

Comparative Study of Two Herbs from India

Alkaloids and Antioxidant Activity in *Withania Somnifera* and *Tinospora Cordifolia*.

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Abstract

Most developing countries continue using traditional herbal medicine over western medicine. Recently, the western world has also started using herbs like *ashwagandha* (*Withania somnifera*) as stress reliever and memory enhancer. *Guduchi* or *giloy* (*Tinospora cordifolia*) is used traditionally in India as a fever reducer and hypoglycemic. Both of these herbs are of Indian origin and contain alkaloids that are associated with anti-inflammatory effects. Yet there is little known about the total amount or type of alkaloids and their anti-oxidant activity. The results of this study show that there is higher amount of polar alkaloids in *Withania s.* than in *Tinospora c.* Interestingly enough, *Tinospora c.* was found to have a higher antioxidant activity than *Withania s.*

Introduction

A medicinal herb *Withania somnifera* commonly known as Indian ginseng or *ashwagandha* has been widely marketed for its stress relieving properties but is also a well-known source of antioxidants. Another medicinal herb, *Tinospora cordifolia*, has also been mentioned to have antioxidant properties although it is most commonly used as a fever-reducing medicine⁴. Both plants have many common structural groups of active compounds such as glycosides, alkaloids, and lactones. Alkaloids, a major group in both plants, are also associated with antioxidant activity among other medicinal properties^{1,5}. *Withania s.* has been used in memory enhancement herbal supplements and in commercial extraction of adaptogens. However, *Tinospora c.* is used traditionally in India more as a fever reducer and a hypoglycemic drug¹.

Researchers have qualitatively analyzed the individual alkaloids in *Tinospora c.* using techniques like HPLC and the antioxidant activity for select few alkaloids is also known^{4,5}. On the other hand, in *Withania s.*, the steroidal lactones like withanoloids have been studied extensively, as these are responsible for the adaptogenic effects^{1,3}. Yet research is still lacking in the quantification of total alkaloids and their cumulative antioxidant activities for both herbal plants. This work explores the antioxidant properties of both herbs, *Withania somnifera* and *Tinospora cordifolia*.

The purpose of the project is to identify the major chemical factor that makes one of these herbs to be a better source of antioxidants. This goal is achieved by extracting the total crude

mixture of alkaloids from both plants using the same method, quantifying the total amount of polar alkaloids, and measuring the antioxidant activities of the extracted filtrates. Crude alkaloid mixture is extracted from both plants using the Stas-Otto method². Chromatographic techniques and SPE are used to isolate the polar alkaloids from the crude mixture. The quantification of total alkaloids is achieved using Dragendorff's reagent followed by UV/VIS spectrophotometric analysis. FTIR (*Fourier Transform Infrared Spectroscopy*) and other supplementary qualitative tests are used in preliminary experiments to identify the functional groups of the isolated mixture. Antioxidant activity measurements are done using the *Ferric Reducing Antioxidant Power* electron transfer based assay.

My motivation for studying the alkaloids and their antioxidant properties comes from the consideration of possible use of these herbs as easily available sources for anti-cancer and anti-inflammatory medicine in the future. The aim of this study is to determine the herb with higher activity and explore the reasons for this with further analysis.

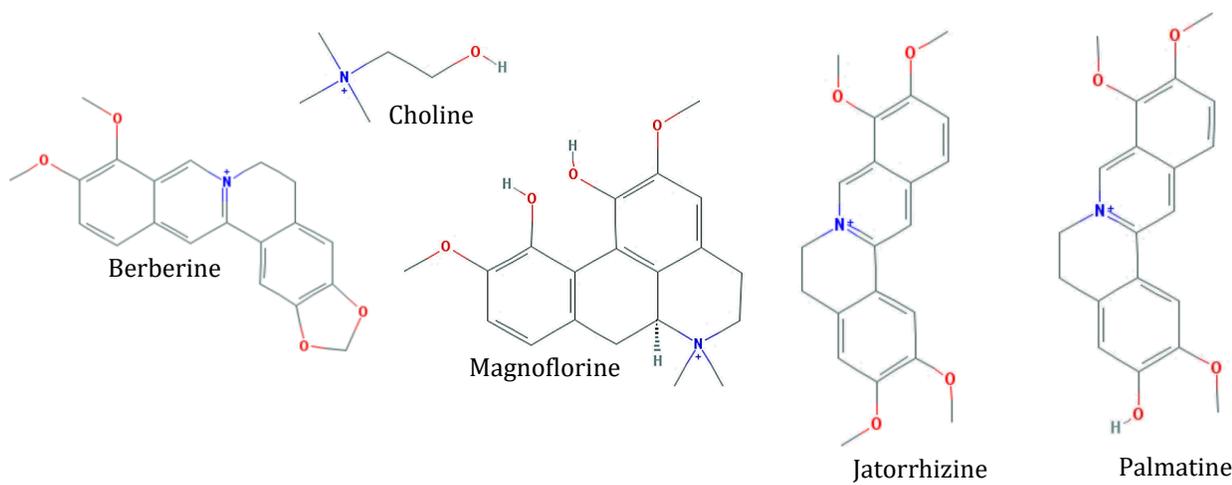


Fig. 1: Structures of known alkaloids in *Tinospora cordifolia*.

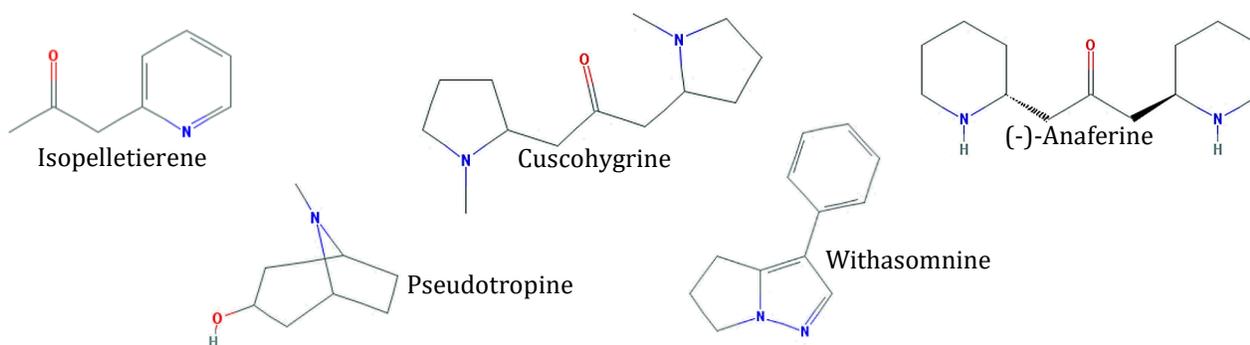


Fig. 2: Structures of known alkaloids in *Withania somnifera*.

Materials and Methods

a.) Extraction and Isolation of alkaloids

Dried root powders of *Withania somnifera* and dried stem powder of *Tinospora cordifolia* were obtained from *Banyan Botanicals* (approved USDA organic source).

Reagents and solutions for extraction were purchased from *Sigma-Aldrich*.

The Stas-Otto method² of extracting alkaloids from plants was followed with a few modifications. Oxalic acid was used instead of tartaric acid since both are multi-protic organic acids. Centrifuge (*Sorvall RC 24*) was used to remove insoluble substances like fats instead of ether extraction (as suggested in literature).

For 10 g of the dried herb powder, 11 mL of methanol was added, and the pH of the mixture was adjusted to 2.8-3.5 using oxalic acid. This mixture was refluxed 3 times for 30 minutes each time. The solvents were filtered out after every reflux and new solvents added. Methanol was evaporated from the filtrate using rotary evaporator (*BUCHI Rotavapor R-3*) set at 77 C for 40 min. A clear brown colored filtrate was obtained after centrifuging at 5000 rpm for 15 min. This filtrate is referred to as aqueous filtrate and was used in the quantification of alkaloids and antioxidant activity tests.

Ionizable alkaloids were isolated by collecting the filtrate that did not bind to the octadecyl column (*LC18* from *Sigma-Aldrich*) after passing the aqueous filtrate. The aqueous phase containing the ionizable alkaloids is referred to as the aqueous fraction which is further used for the quantification and antioxidant activity tests.

b.) Verification of alkaloids

A common established color test was used as quick verification for the presence of alkaloids. Iodine potassium iodide (*Fischer Scientific*) forms a brown precipitate with the alkaloids in the filtrate. FTIR (*Perkin-Elmer*) was used to further verify the presence of nitrogenous organic compounds (alkaloids).

c.) Quantification of alkaloids

Dragendorff's reagent (KBiI_4) and spectrophotometric methods were used to quantify the total alkaloids present in the aqueous filtrate and fraction. The reagents and the procedure used for Dragendorff's test were in general similar to that of Sreevidya and Merhotra⁶, but some

modifications were made. Instead of using the suction filtrate for removing the precipitate formed by Dragendorff's reagent, the supernatant was decanted, and the precipitate was re-suspended in aqueous filtrate and washed with methanol for about 5 times, each time the solvent being separated via centrifugation at 5000 rpm for 15 min. This modified washing method ensured that no precipitate was lost, as it would have if suction filtration were performed.

d.) Antioxidant activity measurement

Ferric Reducing Antioxidant Power, an electron transfer based antioxidant assay was used with *Trolox* as the standard antioxidant. The reagents used, and the procedure followed for this test was from Tomasina⁷.

Data Analysis and Results

a.) Verification of alkaloids

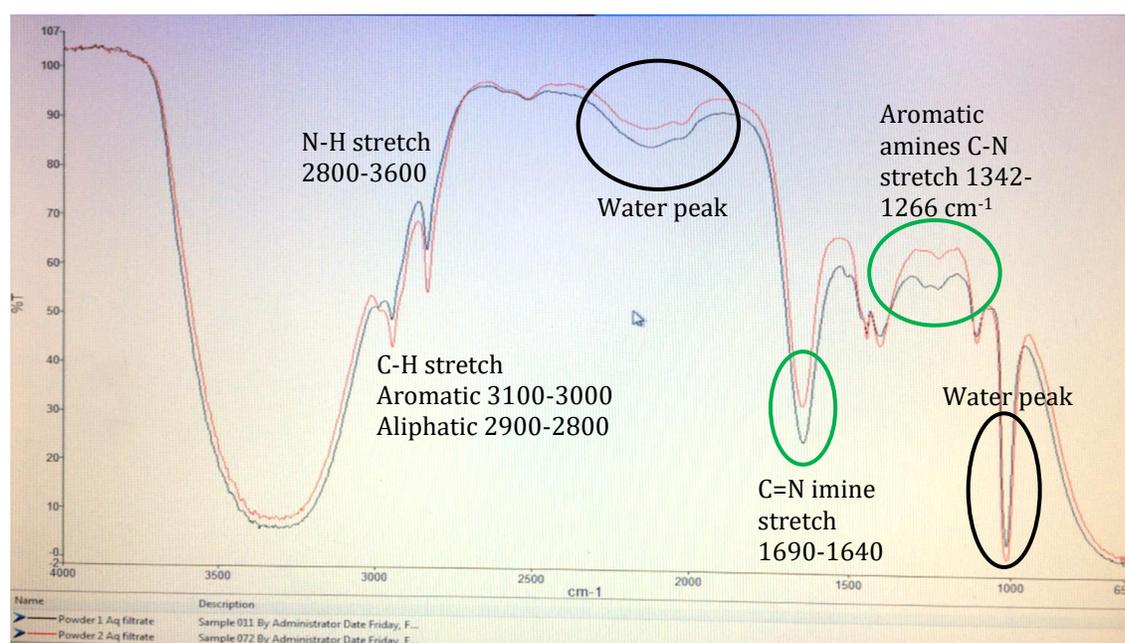


Fig. 3: IR Spectra comparing aqueous filtrates of both herbs (obtained from extraction); red line represents *Withania s.* and black represents *Tinospora c.* (the significant peaks of alkaloid identification are circled in green while water peaks are circled in black).

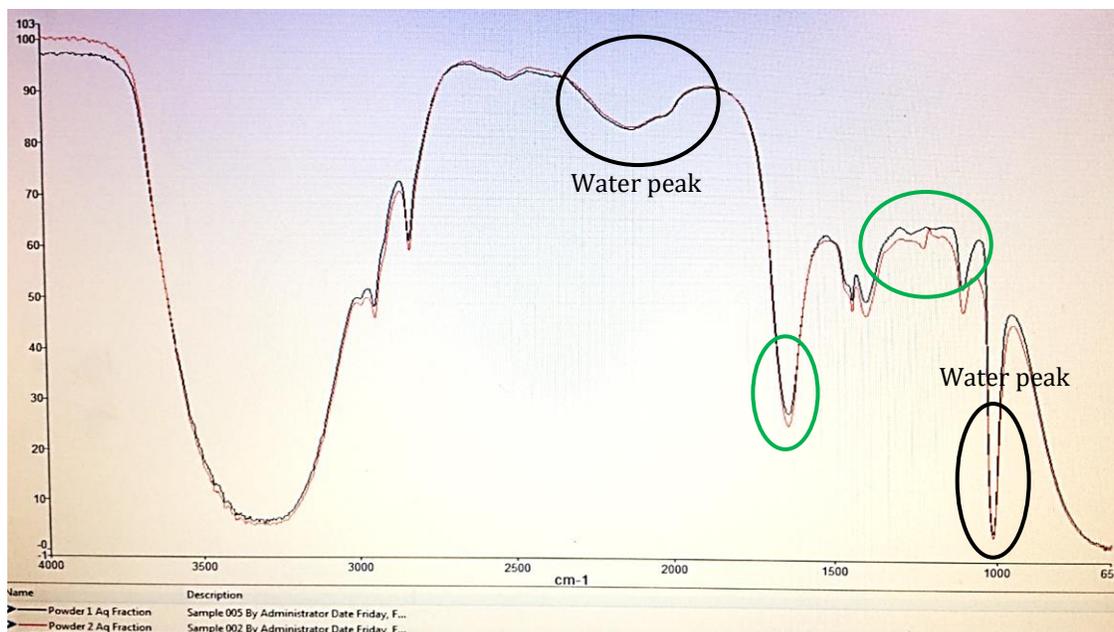


Fig. 4: IR Spectra comparing aqueous fractions of both herbs (obtained from SPE); red line represents *Withania s.* and black represents *Tinospora c.* the significant peaks of alkaloid identification are circled in green while water peaks are circled in black).

b.) Quantification of total alkaloids

The total amount of alkaloids obtained was expressed in grams of Bismuth equivalents since the extract contains a mixture of alkaloids (both known and unknown) with varying molar masses. This is a valid method of analysis since the bismuth ion from Dragendorff's reagent precipitates with alkaloids in a 1:1 ratio as $(BiI_3)(Alk.HI)$.

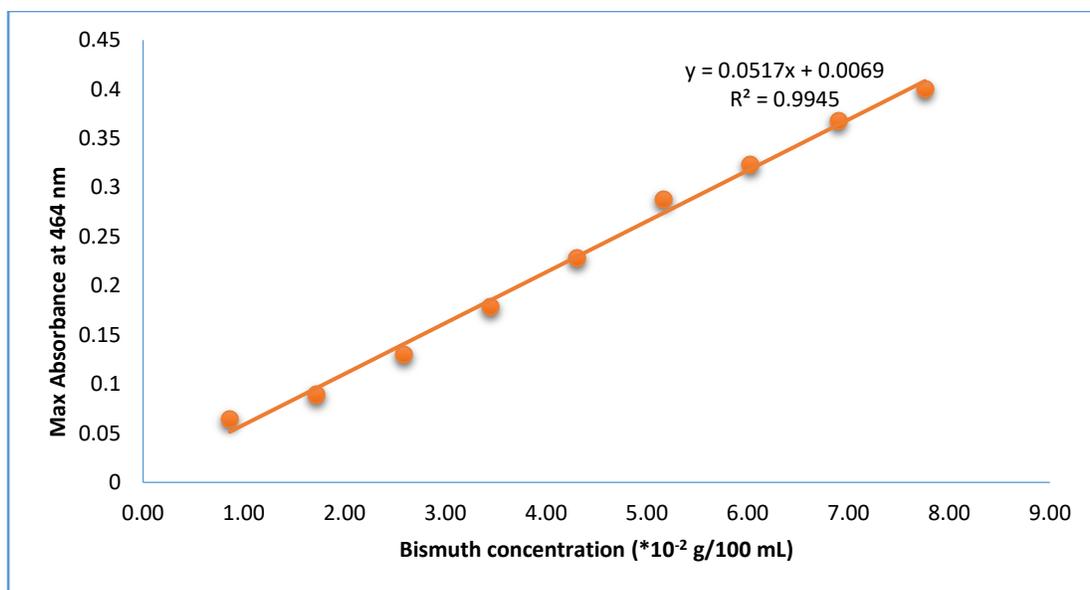


Fig. 5: Standard curve for the quantification of alkaloids (the absorbance of the yellow bismuth-thiourea complex was read at 464 nm).

Sample calculation of Concentration of alkaloids (g Bi equivalent) in aqueous filtrate of *Tinospora cordifolia*:

$$x = \frac{y-b}{m} = \frac{0.633-0.0069}{5.174} = 0.121 \text{ g Bi (equivalent)}$$

$$\frac{0.121 \text{ g Bi}}{\frac{0.5 \text{ mL used}}{2.5 \text{ mL total}} \text{ ppt in HNO}_3} \times \frac{5 \text{ ml used}}{28 \text{ ml total}} \text{ aq filtrate} \times 10 \text{ g powder} = \frac{0.3390 \text{ g Bi equivalents}}{1 \text{ g powder}}$$

c.) Antioxidant activity tests

The amount of antioxidant activity was expressed in Trolox equivalents since Trolox was used as the standard antioxidant of known concentration for the calibration curve.

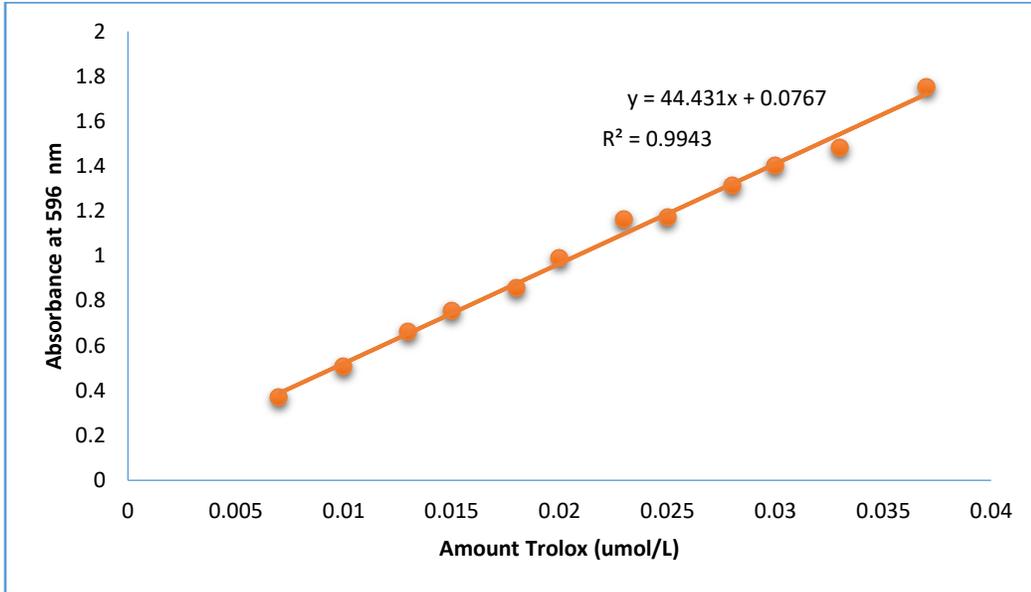


Fig. 6: Standard curve for the FRAP antioxidant assay measuring the absorbance of the blue Fe^{2+} - $(TPTZ)_2$ complex at 596 nm.

Sample calculation of Concentration of antioxidant (in μM Trolox equivalents) in aqueous filtrate of *Tinospora c.*:

$$x = \frac{y-b}{m} = \frac{1.476-0.0767}{44.43} = 0.0315 \mu M \text{ (Trolox equivalent)}$$

$$\frac{0.0315 \mu M \text{ Trolox}}{\frac{10 \mu L \text{ used}}{1500 \mu L \text{ total}} \text{ diluted filtrate}} \times \frac{500 \mu l \text{ used}}{28000 \mu l \text{ total}} \text{ aq filtrate} \times 10 \text{ g powder} = \frac{26.45 \mu M \text{ trolox equivalents}}{1 \text{ g powder}}$$

Tab.1: Summary of results showing total alkaloids (g Bi equivalents) and antioxidants (μM Trolox equivalents) in the aqueous filtrates and fractions from both plants. Aqueous filtrates and fractions are obtained from reflux-extraction and solid phase extraction, respectively.

Plant	Amount of antioxidants (μM Trolox eq/g powder)		Amount of alkaloids (g Bi equivalent/ g powder)		% Ionizable polar alkaloids
	<i>Aq Filtrate</i>	<i>Aq Fraction</i>	<i>Aq Filtrate</i>	<i>Aq Fraction</i>	
<i>Tinospora c.</i>	26.45	19.84	0.3390	0.2052	60.5
<i>Withania s.</i>	11.92	10.14	0.3617	0.2648	73.2

The total ionizable polar alkaloids are the alkaloids that do not bind to the octadecyl column when the aqueous filtrate is passed through the LC18 column. The percentage of these alkaloids was calculated by taking the ratio of the alkaloid amount in fraction sample to that in the filtrate sample as shown:

$$\% \text{ Ionizable polar alkaloids in } \textit{Tinospora c.} := \frac{0.2052}{0.3390} \times 100 = 60.5\%$$

Discussion

Of the two herbs analyzed, *Withania s.* had significantly higher amount of total alkaloids and polar ionizable alkaloids. Based on the structures of the known alkaloids in *Withania s.*, it would be expected that most alkaloid molecules in this herb have small sizes. This might be a possible explanation for the higher amounts of alkaloids precipitated with Dragendorff's reagent. The smaller molecular size (i.e., mass) of the alkaloids results in a higher number of alkaloid molecules per gram of *Withania s.* increasing the number of bound Bi ions (i.e., g Bi equivalents) per gram of *Tinospora c.* assuming that the binding ratio is 1:1. Amount of polar ionizable alkaloids under acidic conditions is found higher in *Withania s.* than of those in *Tinospora c.* when one expects the opposite result looking at the structures (*Tinospora c.* has positively charged quaternary nitrogens already at neutral pH). The unexpected result might be due to the larger molecular size and more extended hydrophobic rings of the alkaloids in *Tinospora c.* Larger hydrophobic portions of *Tinospora c.* molecules are more prone to get caught in the hydrophobic filling material of the SPE column, resulting in more alkaloids being bound to the column. The alkaloids in *Withania s.* have smaller molecules with small hydrophobic rings, hence these would not have been bound to the column. Another possible explanation is that the charged quaternary alkaloids might exist as soluble complex ionic salts in *Tinospora c.* Hence, the nitrogen of the alkaloid could be spatially buried inside the complex in a not easily reachable location by Drangendorff's reagent (DR) resulting in lower amount of alkaloids in *Tinospora c.*

Surprisingly, *Tinospora c.* had the higher antioxidant activity in both aqueous filtrates and fractions. This could be explained by the presence of higher number of antioxidant sites per alkaloid molecule of *Tinospora c.* as compared to *Withania s.*

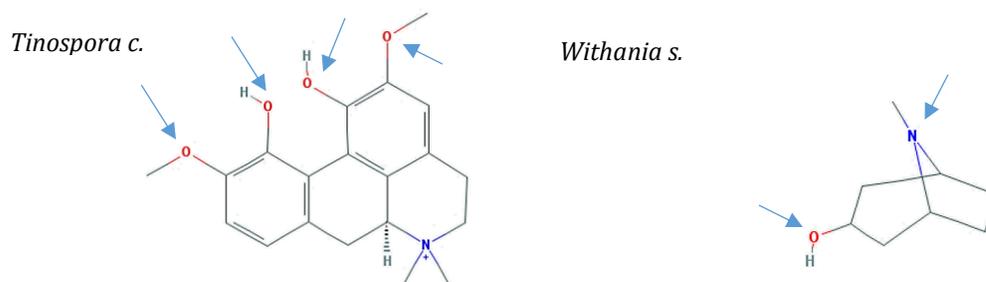


Fig. 3: The two alkaloids from *Tinospora c.* and *Withania c.* showing potential antioxidant sites.

Another possible explanation is that there might be some non-alkaloidal antioxidants that were extracted in the process, which contribute to the antioxidant activity in both plants. *Tinospora c.* might have a higher amount of such non-alkaloidal antioxidants. The possible group of non-alkaloidal antioxidants might be the steroidal lactones and sesquiterpene lactone groups found in *Withania s.* and *Tinospora c.* respectively.

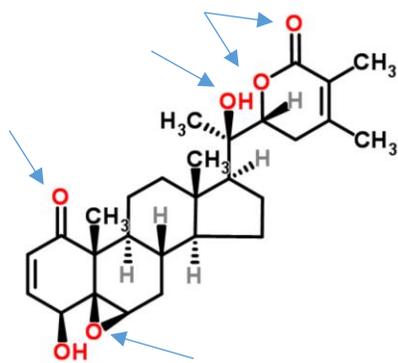


Fig. 7: Withanolide, a steroidal lactone found in *Withania s.*, with potential antioxidant sites indicated with arrows.

Conclusion

This study has shown that a high amount of alkaloids does not necessarily result in higher antioxidant activity; since *Withania s.* was found to have more alkaloids but *Tinospora c.* had the higher amount of antioxidants. The high antioxidant activity in *Tinospora c.* could be a result of the multiple potential antioxidant sites. Therefore, the number of potential antioxidant sites per alkaloid molecule is a more significant governing factor rather than the total number of alkaloid molecules present in the herb.

Other non-alkaloidal organic compounds like lactones (found in both plants) might be contributing to the higher antioxidant activity. Therefore, the higher antioxidant activity in *Tinospora c.* could be a result of the combined effect from the diterpenoid lactones and the alkaloids. *Tinospora c.* might have a higher lactone content than *Withania s.*, which can further explain the common uses of this herb. The diterpenoid lactones found in *Tinospora c.* are well-known organics having fever-reducing and anti-diabetic effects. Hence, it can be concluded that the common uses of these herbs are based on the lactones groups in both plants.

Further studies on quantification of total lactones and their antioxidant activities for both herbs will allow comparison to the findings of this research. Alternatively, modification to this study could be made by optimizing the extraction method such that the maximum possible amount of alkaloids from both plants can be isolated without extracting lactones in the process.

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