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## Untargeted Bioassay Strategy for Medicinal Plants: *In Vitro* Antidiabetic Activity and $^{13}\text{C}$ NMR Profiling of Extracts from *Vitex negundo* L

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### Abstract

Bioassay-guided fractionation is the principal method for the identification of active constituents in medicinal plants. By design, this method aims to identify the most active compound in a complex mixture with the objective of discovering novel drug candidates. Described here is a complementary method for the identification of known bioactive compounds in medicinal plants which is untargeted and which takes advantage of the large NMR database of known natural products and availability of statistical software. This untargeted bioassay strategy is demonstrated as a proof of principle in the determination of the antidiabetic compounds in *Vitex negundo* L. Crude methanol and ethanol extracts, and chloroform, ethyl acetate and aqueous fractions of *V. negundo* L. were prepared and tested for their *in vitro* antidiabetic potential using the glucose diffusion retardation assay and the *in vitro* starch-amylase inhibition assay. The same crude extracts and fractions were profiled using  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectroscopy. The  $^{13}\text{C}$  NMR spectra of twelve known compounds from the semi-polar fraction of *V. negundo* – two iridoids, seven iridoid glucosides, two flavonoids and one flavonoid C-glucoside – were matched from the  $^{13}\text{C}$  NMR spectra of the extracts and fractions. The  $^{13}\text{C}$  NMR match factor values of the twelve compounds were used in the multivariate correlation analysis with antidiabetic activity using the glucose diffusion retardation activity and the starch-amylase inhibition assay. This method was able to correlate the seven iridoid glucosides with the antidiabetic activity, a result that would have been difficult to obtain using bioassay-guided fractionation.

**Keywords:** *Vitex negundo* L.; Untargeted bioassay; Glucose diffusion retardation index;  $\alpha$ -amylase inhibition; Multivariate correlation;  $^{13}\text{C}$  NMR profiling; Match factor

### Introduction

The standard method for the identification of active compounds in medicinal plants is bioassay-guided fractionation. This method, which was first developed in the 1970s has been successfully used for the discovery of novel bioactive constituents from medicinal plants. By design, bioassay-guided fractionation is a targeted approach which aims to identify compounds with strong activity [1]. However, in the case of medicinal plants that contain several active ingredients with moderate to low activity which can be additive or synergistic, bioassay-guided fractionation is not the most efficient strategy. In such cases, many studies on traditional medicinal plants often end at the level of solvent or chromatographic fractions and do not proceed further. Indeed, a number of authors have pointed out this problem and have suggested that alternative methods be developed [2,3].

Metabolomics refers to the global study of all low molecular weight metabolites (MW<1,500 Da) in a biological sample, such as a cell, organ, or a whole organism [4]. The metabolomics approach can be targeted or untargeted. Targeted metabolomics refers to the measurement of all known compounds in the sample while untargeted metabolomics is the comprehensive unbiased profiling of all the observable compounds in the sample, whether known or unknown. The ever-expanding database of natural product compounds with spectroscopic data and the development of metabolomics offer new approaches in the study of medicinal plants [5]. The most common

techniques used in metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy [6]. NMR analysis of crude extracts yields an unbiased, comprehensive and reproducible profile of extracts and fractions with minimal sample preparation and provides structural information from chemical shift data [7].  $^1\text{H}$  NMR is the more common technique used because of its higher sensitivity; however,  $^1\text{H}$  spectra are complex because of overlapping multiplets and are strongly affected by magnetic field strength and solvent. In comparison,  $^{13}\text{C}$  NMR spectra have simpler singlet signals, are independent of magnetic field strength, and are less affected by solvent effects making it more reproducible and easier to interpret; however,  $^{13}\text{C}$  NMR requires much longer acquisition times [8]. MS, on the other hand, provides information on molecular masses or fragment masses and is much more sensitive, but is more useful in a targeted metabolomics approach [9].

Because untargeted metabolomics generates a lot of data, it must be coupled to chemometric techniques to reduce the amount of data. Principal components analysis (PCA) is a useful statistical technique for reducing the number of variables without loss of information which enables one to more easily find patterns. PCA clusters data based on their characteristic similarities and dissimilarities with no prior knowledge of grouping [10].

An untargeted bioassay strategy using natural product databases provides an efficient way of profiling and dereplicating medicinal plants before proceeding to bioassay-guided fractionation to look for novel compounds. In this work, we illustrate the strategy of untargeted bioassay for the study of medicinal plants. Briefly, the untargeted bioassay strategy that is used in this work involves the preparation of

several non-identical extracts and fractions which are bio assayed and profiled using  $^{13}\text{C}$  NMR. In the case of  $^{13}\text{C}$  NMR, the process of searching for matches between spectra in the database and extracts can be facilitated using automated systems which are able to compare the chemical shifts and intensities in the NMR spectra of the extracts with those from the database to obtain a match which can be scored according to closeness of fit. A rapid and accurate method for calculating  $^{13}\text{C}$  NMR for use in automated structure elucidation was proposed using artificial neural networks, this is the method used in commercial software, such as ACD Labs software Spectrus™. The results can also be manually evaluated as needed which can reveal partial structures [11].

The bioassay results and the matched compounds from  $^{13}\text{C}$  NMR spectra are then subjected to multivariate analysis to reveal correlations between biological activity and matched compounds. This method may be considered as an initial screen and dereplication prior to a search for novel compounds using bioassay-guided fractionation.

Diabetes is the third leading cause of death after heart disease and cancer [12] and medicinal plants can provide a safe and affordable option for the management of diabetes [13]. Carbohydrate digestion starts in the mouth by the hydrolyzing enzyme amylase and continues in the stomach with gastric acid and digestive enzymes. Lower postprandial glucose can be achieved if the rate of its absorption in the small intestine can be decreased [14]. This dialysis experiment mimics the events that occur in the small intestine and is the basis for the *in vitro* experiment of glucose retardation that will be used in this study. The glucose diffusion retardation index (GDRI) is a useful *in vitro* method for the determination of the effect of a plant sample on the delay in glucose diffusion in the gastrointestinal tract. The lower the GDRI value the greater the inhibition activity of the sample. The glucose diffusion model is a simple water bath incubation setup that has been used by many researchers to evaluate the effect of potential antidiabetic agents before initiating long and costly clinical trials [15-17].

*Vitex negundo* L. has been known since ancient times as a medicinal plant for various ailments [18] and a number of flavonoids and iridoids have been isolated from its semi-polar fractions (Figure 1) [8,19,20]. Both *in vitro* and *in vivo* studies have shown that *V. negundo* has promising anti-diabetic properties, in particular, alpha-amylase inhibitory activity [21], regulation of plasma and tissue glycoprotein components in streptozotocin-treated rats [22], and antidiabetic activity using alloxan-treated rats [23]. Devani et al. [24] reported that the chloroform, ethyl acetate, and n-butanol crude extracts of *V. negundo* showed alpha-amylase inhibition. Sundaram et al. [25] reported the isolation of an iridoid glucoside, 2'-para-hydroxybenzoyl mussaenosidic acid (4, also known as negundoside), that showed its

antihyperglycemic effect by improving insulin secretion in diabetic rats. Although the study of Sundaram et al. [25] identified a specific compound, it is possible that other compounds may also be active.

This study aims to apply the untargeted bioassay strategy using  $^{13}\text{C}$  NMR for the identification of anti-diabetic compounds in *V. negundo* using an *in vitro* glucose diffusion retardation and alpha-amylase inhibition assays. *V. negundo* will be separated into crude extracts and solvent fractions which will be assayed; these will not be subjected to extensive bioassay-guided fractionation. The results of the assays and the match factors for each of the twelve *V. negundo* compounds with the  $^{13}\text{C}$  NMR spectra of the crude extracts and solvent fractions will be correlated using multivariate methods to determine which compounds may be active. The results of the untargeted approach will be compared with previously published work on the anti-diabetic compounds in *V. negundo* which used the method of bioassay-guided fractionation.

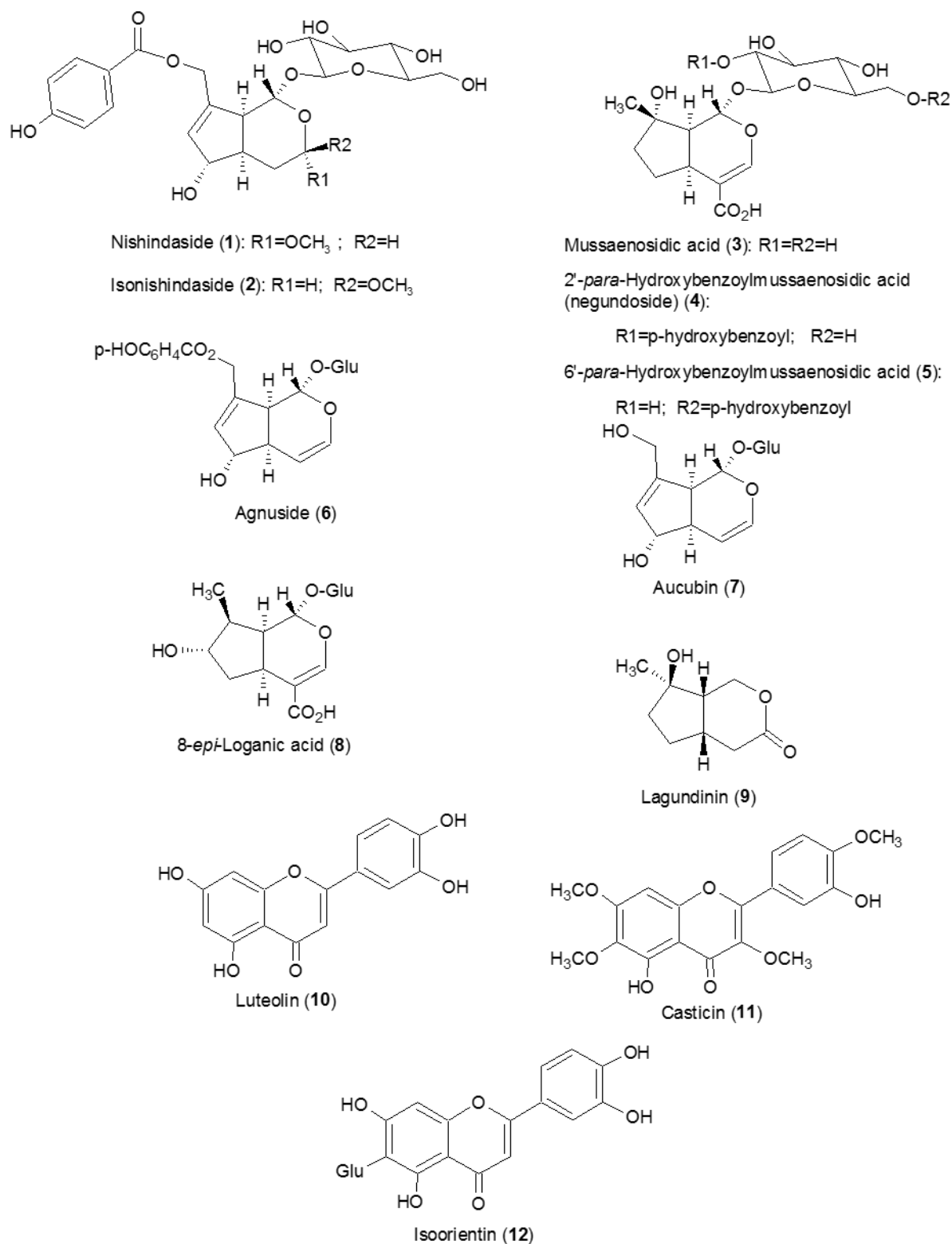
## Experimental

### Materials and reagents

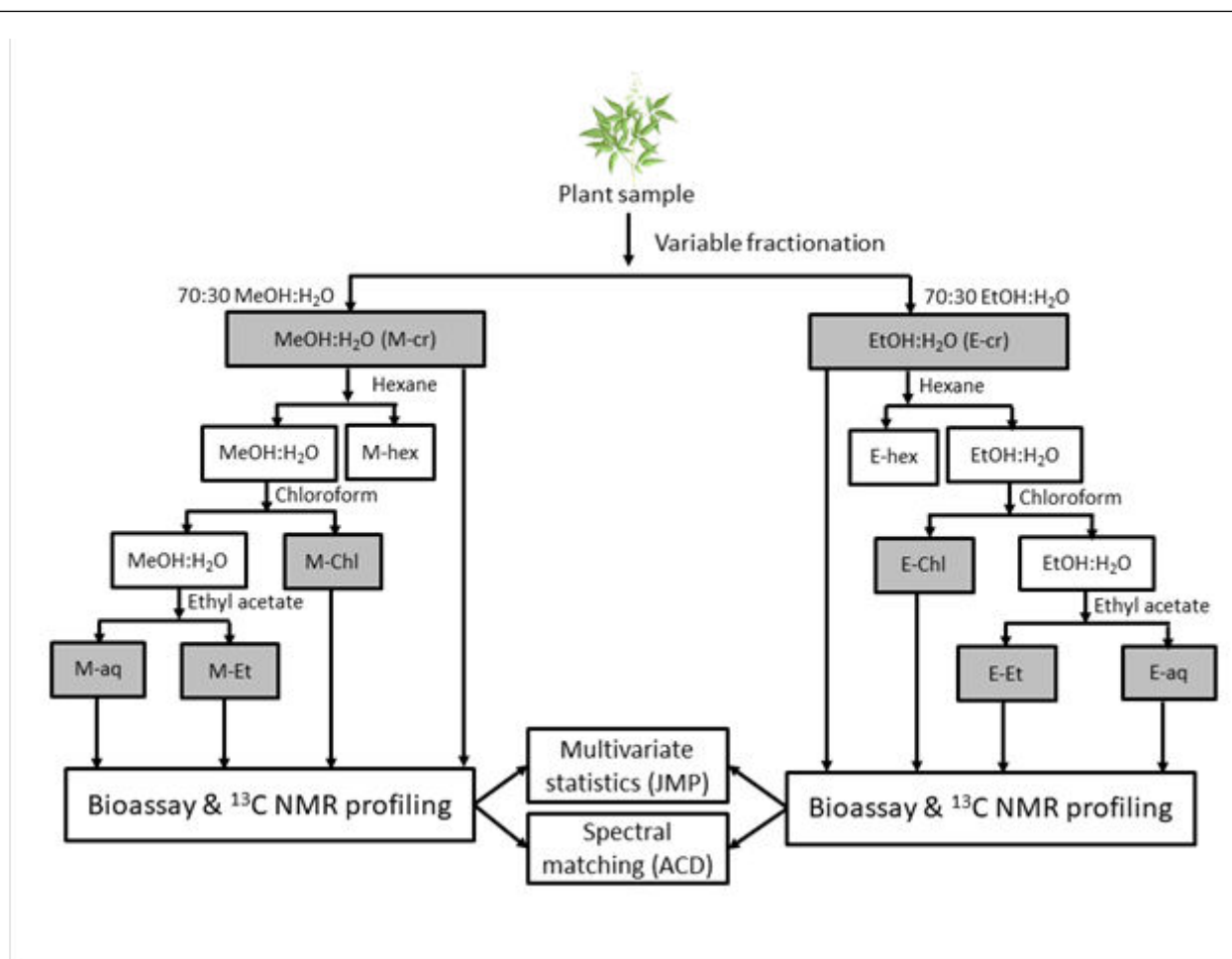
The following materials and reagents were used: dialysis membrane tubing, 1000 Da molecular weight cut-off (MWCO) (Spectra/Por®); alpha-amylase (400 U/mL, obtained from (University of the Philippines Los Baños, Laguna); 3,5-dinitrosalicylic acid, 98% (DNS) (Sigma-Aldrich); dimethyl sulfoxide-D<sub>6</sub>, 99.9% (Cambridge Isotope Laboratories, Inc.); and 1,4-dioxane (Merck Darmstadt).

### Plant material and extraction

*V. negundo* leaves were collected from the University of the Philippines Los Baños, Laguna. The leaves were dried with warm air, ground with a blender to a coarse powder, and sieved (590 microns, U.S. Standard Sieve Series). Crude extraction was done by maceration of the powdered plant (100 g) for 48 hours with 500 mL of 70:30 methanol:water. The solution was filtered and concentrated in vacuo. The concentrated crude extract was then subjected to liquid-liquid sequential fractionation using 150 mL of hexane, chloroform, and ethyl acetate. The hexane fraction was discarded. This gave four samples as follows: crude methanol (M-cr); methanol-chloroform (M-Chl); methanol-ethyl acetate (M-Et); and methanol-aqueous (M-aq). The same procedure was done using 70:30 ethanol:water extraction mixture, giving another 4 samples: crude ethanol (E-cr); ethanol-chloroform (E-Chl); ethanol-ethyl acetate (E-Et); and ethanol-aqueous (E-aq). All the samples were dried under a stream of nitrogen gas and then freeze dried. A total of 8 samples were used for the bioassay runs and  $^{13}\text{C}$  NMR profiling measurements. The workflow is described in Figure 2.



**Figure 1:** Iridoids and flavonoids that have been identified from the semi-polar fraction of *V. negundo*.



**Figure 2:** Workflow for the fractionation, *in vitro* assay and  $^{13}\text{C}$  NMR profiling. The use of aqueous methanol and aqueous ethanol extraction solvents is meant to generate variability in the composition of the extracts and fractions. Shaded boxes refer to extracts and fractions that were subjected to bioassay and  $^{13}\text{C}$  NMR profiling.

### Determination of glucose concentration

The 3,5-dinitrosalicylic acid (DNS) reagent was used to quantify glucose in the external solution of the dialysate (Wang, 2015). Glucose was used as a standard to estimate the reducing sugars present in the samples using a 96-well microplate reader (BioTek™ Eon™, Gen 2.05 software) at 540 nm. A standard curve was established using the following glucose concentrations: 0.25, 0.50, 1, 2, 3, 5, 7, 10 and 15 mM.

### Retardation of glucose diffusion by plant extracts

The diffusion of free glucose was determined using the method of Ahmed et al. [26] with some modifications. *V. negundo* plant extracts were weighed and dissolved with 200  $\mu\text{L}$  to make 0.1% w/v (1 mg/mL). Separate 25 mL solutions containing 0, 7, 10 and 15 mM glucose and plant extract were placed inside the dialysis tubing membrane (11.5 cm  $\times$  38 mm). A membrane with smaller MWCO (1000 Da) was used, compared with most literature references that used larger cutoff values (2,000 to 14,000 Da) [27-31]. The test solution was dialyzed against 200 mL of deionized water at 37 degrees Celsius at 100 rpm using a water bath shaker. The glucose concentration in the external solution was

measured at time intervals (1, 2, 3, 4 and 5 hours) using the DNS method.

### Glucose diffusion retardation index

The glucose diffusion retardation index (GDRI) was calculated using the following formula:

The higher the GDRI value the greater the inhibition activity of the sample.

Two negative control runs were performed: the first did not contain any plant extract; the second did not contain any glucose. Corrections were made by subtracting the results of the negative control runs from those of the test runs.

### Inhibition of alpha-amylase by plant extract

The study of the inhibition of alpha-amylase by *V. negundo* plant extract was adapted from Ou et al. [28] with modifications. To test for the effect of the plant extracts, 5 mL (50 U/mL) alpha-amylase and 0.1% (w/v) of *V. negundo* plant extract in DMSO were mixed. Twenty milliliters of the above starch solution were then added and dialyzed



against 200 mL of deionized water at 37 degrees Celsius. The glucose concentration at the external solution was measured at time (1, 2, 3, 4 and 5 hours) using the DNS method. The negative control was prepared using 300  $\mu\text{L}$  DMSO in place of the plant extract. Glucose was used for the calibration curve. Corrections were made by subtracting the results of the negative control runs from those of the test runs. It should be noted that upon generation of the glucose from starch hydrolysis, this may be further retarded by intermolecular binding with compounds from the plant extract.

### NMR profiling

The  $^{13}\text{C}$  NMR spectra were recorded on a JEOL Lambda 400 spectrometer at 100 MHz. The chemical shifts are reported in ppm ( $\delta$ ) units downfield from TMS. DMSO- $\text{D}_6$  was used as the solvent and 1,4-dioxane was used as the internal standard (IS). 16.2  $\mu\text{L}$  of 1,4-dioxane and 10 mL of DMSO- $\text{D}_6$  were mixed and then 0.5 mL (~0.6 g) of this IS solution was added to the samples (100 mg each).

### Statistical analysis and data processing

Four readings were gathered from each of the diffusion test parameters and values were expressed as: mean  $\pm$  standard deviation (SD). Differences between the samples and control test were analyzed using analysis of variance (ANOVA) followed by Tukey's post hoc test (SPSS version 16.0). Results with p-values less than 0.05 were considered significantly different. Other statistical analyses were performed using JMP<sup>™</sup> software package.

$^{13}\text{C}$  NMR chemical shifts higher than 4% intensity were selected from each spectrum. ACD Labs Spectrus<sup>™</sup> software was used to determine the presence of the 12 known *V. negundo* compounds in the  $^{13}\text{C}$  NMR spectrum of each sample (extract and fraction).

The generated match factor for each compound, the glucose diffusion retardation activities, and the starch-amylase inhibition data (both expressed as GDRI) were loaded as a JMP table. This procedure correlates the match factors of each compound with the activity data to determine which compounds are responsible for the activities of the samples.

## Results and Discussion

An untargeted extraction and fractionation method using 70:30 MeOH:water and 70:30 EtOH:water was used to produce eight samples which were profiled by  $^{13}\text{C}$  NMR and subjected to two *in vitro* antidiabetic assay methods. The use of two sets of extraction solvents was meant to generate overlapping variability, which increases the ability of the method to produce the correlations between the chemical profile and activity.

### Glucose diffusion retardation test

The glucose concentrations in the external solution of all extracts were significantly different with the control for all runs ( $p < 0.001$ ) at 7 mM glucose concentration. The trendlines for the GDRI values of the glucose diffusion retardation tests with glucose concentrations of 7mM, 10mM, and 15mM with 0.1% *V. negundo* extract are shown in Figure 3. The following overall observations can be made:

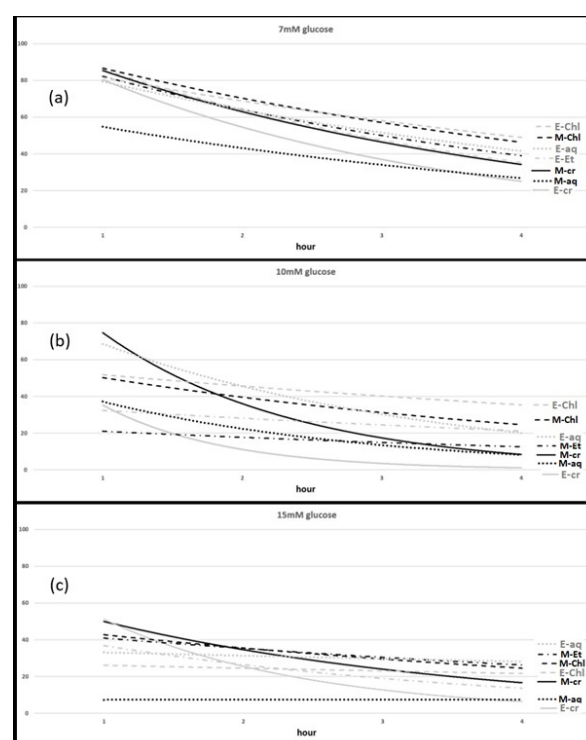
Although a direct comparison between the 1000 MWCO and larger pore dialysis membranes was not done, our results indicate that this

smaller pore size is able to give good results and is presumably more selective than membranes with larger pore sizes.

The GDRI values for all extracts were higher during the first hour and dropped to lower values at 4 hours. This indicates that the interaction is reversible and approaches equilibrium over time [28].

The GDRI activity was higher at 7mM glucose concentration and tended towards lower values as the glucose concentration increased. Since the GDRI is a test for the extent of intermolecular binding of glucose with compounds in the plant extract, this result is consistent with the saturable nature of this activity.

The E-Chl and M-Chl showed the highest activity in the GDRI test. This means that the compounds with strongest glucose binding activity are found in these fractions.



**Figure 3:** Trendlines for glucose diffusion retardation test using 0.1% *V. negundo* extract and glucose concentrations of: (a) 7mM; (b) 10mM; and (c) 15mM.

### Starch- $\alpha$ -amylase inhibition test

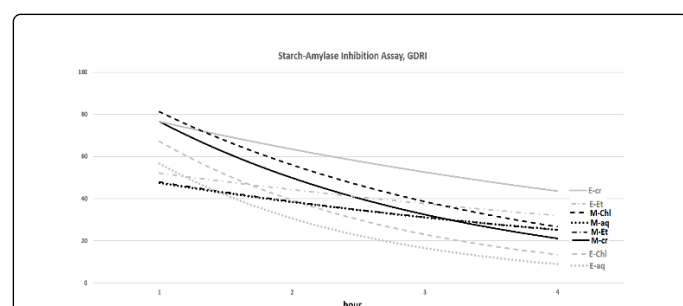
This test sought to determine the effect of 0.1% *V. negundo* extract on the activity of  $\alpha$ -amylase for starch hydrolysis. The trendlines for the starch-amylase inhibition tests are shown in Figure 4. The glucose concentrations in the external solution with extracts during the first two hours versus control was observed to have a significant difference with control ( $p < 0.001$ ). The following overall observations can be made:

- The amylase inhibition values for all extracts were higher during the first hour and dropped to lower values at 4 hours. This indicates that enzyme inhibition is reversible.

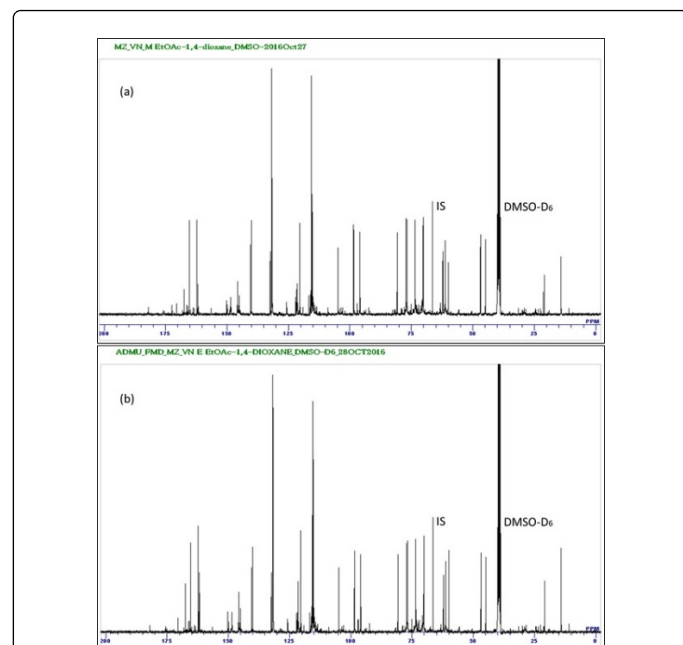
- The crude ethanol (E-cr) showed the highest retardation activity, followed by the ethanol-EtOAc (E-Et) and methanol-chloroform (M-Chf) fractions.

### $^{13}\text{C}$ NMR profiles of extracts

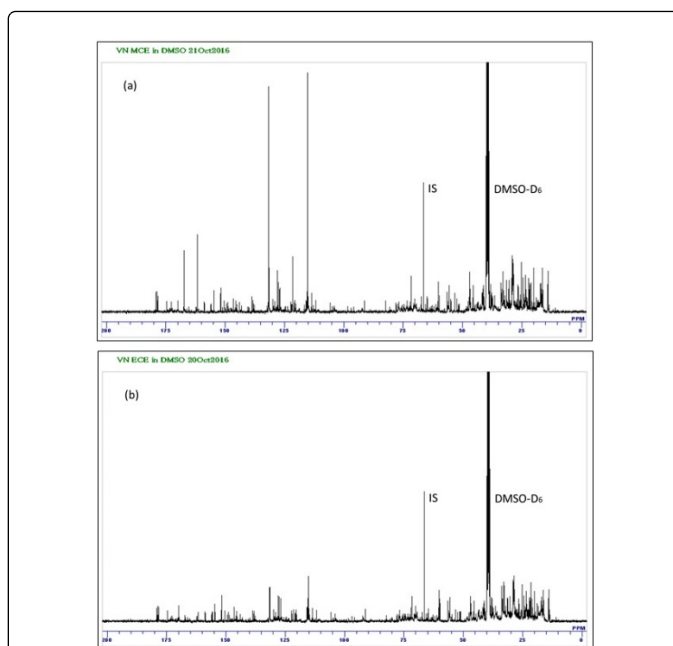
Figures 5-7 show the  $^{13}\text{C}$  NMR spectra of the various freeze-dried fractions in DMSO- $\text{D}_6$  NMR solvent. A measured amount of internal standard (1,4-dioxane) was added to all of the NMR samples which allows for quantitative comparison of peak intensities in the spectra of all the samples. Although the  $^{13}\text{C}$  NMR spectra of the corresponding methanol and ethanol fractions look similar, their peak intensities vary, indicating that their constituents differ in amount. These subtle differences are the basis for the variations that this method is intended to generate.



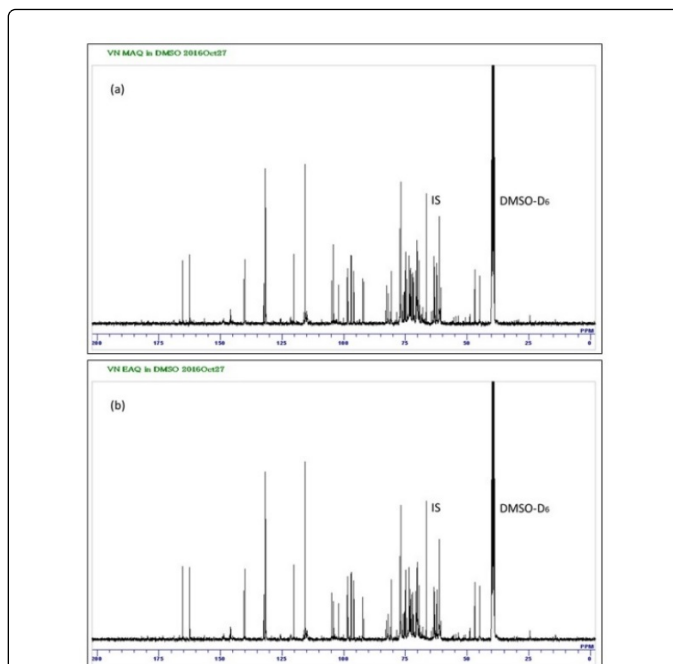
**Figure 4:** Trendlines for starch- $\alpha$  amylase inhibition test using 0.1% *V. negundo* extract concentration.



**Figure 5:**  $^{13}\text{C}$  NMR spectra of: (a) M-Et; and (b) E-Et (NMR solvent: DMSO- $\text{D}_6$ ; IS: 1,4-dioxane).



**Figure 6:**  $^{13}\text{C}$  NMR spectra of: (a) M-Chf; and (b) E-Chf (NMR solvent: DMSO- $\text{D}_6$ ; IS: 1,4-dioxane).

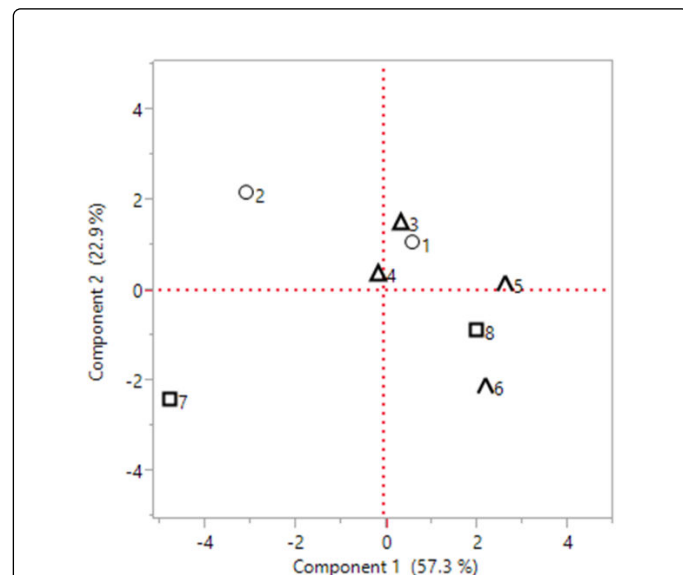


**Figure 7:**  $^{13}\text{C}$  NMR spectra of: (a) M-aq; and (b) E-aq (NMR solvent: DMSO- $\text{D}_6$ ; IS: 1,4-dioxane).

### Principal Components Analysis (PCA)

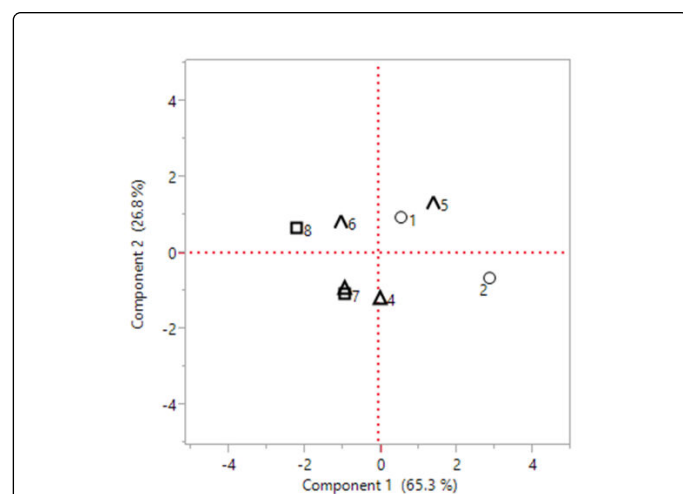
PCA reduces the variables in the results of the glucose diffusion retardation experiments, the starch-amylase inhibition experiments at 0.1% *V. negundo* extract concentration, and the  $^{13}\text{C}$  NMR spectra of

the fractions. Figure 8 shows the PCA of data from glucose diffusion retardation activities at 0.1 % *V. negundo* extract concentration which shows that fractions M-Chl, E-aq, and E-Chl cluster on the positive side of the PC1 axis indicating a strong influence on the activity. The aqueous-methanol residual fraction (7) appears as an outlier in terms of its GDRI activity. This PCA accounts for about 80% of variability which indicates that it is a good model for the GDRI activity.



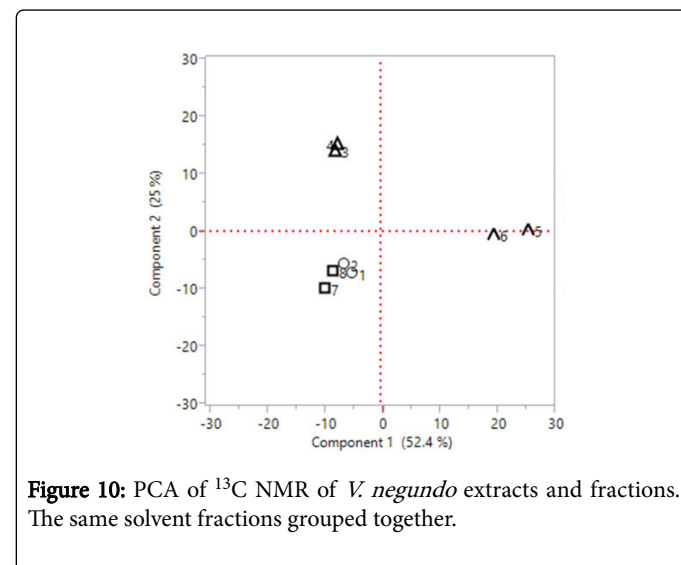
**Figure 8:** PCA of glucose diffusion retardation index (GDRI) using 0.1% *V. negundo* extracts and fractions. Sample 7 (M-aq) is a possible outlier.

Figure 9 shows the PCA of the starch-amyase inhibition activity. The spread of the data shows less distinct groupings, which may suggest that the differences among the activities of the fractions is not that large. This PCA accounts for about 92% of the variability which indicates that it is a good model for the starch-amyase inhibition activity.



**Figure 9:** PCA of starch-amyase inhibition data using 0.1% *V. negundo* extracts and fractions.

Figure 10 shows the PCA of the  $^{13}\text{C}$  NMR spectra of the various plant fractions. This PCA shows three distinct groupings which are consistent with the solvents used: (M-Chl and E-Chl), (M-Et and E-Et), and (M-cr, E-cr, M-aq, and E-aq). This PCA accounts for about 77% of the variability which indicates that it is a good model for the  $^{13}\text{C}$  NMR spectra.



**Figure 10:** PCA of  $^{13}\text{C}$  NMR of *V. negundo* extracts and fractions. The same solvent fractions grouped together.

### $^{13}\text{C}$ NMR compound identification using match factors

The  $^{13}\text{C}$  NMR of the extracts were analyzed using ACD Labs Spectrus™ software to obtain matches with the 12 known iridoids and flavonoids from the semi-polar fraction of *V. negundo*. This software calculates the  $^{13}\text{C}$  NMR spectrum of the compound structure being matched using a neural model and a library of  $^{13}\text{C}$  NMR spectra and generates a match factor for each compound which depends on whether all  $^{13}\text{C}$  peaks of a compound are found and what the closeness of these peaks are to the predicted spectrum. A match factor of >0.75 is considered as a positive match [32].

Table 1 gives the values of the match factors which were generated using ACD Labs Spectrus™ for the 12 compounds in each of the fractions. Match factors >0.75 (shown in black color) are considered as present in the fraction. The calculated match factors indicate that the iridoid glucosides are present in most of the semi-polar fractions, with the chloroform fractions (M-Chl and E-Chl) showing the highest match factors. Among the iridoids, negundoside (4), 6''-p-hydroxybenzoyl mussaenosidic acid (5), and agnuside (6) were present in all of the fractions.



No	Compounds	Match Factors for Samples							
	Name	M-cr	E-cr	M-Et	E-Et	M-Chi	E-Chi	M-aq	E-aq
1	Nishindaside	0.87	0.88	0.84	0.84	0.90	0.92	0.54	0.81
2	Isonishindaside	0.87	0.89	0.85	0.85	0.91	0.93	0.55	0.81
3	Mussaenosidic acid	0.86	0.86	0.92	0.92	0.92	0.88	0.79	0.87
4	Negundoside	0.89	0.89	0.94	0.94	0.96	0.92	0.81	0.88
5	6"-p-hydroxybenzoyl mussaenosidic acide	0.88	0.88	0.87	0.91	0.94	0.91	0.82	0.88
6	Agnuside	0.91	0.91	0.83	0.86	0.96	0.94	0.82	0.84
7	Aucubin	0.88	0.92	0.73	0.92	0.95	0.93	0.67	0.84
8	8-epi loganic acid	0.92	0.9	0.45	0.90	0.97	0.97	0.28	0.73
9	Lagundinin	0.85	0.85	0.3	0.85	0.90	0.97	0.14	0.30
10	Luteolin	0.24	0.25	0.94	0.94	0.73	0.64	0.91	0.24
11	Casticin	0.06	0.24	0.42	0.57	0.98	0.95	0.26	0.15
12	Isoorientin	0.22	0.33	0.93	0.97	0.78	0.77	0.95	0.28

**Table 1:** Match factors generated using ACD Labs Spectrus™ based on the  $^{13}\text{C}$  NMR for the twelve compounds in each of the fractions. Match factors >0.75 (shown in black color) are considered as present in the fraction.

### Multivariate correlations of GDRI activity with identified compounds

Table 2 gives the values of the multivariate correlations for the GDRI test for each of the 12 compounds at the 1st and 4th hour for glucose at 7, 10, and 15 mM concentration. The correlation values suggest the following: 1. The GDRI activities of the iridoid glucosides are higher than the flavonoids; 2. The activity is highest at 7mM glucose concentration. This is consistent with saturation of activity at higher glucose concentrations; and 3. The activity is highest at the first

hour and falls over time. This is consistent with Figure 3 and suggests tendency towards equilibrium condition. The following glucosylated iridoids showed highest activities in the GDRI test: mussaenosidic acid (3), negundoside (4), and 6"-p-hydroxybenzoyl mussaenosidic acid (5). On the other hand, the non-glucosylated iridoid, lagundinin (9), and the flavonoids (10-12) gave low correlation values. It is of interest to note that negundoside (4), which was previously identified as an antidiabetic compound (Sundaram et al.), was also identified as active in the GDRI test.

No.	Compounds	Multivariate Correlations					
		7mM glucose		10mM glucose		15mM glucose	
	Name	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr
1	Nishindaside	0.70	0.26	0.48	0.35	0.60	0.03
2	Isonishindaside	0.71	0.26	0.45	0.36	0.60	0.03
3	Mussaenosidic acid	0.84	0.39	0.17	0.60	0.52	0.33
4	Negundoside	0.85	0.42	0.23	0.63	0.54	0.27
5	6"-p-hydroxybenzoyl mussaenosidic acide	0.85	0.51	0.37	0.67	0.49	0.30
6	Agnuside	0.61	0.33	0.47	0.37	0.49	-0.03
7	Aucubin	0.68	0.21	0.36	0.37	0.56	-0.06
8	8-epi loganic acid	0.68	0.21	0.44	0.34	0.60	-0.03
9	Lagundinin	0.53	0.51	0.55	0.66	0.27	0.19
10	Luteolin	-0.21	0.14	-0.45	0.38	-0.49	0.06

11	Casticin	0.45	0.66	0.18	0.85	-0.11	0.26
12	Isoorientin	-0.21	0.19	0.43	0.44	-0.54	0.03

**Table 2:** Multivariate correlations for the GDRI test for each of the 12 compounds at the 1st and 4th hour for glucose at 7, 10, and 15 mM concentration at 0.1% *V. negundo* concentration.

### Multivariate correlations of starch-alpha-amylase activity with identified compounds

Table 3 gives the multivariate correlations for the starch-amylase inhibition test for each of the 12 compounds at the 1st and 4th hour at 0.1% *V. negundo* concentration. The correlation values suggest the following: 1. The activities of the iridoid glucosides are higher than the flavonoids; and 2. The activity is highest at the first hour and falls to equilibrium values at the 4th hour consistent with Figure 4. In this test, agnuside (6) showed the highest activity, followed by the other glucosylated iridoids, including negundoside (4). The flavonoids (10-12) gave low correlation values in this test.

No.	Compounds Name	Multivariate Correlations Alpha-amylase	
		1 hr	4 hr
1	Nishindaside	0.67	-0.23
2	Isonishindaside	0.67	-0.21
3	Mussaenosidic acid	0.41	-0.07
4	Negundoside	0.55	-0.06
5	6"-p-hydroxybenzoyl mussaenosidic acide	0.64	-0.16
6	Agnuside	0.88	-0.10
7	Aucubin	0.66	-0.07
8	8-epi loganic acid	0.67	-0.16
9	Lagundinin	0.48	-0.36
10	Luteolin	-0.39	-0.32
11	Casticin	0.39	-0.05
12	Isoorientin	-0.36	0.32

**Table 3:** Multivariate correlations for the starch-alpha-amylase inhibition test for each of the 12 compounds at the 1st and 4th hour at 0.1% *V. negundo* concentration.

### Conclusions

The present study provides *in vitro* confirmation of the potential of *V. negundo* as a hypoglycemic agent. The crude extracts and the fractions of *V. negundo* were prepared via untargeted extraction which had the objective of increasing variability among the samples to be tested. The extracts were evaluated using an *in vitro* model for the retardation of the diffusion of glucose across the intestinal membrane into the bloodstream and the inhibition of amylase activity on starch. The methanol-chloroform and ethanol-chloroform fractions showed the highest activity in retarding glucose diffusion while the crude

methanol extract and methanol-chloroform fraction showed highest starch-amylase inhibition. Correlation analysis of the match factors of the twelve known *V. negundo* compounds from the <sup>13</sup>C NMR profiles of the samples with activities suggests that iridoid glucosides may be responsible for the glucose retardation activity and starch-amylase inhibition activity. Our work indicates that the iridoid glucosides, in particular, negundoside (4), 6'-para-hydroxybenzoylmussaenosidic acid (5), agnuside (6), aucubin (7), and 8-epi-loganic acid (8), have anti-diabetic activity. Negundoside (4), which was previously identified as an anti-diabetic compound, was also identified in this study. Thus, this work serves as a proof of principle that this is a viable and valid method.

This work can be extended in a number of ways. Other known compounds from the plant can be added to determine whether these can be matched to the spectra and activity of the fractions. The identified compounds can be quantified, and further studies can be conducted to determine whether the activity is additive or synergistic. If the compound is present at low quantities, further fractionation can be done.

This work demonstrates that if a biological activity is due to known compounds, an untargeted approach using <sup>13</sup>C NMR profiling combined the appropriate assay can be used as a first step. This method takes advantage of NMR spectral software and PCA to assist in the identification of known active compounds. However, bioassay-guided fractionation will be required in cases where the activity is due to a novel compound.

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