A FACILE METHOD FOR TESTING ANTIOXIDANT CAPACITY AND TOTAL PHENOLIC CONTENT OF *NOTOBASIS SYRIACA* AND *SCOLYMUS MACULATUS* EXTRACTS AND THEIR ANTIFUNGAL ACTIVITY

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Keywords: *Notobasis syriaca; Scolymus maculatus; Rhizopus stolonifera;* total phenolic content; antioxidant capacity.

In this study, three extracts of the aerial parts of *Notobasis syriaca* and *Scolymus maculatus* were prepared. Each extract was tested for antifungal activity against *Rhizopus stolonifer* (black mold), and its total phenolic content (TPC) and antioxidant activity were measured. As for these measurements, we report here a facile method that we developed. Our results show moderate antifungal activity for both plants extracts, notably high TPC and antioxidant capacities. They are also in very good agreement with the partial published data, and our new method is consistent and validated by very well known, yet complicated or expensive methods.

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Introduction

Notobasis syriaca (Syrian thistle, NS) and *Scolymus maculatus* (Spotted golden thistle, SM) are two of the spiny, most widespread plants of the Middle eastern region, Mediterranean basin and Western Asia, yet, the habitat of SM is wider and includes most of Asia. Both plants belong to the Asteraceae family, but to different *genera*: NS to *Notobasis* (2 different subspecies)[1] and SM to *Scolymus* (7 different subspecies).[2] NS was discovered in archeological excavations, and there are evidences that humans used this plant, mainly as food, as early as 23000 years ago.[3] Interestingly enough, and despite being very sweet tasty plant (young stems), there are no published studies of archeological findings that indicate early use of SM by humans, similar to NS.

Present time traditional societies, extensively use both plants. NS is used as food in Cyprus and Italy,[4,5,6] in Jordan it is used to treat diabetes (method not described), as food and for cheese production from milk (dry immature flowrs),[7] and in Turkey, ground seeds are eaten to treat liver diseases.[8] SM is widely used as a sweet snack in the Palestinian traditional society, in Spain,[9] in Italy,[6,7] and in Arab folk medicine, stem decoction is used to treat intestine and kidney inflammation.[10]

Despite the fact that in recent years there is renewed recognition of natural products as an impotrant source for drug discovery,[11] these two plants were very limitedly studied by modern research so far. One of the earliest published studies of the medicinal activities of NS, tested the antifungal activity (*Alternaria solani*, *Hetminthosporium sativum* and *Rhizoctonia solani*) of shoot aqueous extract and found it moderate.[12] Antimicrobial activity of ethanolic extract of aerial parts of NS was tested against six types of bacteria, including *P. acnes*.[13] The results show relatively low activity.

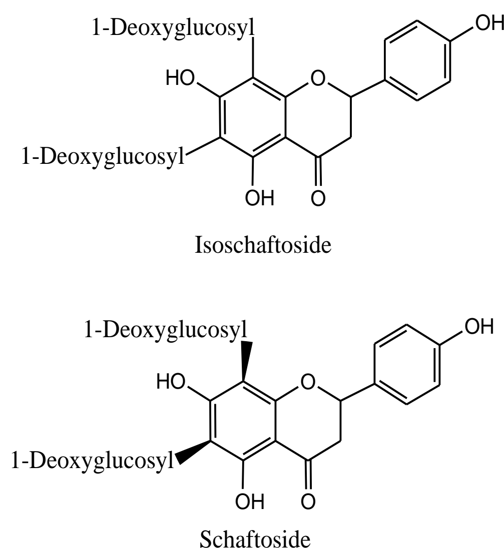


Figure 1. Structures of steric isomers schaftoside and isoschaftoside found in *N. syriaca*

The complete chemical composition of NS was never published, even though some partial results were reported. Mericli and Dellamonica studied the flavonoid content of the plant and they reported the presence of nine different compounds in the aerial parts, including schaftoside and isoschaftoside (Figure 1), but none of these compounds was new.[14] An interesting study was published in 2007 and has high relevance to our research since it tested the TPC and antioxidant capacity of aqueous and methanolic extracts of NS.[15] The results are surprisingly low, compared with other reports and our current study (see results and discussion sections). El-Hela and his colleageas tested TPC and antioxidant capacity of extactly same extracts indicated in reference 15. Their findings are notably higher.[16] They also

tested them larvicidal activity of these extracts against *Aedes aegyptii*, and they were found moderately active.

Contrary to published studies about NS that did not report new compounds, a research that investigated the chemical composition of *Phomopsis* sp., an endophytic fungus of NS, reported several new compounds, including phomosine K (Figure 2) that had strong antibacterial activity.[17]

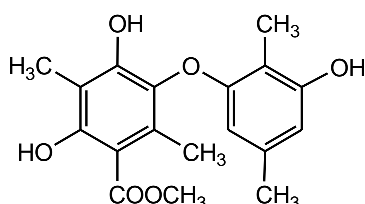


Figure 2. Phomosine K, a novel compound found on endophytic fungus of *N. syriaca*

Earlier this year, we published some results in collboration with other research group, where we studied the anti-inflammatory activity of the aqueous extract of the aerial parts of NS.[18] Based on these successful findings, we performed the current study and we are continuing the investigation of this plant.

Research findings related to SM are even much less than these of NS. In one of the only two published studies, TPC and antioxidant capacity of methanolic and aqueous extracts were tested.[15] The results are lower than ours and higher than the only second published research. This was presented as a poster by Rayan *et al.* and never published as a research article.[19] This group did not report new compounds in the three extracts that they prepared from aerial parts of SM: methanolic, ethyl acetate and hexane. Expectedly, methanolic extract has the highest antioxidant capacity. An enzyme isolated from SM, was reported by M. Benchiheub and her colleagues as successful milk-clotting agent.[20]

Black mold (*Rhizopus stolonifer*) is one of the most common fungi, and it has been a research focus since its vast damages to human health, especially to food, but also because of the potential of isolating unique biologically active compounds from it.[21] It was also reported to assist transformations of organic compounds and peptides.[22] Dozens of treatments were suggested to combat the damages of *R. stolonifer*. While some methods consisted of synthetic compounds,[23] the latest publications report the use on nanochemicals.[24] But synthetic compounds have adverse side effects, so most proposed antifungal treatments of *R. stolonifer* are plant products. Among these, essential oils,[25] extracts[26] and single natural products.[27,28,29]

Total phenolic content (TPC) and antioxidant capacity of plant materials, are among their most important properties. Several methods were developed to determine TPC, where the most commonly used is the Folin-Ciocalteu assay.[30] But variations of other methods are also used, such as in our previous work (Kumar and Jain method).[31] These methods involve many samples (or many dilutions of one sample), spectroscopic measurements and costly reagents. Testing the strongly related property of antioxidant capacity of plant materials, involves many more experimental methods, where some use costly materials and spectroscopic measurements,[31] and some use simpler methods such as potentiometic/redox titrations.[32,33,34] Some of these and other procedures are very simple, and we used their potential to develop a new, facile method to measure TPC and antioxidant capacity of plant extracts by single titration.

Experimental

Chemicals

Gallic acid and ascorbic acid were purchased from Merck KgaA (Germany). All other chemicals were purchased locally in at least analytical grade.

Plant material and extractions

Both the studied plants (*Notobasis syriaca*, NS, and *Scolymus maculatus*, SM, aerial parts) were harvested from the wild near our laboratory in Kfar-Qari (northern Israel). The green materials were washed with distilled water and air dried for 4 weeks. The dry matter of each plant was ground into a fine powder and stored at -12 °C in sealed containers.

The plant material (500 g) were stirred in 1000 mL of solvent (water, ethanol, ethyl acetate) for 24 h at 50 °C. Suspensions were allowed to cool to room temperature and filtered (Munktell quant. Grade 393) to obtain clear solutions. These were evaporated to dryness with rotary evaporator: aqueous extracts at 60 °C, ethanol and ethyl acetate extracts at 50 °C. All six extracts were solids, and they were stored in screw-capped vials at -12 °C.

Tests for Alkaloids

Presence of alkaloids in extracts was tested according to I. P. Udeozo et al., using the Wayner's reagent, with no modifications.[35] In a 100 mL volumetric flask, 2 g of iodine (I_2) and 6 g of potassium iodide (KI) were dissolved in distilled water and made up a 100 mL solution.

A sample of 0.1 g of each plant extract was placed in 20 mL test tube and dissolved in 10 mL ethanol. To the tested solution, 5 mL of Wayner's reagent were added, the tube was capped and the suspension was vigorously shaked for 30 seconds.

Antifungal activity tests

Antifungal assay was performed according to the method we reported in our previous publication, with no modifications.[31] *Rhizopus stolonifer* was grown on whole wheat bread and extracted with water. The center of each Petri dish was inoculated with 5 mm diameter disc of fungal mycelium, taken from pure culture (7 days old). Then, all inoculated dishes were incubated at 25 °C for 6 days and the radial mycelial growth was measured. The antifungal activity of each extract was calculated in terms of inhibition percentage of mycelia growth by using the following equation (1).

$$\% \text{ Inhibition}=[(dc - dt)/dc] \times 100 \quad (1)$$

where dc is the average increase in mycelia growth in control and dt is the average increase in mycelia growth in treated samples with extracts.

In all experiments, the control was the extraction solvent and we performed the antifungal tests using two concentrations for each extract, 10 % and 20 % (w/w).

Preparation of oxidative solution

Solution of sulfuric acid was prepared by diluting 0.89 mL of 96 % solution of concentrated sulfuric acid (approx. 18.11 M) with distilled water to final volume of 1000 mL. The resulting sultion was standardized by pH titration with 0.01 M of sodium hydroxide (NaOH) solution. The acid concentration was 0.016 M.

The 0.004 M solution of potassium permanganate ($KMnO_4$) was prepared by dissolving 0.633 g of the salt in 800 mL of distilled water at 60 °C. The solution was allowed to cool to room temperature, filtered and completed to 1000 mL.

The final oxidative solution was prepared by combining the acid (0.016 M) and permanganate solution (0.004 M), which was stored in 4 °C in a sealed flask.

Calibration curve of ascorbic/gallic acid

A stock solution of 600 mg of ascorbic/gallic acid in 6 mL of distilled water was prepared (100 mg mL^{-1}). By dilution with distilled water, the following concentrations of tested solutions were prepared: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg 10 mL^{-1}.

In a 250 mL Erlenmeyer flask that contained a magnetic stir bar, one of the ten test solutions was titrated with the oxidative solution, with pH monitoring by JK-PHM-005 pH-meter. Titration speed was 2 mL min^{-1} with continuous gentle stirring. End point was determined when $\Delta pH > 0.1$ after half way of the titration (see discussion).

Titration of plant extracts

In a 100 mL Erlenmeyer flask that contained a magnetic stir bar, 100 mg of dry plant extract were suspended with 10 mL of distilled water and stirred for 5 minutes. The solution/suspension was titrated with the oxidative solution, with pH monitoring. Totration speed was 2 mL min^{-1}, with continuous gentle stirring.

Validation of our method

TPC was also determined by the method we used in our previous work,[31] with no modifications. The sample mixture that contains 3 mg of extract (or standard gallic acid solutions) dissolved in 1 mL of solvent, was obtained by dilution of 0.3 g of extract in 10 mL stock solution 10 folds. Then it was added to 10 mL volumetric flask containing 8 mL of dd H_2O. After that, 1mL of Folin-Ciocalteu's reagent was added to the mixture. After 3 min, 1 mL of 35 % Na_2CO_3 solution was added with mixing to reach the reaction system to 10 mL. The reaction mixture was mixed thoroughly and allowed to stand for 90 min at 25 °C in the dark. Absorbance of all the sample solutions against a blank was measured at 725 nm. Calibration curve was constructed with different concentrations of gallic acid (2–12 μg mL^{-1}) as the standard and double-distilled water was used as reagent blank. The results were expressed as mg gallic acid equivalents (GAE) g^{-1} of dried extract.

Antioxidant capacity was determined according to the method we described in our previous publication, with no changes.[31] 0.1 mL of aliquot of test solution (100 mg extract) was added to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The blank was 0.1 mL of ethanol. The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min, then allowed to cool to room temperature. Absorbance of the aqueous solution of each was measured at 695 nm. The antioxidant capacity was expressed as an equivalent of ascorbic acid (mg of ascorbic acid g^{-1} of dried extract).

Results

Yields of extractions

The yields of the extractions of both NS and SM with water, ethanol and ethyl acetate are shown in Table 1.

Table 1. Yields of extractions of *N. syriaca and S. maculatus* with three different solvents[a]

Plant	Water		Ethanol		Ethyl acetate	
	mass	%[a]	mass	%	mass	%
NS	42.1	8.42	42.8	8.56	23.7	4.74
SM	46.9	9.38	48.0	9.60	21.5	4.30

[a]For each extraction, 500 g of dry plant powder were extracted (aerial parts).

Alkaloid presence

All six extracts showed negative results in alkaloid presence test with Wayner's reagent.

Statistical analysis

Except for extractions (Table 1) and alkaloid presence tests, that each was done in a single experiment, all data presented below, are average values of three experiments that we peformed for each test.

Antifungal activity

Antifungal activity was measured as the of inhibition percentage of mycelia growth of *Rhizopus stolonifer*. Two concentrations of extracts were used, 10 % and 20 % (w/w) in the extraction solvent and the results are shown in Table 2 and Figure 3.

Table 2 Antifunfal activity of *N. syriaca* and *S. maculatus* extracts against *R. stolonifer*

Plant	Inhibition of extracts (%)[a]					
	Aqueous		Ethanolic		Ethyl acetate	
	10 %	20 %	10 %	20 %	10 %	20 %
NS	17.2	19.4	24.3	26.8	25.9	27.2
SM	12.3	14.4	19.2	21.8	18.8	22.3

[a]Extraction solvent in each experiment was used as control and resulted in 0 % inhibition.

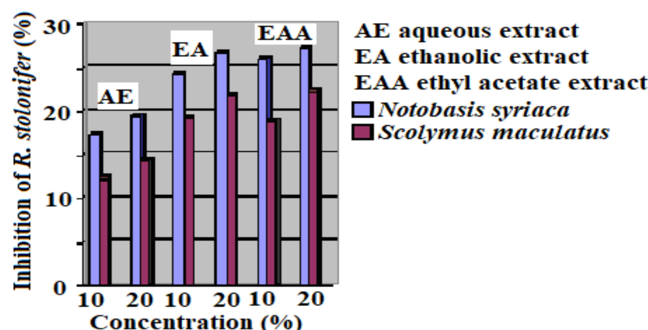


Figure 3. Inhibition (%) of *R. stolonifer* by extracts of *N. syriaca* and *S. maculatus*

Calibration curves

Concentrations of titrated ascorbic/gallic and volumes of oxidative solution needed are shown in Table 3, and calibration curves are shown in figure 4.

Total phenolic content and antioxidant capacity

Each (100 mg) extract were titrated with the oxidative solution. End point volume was multiplied by 10 in order to reach the value for 1 g of dry extract, and the final values were calculated as shown in Table 4 and Figure 5.

Validation of our method

Total phenolic content and antioxidant capacity were also measured by the known methods, described in the experimental section. The results of these measurements, as well as the results measured by our method are shown in Table 5.

Discussion

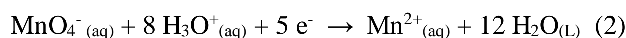
All the six qualitative tests for the presence of alkaloids in the extracts showed negative results that is no alkaloids present in *N. syriaca* or *S. maculatus*. In fact, this was expected since a closely related (and sometimes confused with) plant, *Silybum marianum* (Milk thistle), contains no alkaloids.[36,37] The cultivated Artichoke (*Cynara carduncul*us var. *scolymus*) is also closely relted to NS and SM, and it does not contain alkaloids.[38]

Antifungal activity of both plants, NS and SM, was found moderate and even weak, not only in comparison with plants with strong antifungal activity,[39] but also with other plants from other genera, such as *Carthamus tenuis* and *Cephalaria joppensis*.[31] This can be partially understood on the basis of the very limited presence of volatile, aromatic compounds, that we discovered while we tried to prepare essential oils of NS and SM, and we obtained negligible yields.

Total phenolic content and antioxidant capacity are of the most important medicinal properties of plants products, and they are strongly related.[40] Phenols and polyphenols are among the most powerful antioxidants in the plant kingdom.[41]

Based on this, we managed to develop a unified test for both properties. Our considerations included (1) use of strong oxidative solution, yet, not too strong that can cause oxidation way beyond common reagents used for this matter, (2) a facile technique that involves simple instruments and very simple and few preparations of samples and (3) Use of cheap, commercially available reagents.

We selected acidic solution of potassium permanganate. This is a strong oxidant (E^0= +1.51 V) but in low concentration, this strength is even lower, so it will not exceed the power of common oxidants that are used in other methods (see experimental section: validation of our method). The molar ratio between per manganate and acid was designed according to the stoichiometry of the reaction:



$$MnO_4^-{}_{(aq)} + 8\ H_3O^+{}_{(aq)} + 5\ e^- \rightarrow Mn^{2+}{}_{(aq)} + 12\ H_2O_{(L)} \quad (2)$$

So the ratio of MnO_4^- : H^+ is 1:8. Sulfuric acid was chosen due to its stability and strength.

Our theoretical assuptions of the stoichiometry of oxidation of ascorbic acid by this oxidant, were based on the previous studies,[42] and we designed the tests for MnO_4^- : ascorbic acid = 2:5. As for gallic acid, the stoichiometry is more complicated since there are several competing reactions, even though, one of them occur as major path of the oxidation.[43] This reaction is shown in Figure 6.

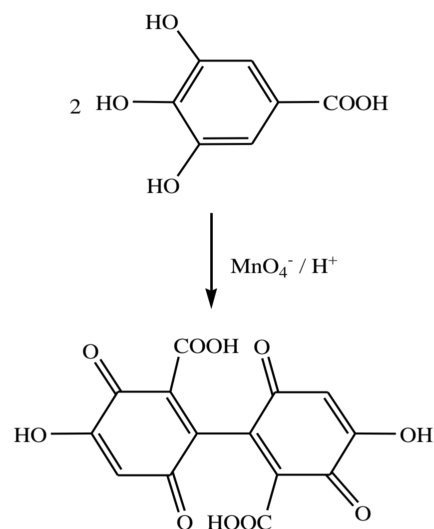


Figure 6. Major reaction of oxidation of gallic acid by acidic permanganate

Table 3. Titrated concentrations of ascorbic/gallic acid and oxidative solution volumes.

Titrated Concentration[a]		10	20	30	40	50	60	70	80	90	100
Oxidative Solution Volume[b]	AA[c]	10.2	26.6	33.1	42.8	58.4	65.2	80.6	91.4	103	109.4
	GA[d]	20.4	52.4	63.2	79.6	100	108.2	145.2	180.8	199	224.8

[a]mg 10 mL^{-1}, [b]mL, [c]Ascorbic acid, [d]Gallic acid.

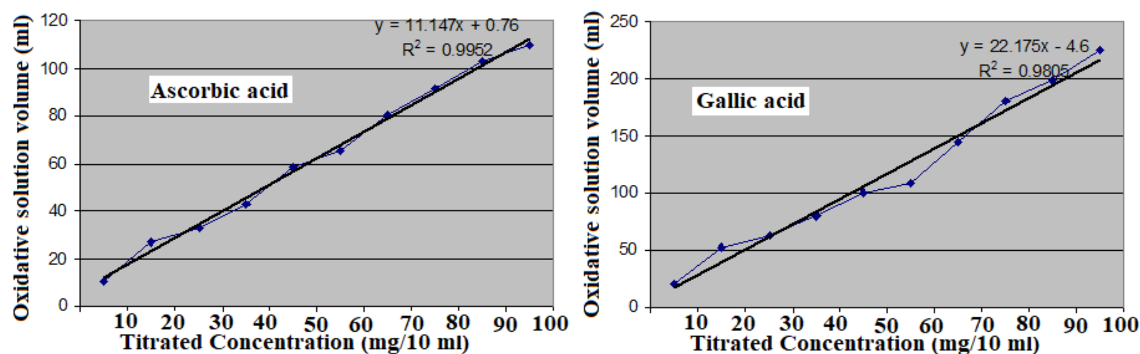


Figure 4. Calibration curves of oxidative titration of ascorbic/gallic acid with permanganate.

Table 4. Total phenolic content (TPC) and antioxidant capacity (AOC) of extracts of *N. syriaca* and *S. maculates.*

Plant	Extract	Volume of oxidizing solution	TPC[a]	AOC[b]
Notobasis syriaca	Aqueous	37.8	17.25	33.84
	Ethanolic	29.7	13.6	26.58
	Ethyl acetate	16.1	7.47	14.38
Scolymus maculatus	Aqueous	39.6	18.02	35.37
	Ethanolic	29.1	13.33	26.04
	Ethyl acetate	14.9	6.39	13.30

[a]mg of gallic acid g^{-1} of dry extract (according to 10y=22.175x-4.6); [b]mg of ascorbic acid g^{-1} of dry extract (according to 10y=11.147x+0.76)

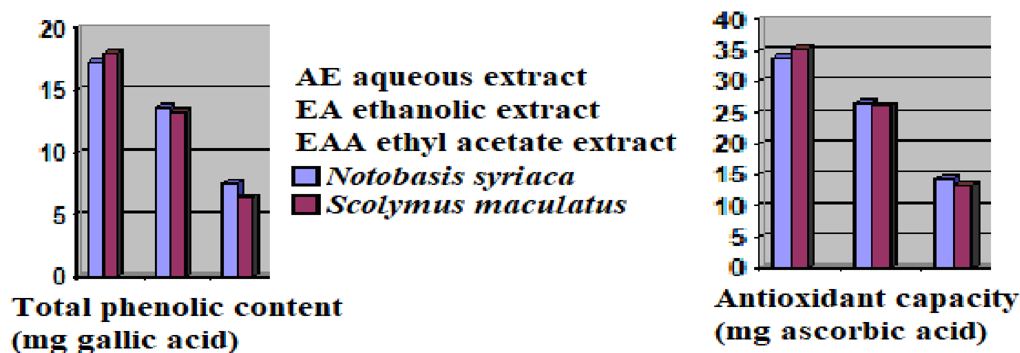


Figure 5. Total phenolic content and antioxidant capacity of *N. syriaca* and *S. maculates.*

Table 5. Total phenolic content and antioxidant capacity of *N. syriaca* and *S. maculatus*, measured by our method and known methods[a].

Plant	Extract	Total phenolic content[b]		Antioxidant capacity[c]	
		Our method	Known method	Our method	Known method
Notobasis syriaca	Aqueous	17.25	16.84	33.84	31.88
	Ethanolic	13.6	13.2	26.58	24.23
	Ethyl acetate	7.47	7.39	14.38	13.87
Scolymus maculatus	Aqueous	18.02	16.1	35.37	32.11
	Ethanolic	13.33	12.55	26.04	23.74
	Ethyl acetate	6.39	6.25	13.30	12.13

[a]See experimental section, [b]mg of gallic acid g^{-1} of dry extract, [c]mg of ascorbic acid g^{-1} of dry extract.

The formation of the bisphenyldiquinone product, involves loss of three electrons from each molecule of gallic acid. Since lower oxidations also take place but in minor amounts, we assumed that the average ratio of MnO4- : gallic acid = 1:1.2.

Titrations were monitored by pH-meter. Despite being slowly responsive, especially while titrating polyphenols,[44] this method is used for various purposes, and our work was based on a successful example.[45] To overcome the slow response, we conducted very slow titrations (2 mL min-1). Initial pH gallic acid solution was 3.64. In the beginning of the titrations, pH dropped rapidly (0.1-0.15 units drop-1), but then it was much slower (0.01 units drop^{-1}). At the end point (pH=1.39) the pH dropped by 0.2 units. As for ascorbic acid, the initial pH was 3.45 and the final, 1.21. The titration advanced in the same course as gallic acid but was slightly faster.

Our results, using this very simple method are very good. In case of antioxidant capacity, there is a high proximity between our results, the results that we obtained by other know methods (Table 5) and published results.[15,16] The results reported by Rayan et al.,[19] are lower than our results and the results reported in references 15 and 16, so we will not take them into account. All results obtained by our method, were higher that those obtained by other known methods, and the highest difference was (2.3 mg, ethanolic extract of SM) around 9.24 %.

As for TPC, differences between our method and known methods were even higher than these of antioxidant capacity, despite the fact that they are still reasonable. And again, the highest difference was SM extract, but this time, aqueous (Table 5, 11.25 %). As far as we can explain this, our understanding is that the sweet taste of SM is a result of the presence of saccharides, and these may have been oxidized and made the results obtained by our method higher, compared to other methods. We base our explanation on similar published results, where carbohydrate content affected TPC and antioxidant capacity measurements.[46,47]

Conclusions

Notobasis syriaca and *Scolymus maculatus*, are edible plants and possess high amounts of polyphenols and other antioxdants. Chemical composition of both plants is still unkown and should be investigated. Only antioxidant capacity, total phenolic content and inti-inflammatory (NS only) activities are known. Other properties must be studied. We developed a new, very simple method for measuring TPC and antioxidant capacity, that has shown good agreement with other known methods. *Notobasis syriaca* and *Scolymus maculatus*, do not contain alkaloids. The essential oil content of *Notobasis syriaca* and *Scolymus maculatus*, is negligible.

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