recommended daily allowances by the National Academy of Sciences Food and Nutrition (NASFN) was 0.675 with a *p* value of 0.066 for a significance level of 0.05. The correlation between *E. tirucalli* vitamin levels and the NASFN recommended daily allowances was 0.638 with a *p* value of 0.089 for a significance level of 0.05. It was speculated from the results that *E. burkei* and *E. tirucall*i have therapeutic and nutritional potential for humans because they can supply the recommended daily levels vitamins if exploited appropriately either for therapeutics or dietary supplementation.

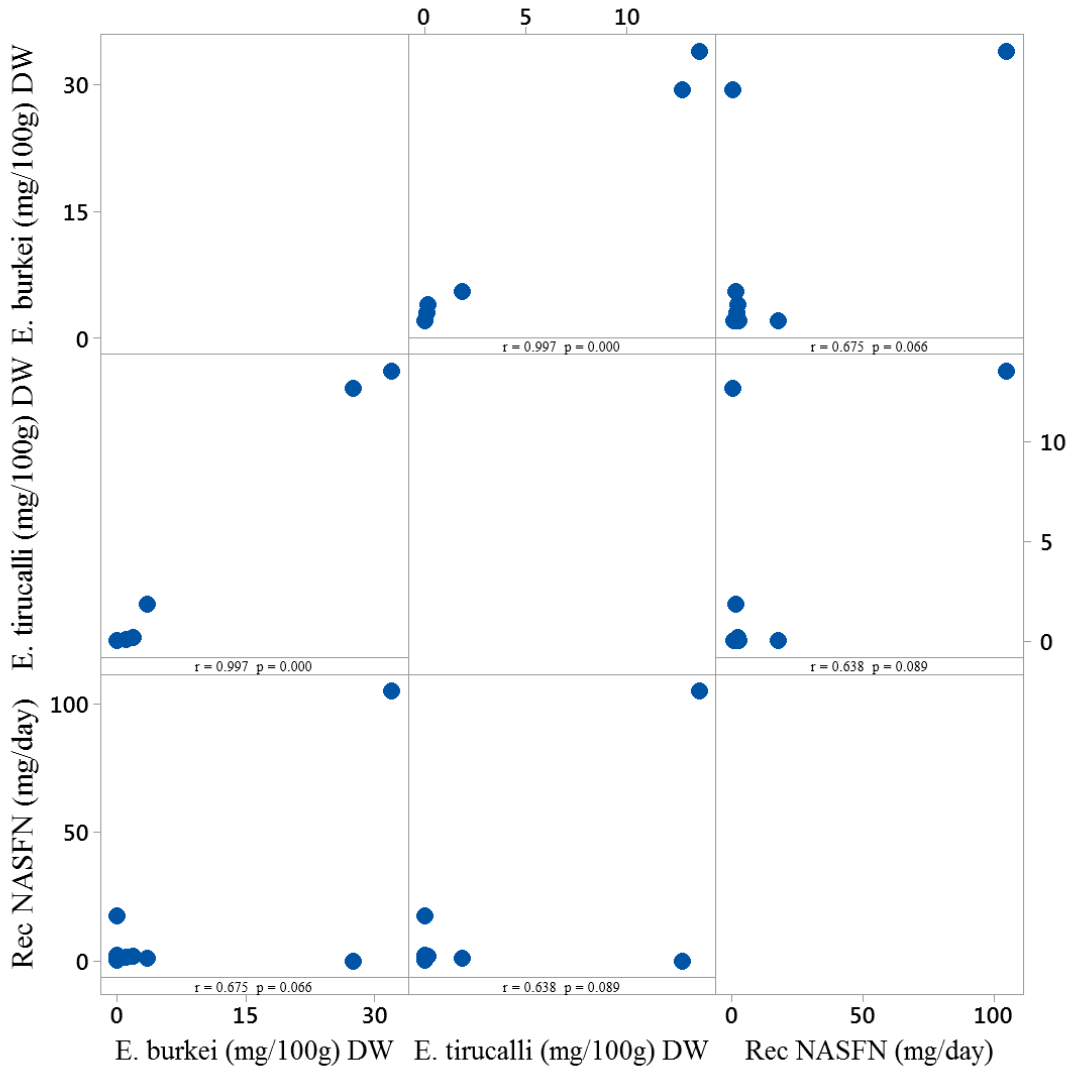

Fig. 5. Pearson correlation coefficients matrix plot of E. burkei, E. tirucalli vitamin contents in (mg/100g) DW and recommended daily allowed vitamin levels by NASFN (Rec NSFN) in mg/day

In this study it has been demonstrated that *E. burkei* roots contain a high concentration of free vitamin C. Carr and Maggini (2017) reported that vitamin C acts as an enzyme co-factor for the biosynthesis of many biochemical processes. In addition, Combs (2008) showed that vitamin B_6 breaks down amino acids and aid in the development of haemoglobin components.

3. Experimental

3.1. Chemicals and Reagents

Chemicals and reagents were purchased from Sigma-Aldrich, Poole, (Dorset, Dublin, Ireland). Methanol, vitamin B1, B2, B3, B4, B6, B9, B12 standards were purchased from Rochelle Chemicals (Johannesburg, South Africa) while vitamin C standard was from Merck (Carolina, USA). Formic acid (99%), potassium hydroxide pellets and potassium hydrogen carbonate salt were from uniLAB (Mandaluyong, Philippines). All water-soluble vitamin standards were of analytical grade. Methanol and all other reagents/solvents were of HPLC grade. Double distilled water used for HPLC analysis was prepared by an Elix® Millipore deionizer (Saint-Quentin-en-Yvelines Cedex, France). Sep-Pak C18 solid phase extraction (SPE) cartridges (1 g) were purchased from the Waters Corporation (Dublin, Ireland)

3.2. Instrumentation

The Agilent Technologies 1260 Infinity Series Liquid Chromatography system, (Mainz, Germany) used throughout consisted of a G 1312C, a degasser, binary pump, a G 1316A column thermostat and a G 1315D UV variable wavelength detector. An Agilent Technologies S 6020 manual injector fitted with a 20 µL loop was used throughout. Instrument control and data acquisition/analysis were done using Chemstation (Agilent Technologies) software. All experiments were done at 25 °C. UV data acquisitions were done at 266 and 270 nm, depending on the vitamins of interest. The pH of aqueous mobile phase was measured using a Nanna Hi 2211 pH meter (Europe, USA). The analytical column, Synergi C18, 150 x 4.6, 4 µm particle size (Phenomenex Co in USA) was used throughout. A glass vacuum-filtration apparatus obtained from Alltech Associates was employed in the filtration of the mobile phase solutions, using 0.45 µm membrane filters obtained from Millipore, Bedford, MA, USA. All solutions were degassed before use. To achieve better extraction each sample was vortex using a vortex instrument (Scientific industries, Inc. Bohema, NewYork, USA). Extractions were performed with ice-cold ultrasonic bath (SCIENTECH ultrasonic cleaner, South Africa; model: 704), which worked at low kHz, high kHz frequencies with a variable sonic power output, and was fitted with a digital timer to set up time and a temperature controller to control the temperature.

3.3. Sample treatment and drying.

Fresh and matured *E. burkei* and *E. tirucalli* roots were collected from their natural habitats in Moshupa village in the South-Western region of Botswana. The plants were identified and authenticated by a Botanist in the Department of Biological Sciences at the University of Botswana. Each of the plant samples was washed extensively in distilled water in order to remove superficial dust and sand. After washing, the roots were air dried until constant weights were obtained in subdued light to prevent environmental factors from impacting the vitamins for at least 21 days. The samples were further ground into fine powder with a food grade blender machine. The powdered samples were kept in separate air-tight dark containers to shield them from light until use.

3.4. Stock standard solutions of water-soluble vitamins

Stock standard solutions of water-soluble i.e. vitamins B1 (thiamine), B2 (riboflavin), B3 (niacin), B4, B6 (pyridoxine), B9 (folic acid), B12 (cobalamin) and C (ascorbic acid) were prepared individually by accurately weighing 0.025 g of each and quantitatively transferring to 25 mL volumetric flasks and dissolving with double distilled water to make stock solutions of 1.0 mg/mL i.e. 1000 ppm of each. Since vitamin C was limited, it was freshly prepared at a higher concentration of 4000 mg/mL at the time of use. A 0.45 µm Nylon membrane filter type, HNWP, Millipore (Dublin, Ireland) were used as required to remove undissolved particles from all solutions before injection on the HPLC system. The solubility of vitamin B2 (riboflavin) and vitamin B9 (folic acid) were poor in water and as such 0.5 mg/mL (500 ppm) of vitamin B2 was prepared using 5mM of KOH and 20 mM $KHCO_3$ for vitamin B9. Working standard solutions were prepared from these solutions and diluted

with water to obtain appropriate calibration standards prior to analysis. All the stock and working standard solutions were prepared in dark brown bottles and stored at + 4 ^{0}C when not in use except vitamin C (ascorbic acid) which was prepared at the time of use.

3.5. Calibrations

The calibration curves were constructed based on six measured concentration levels of external standards as shown in Table 2. The standards addition method i.e. the matrix matching approach was used in order to eliminate matrix effects. Peak height versus concentration for each vitamin were analysed by linear least-square regression and the regression equations obtained from the calibration curves, were used to estimate each water-soluble vitamin content in the samples.

Table 2. Dilution scheme for preparation of calibration standards.

Standard Solution	**1**	**2**	**3**	**4**	**5**	**6**
Volume of mixed stock solution for 10 mL preparation (mL)	0.5	1.0	1.5	2.0	2.5	3.0
Concentration of Vitamin B_1, B_3, B_4, B_6, B_{12} (µg/mL)	2.5	5.0	7.5	10.0	12.5	15.0
Concentration of Vitamin C (µg/mL)	5.0	10.0	15.0	20.0	25.0	30.0
Concentration of Vitamin B_2, B_9 (µg/mL)	0.75	1.5	2.25	3.0	3.75	4.5

Detection was done at 266 nm for vitamin B_1, B_3, B_4, B_6, B_{12} and C and 270 nm for vitamin B_2 and B_9 at room temperature.

3.6. Sample Preparation for Extraction Efficiency Estimation

Samples were protected from direct exposure to light and kept in ice to minimize vitamin degradation during the extraction process. The extraction efficiencies expressed as percent (%) recoveries of the free water-soluble vitamins were gauged using dried *Moringa oleifera* plant species as a model plant sample. Using the same ultrasound conditions, three extracting solvents of varying polarities were tested i.e. (i) 10 mM ammonium acetate solution (ii) de-ionised water with 0.2 g of sodium chloride and (iii) 10 mM ammonium acetate in methanol at a ratio of 50:50. These were tested for their extraction efficiencies using a cold UAE procedure optimized in this laboratory. The solvents were selected because they are less expensive, have low toxicity to the bioassay and are compatible with HPLC systems and detectors.

Extractions were carried out using a method developed in this laboratory (Abibu *et al*., 2019). In a nutshell, two sets of extractions were carried out for each sample. In the first instance the sample was spiked with a known concentration standard mixture of the target compounds then extracted. These were labeled pre-extraction matrix spikes abbreviated (PrEMs) and in the other, the same sample was extracted and spiked with the same concentration of a vitamin mixture containing the target vitamins after extraction. These were the post extraction matrix spikes (PoEMs). The peak heights of the PrEMs and PoEMs after the extraction and injection into the HPLC system were then compared. The % recoveries of the vitamins were calculated by using Equation 1.

$$\% \text{ Recovery} = \frac{\text{Concentration of vitamins in PrEMs}}{\text{Concentration of vitamins in PoEMs}} \text{X } 100 \qquad (1)$$

2.5 g of sample were weighed into a 50 mL centrifuge tube, and 1 mL 0.00108 M phenol solution as an internal standard was added. This was followed by addition of 20 mL of extracting solvent followed by vortexing for 5 minutes and extraction using an ice cold ultrasonic bath for 15 minutes. In order to release free form water-soluble vitamins, 10 mL of chloroform was added to the extracts to precipitate proteins, sugars and lipid. The precipitation process involved vortexing the sample extracts for an additional 1 minute and centrifuging for 10 minutes at 5000 rpm to remove suspended material. The supernatant was filtered through a Whatman No. 1 filter paper to remove large suspended particles. Smaller particles were removed by filtration using a 0.45-µm Teflon filter. The filtrate collected was passed through silica based solid phase extraction (SPE) cartridges as described in section 2.6.1 to trap the vitamins as a final sample clean-up step before injection into the HPLC system. The same procedure was repeated by changing extraction solvents using a fixed sample weight and frequency of sonication. The extraction conditions of the UAE were also optimized prior to this to achieve best extraction efficiencies. All experiments were performed in triplicates.

3.6.1. Sample clean-up

In order to remove co-extracts from sample matrix and to improve HPLC separations and detections, SPE C18 cartridge was used for sample clean-up. Before use, the cartridge was optimized for sample load volume, elution flow rate and elution solvent volume. It was previously conditioned by flushing with 10 mL methanol and 10 mL hydrochloric acid (pH 2.7) to activate the stationary phase and prevent ionization of analytes. 5 mL of the sample extract were then loaded. The sample was eluted with 5 mL of mobile phase (0.01 % formic acid: methanol, 50:50) at a flow rate of 1.2 mL/min. The eluate was collected in an amber coloured bottle to prevent analytes from degradation and a fraction of it injected into the HPLC system.

3.7. Data analysis

Data analysis was accomplished using Chemstation Version C.01.08 [210], Microsoft Excel and MINITAB Version 19.

4. Conclusion

It has been demonstrated that 10 Mm ammonium acetate in cold ice under ultrasound extraction (UAE) is a good extraction solvent for water-soluble vitamins in plant materials. The root extract of *E. burkei* was shown to contain five (5) water-soluble vitamins i.e. vitamins B_1, B_2, B_4, B_6 and C in significant quantities while *E. tirucalli* contains six vitamins i.e. vitamin B_1, B_2, B_4, B_6, B_9 and C also in significant amounts. These findings suggest that *E. burkei* is a better source of water-soluble vitamins than *E. tirucalli*. All group B vitamins including ascorbic acid are responsible for various and vital functions in several metabolic processes such as immune booster and maintenance of body cells. The plants in this study could have medicinal as well as nutritional benefits. From literature, this is the first report of the content of vitamins in the two indigenous medicinal plants *E. burkei* and *E. tirucalli* from Botswana.

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Conflict of interest statement

The authors have declared no conflict of interest.

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