

Comparative *in-vitro* antioxidant, anti-inflammatory and anti diabetic activity of standardized polar extracts of *S alata*

Abstract

Background: *S alata* Royle (Gentianaceae) is used in Indian Traditional system of medicine and plant was known for its tonic, febrifuge, laxative and antimalarial properties.

Objective: The aim of this study was to examine the possible *In-vitro* antioxidant, anti-inflammatory, and antidiabetic effects of the ethanolic and aqueous extracts from *S alata*.

Materials and methods: Antioxidant activities of ethanolic and aqueous extracts were determined by ferric reducing power assay (FRAP). The anti-inflammatory activities of both the extracts of *S alata* were studied by antiproteinase inhibition assay and antidiabetic activity was investigated by α -amylase inhibition assay.

Results: The ethanolic extract of *S alata* has shown maximum antioxidant and anti-inflammatory activities when compared to that of aqueous extract. The aqueous extract of *S alata* also exhibited significant ($P < 0.005$) inhibition activities on α -amylase enzyme. The results obtained indicate that the extracts possessed significant level of activity; the highest concentration of extract was high effective as an antioxidant, anti-inflammatory agent and anti-diabetic. However, these effects need to be confirmed using *in vivo* models and clinical trials for its effective utilization as therapeutic agents.

Keywords: *S alata*, traditional, *in-vitro*, antioxidant, anti-inflammatory, antidiabetic, extracts

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Introduction

Plant-based products have been known as both nourishment and medicines since ancient times. From the very beginning of the civilization, there is an outrageous relationship between people and plants. In antiquated period the arrangement of treatment was not enhanced like today. The antiquated individuals used to use several parts of plants in various treatment purposes. Plants were not only utilized as medicine, but also in a number of their daily jobs (e.g., fishing, hunting, etc.) purposes. Eventually, plants are the ultimate care taker of environment in a sense. Since acetylsalicylic acid, a synthetic derivative of salicin produced from willow bark, was first presented in 1897,¹ natural products have become major sources for chemical compounds used as starting materials. A solitary part of plant may comprise of various therapeutic esteems, but it has been proved that direct intake of crude plant is not good, as it contains both essential and nonessential components. The nonessential ones may not be required by the body in mending purposes or in other circumstances; the nonessential components may in actuality be toxic to the body under some cases. Even the intake of the essential components via the crude extract may lead to an improper dose.² Within current days considerable research has been progressed in the exploitation of therapeutic plants, all the rage the therapy of various stress-related disorders caused beside metabolism of oxygen leads to the generation of free radical.³ Free radicals cause weariness of immune system antioxidants, change in gene expression and induce abnormal proteins and add to in excess of one hundred issue in individuals including arthritis, atherosclerosis, ischemia and reperfusion injury of many tissues, central nervous

system injury, diabetes, gastritis, cancer and AIDS. Oxidative stress may have critical impact in the glucose transport protein (GLUT) or at insulin receptor.⁴ The correct part of oxidative stress in the etiology of human diabetes is however not known. Longstanding diabetes is related with modifications in mitochondrial metabolism that outcome in both expanded arrangement of reactive oxygen species (ROS) and disappointment of bioenergetics. Specifically, diabetes causes dysfunction of mitochondria in those tissues very subject to aerobic metabolism, for example, heart, cerebrum and skeletal muscle. The level of mitochondrial failure has been corresponded with the term of diabetes.⁵ Scroungers of oxidative stress may have an impact in lessening the expanded serum glucose level in diabetes and may mitigate diabetes and in addition diminish its auxiliary entanglements.⁶ Scroungers of oxidative stress possibly will undergo an impact in reducing the raised serum glucose level in diabetes and in addition diminish its supporting entanglements. Inflammation is normal reaction of the mammalian body to an assortment of hostile specialists including parasites, pathogenic microorganism, poisonous concoction substances and physical harm to tissue. Among its arbiters, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are vital enzymes that manage inflammatory processes.⁷ The procedure related with the inflammatory reaction are mind boggling yet vital perspectives which have been exploited for screening of anti inflammatory compound are the different elements of neutrophils, the metabolic results of arachidonic acid and the pretended by reactive oxygen species (ROS).⁸ Likewise, macrophages assume a focal part in the advancement of vascular inflammation and the development of atheroma. The action of macrophages is higher

in diabetic populace than that in healthy subjects.⁹ These findings complement the pathogenetic part of high glucose in macrophage actuation amid the process of vascular inflammation. *S alata* Royle (Gentianaceae) is an annual herb usually grows in west and north west himalayas especially in Kashmir to Kumaon, Mussorrie, Dehradun and Nanital area.¹⁰ An imbue of plant was known for its tonic, febrifuge and purgative properties. The plant contains three xanthenes i.e swertiaperennine, swertianin and decussating with properties of curing malaria.¹¹ It likewise contains oleanolic acid, ursolic acid, swertiamarin, amarogentin and mangiferin which indicates good antioxidant activity.¹² The writing study demonstrated meager data accessible on this plant and along these lines incited us to dissect the common ayurvedic plant. The present study involves evaluation of antioxidant activity by FRAP assay, anti-inflammatory activity by antiproteinase assay and antidiabetic assay by alpha amylase assay.

Material and methods

Plant material and extraction

The plant material was procured by an Ayurvedic Pharmaceutical Company, New Delhi and authenticated by Dr. H.B Singh, NISCAIR (National Institute of Science communication and Information Resources) Pusa Gate, New Delhi. The voucher specimen (NISCAIR/RHMD/2013/2185/191) of the test specimen has been deposited in the herbarium of NISCAIR for future advertence. The fresh aerial parts were dried under the shade and powdered in a mixer. The coarse powdered material (500g) of *S alata* was extracted by ethanol and water. Each time before extraction with next solvents, the coarse powdered drug was dried in hot air oven below 50°C. The extracts were evaporated to dryness under reduced pressure with a rotary evaporator (Heidolph) at a temperature of 40°C. The dried extracts were kept in tightly packed container under refrigeration until used for evaluating biological activity.

In-vitro anti-oxidant activity

Reducing power assay

The reducing powers of the extracts were determined by the method.¹³ Different concentration (20, 40, 60, 80, 100 and 200µg/mL) of extracts were prepared in 1mL of distilled water was infused with phosphate buffer (2.5mL, 0.2M, pH6.6) and potassium ferricyanide [K₃Fe (CN)₆] (2.5mL, 1%). The mixture was incubated at 50°C for 20min. A portion (2.5mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000RPM for 10min. The upper layer of the solution (2.5mL) was mixed with distilled water (2.5mL) and FeCl₃ (0.5mL, 0.1%) and the absorbance was measured at 700nm. Ascorbic acid was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the average of three observations. This assay is known as a strong and useful method for estimating a wide concentration range of antioxidant activities and capacities.¹⁴

In-vitro anti-inflammatory activity

Antiproteinase activity

This test was done according to the method depicted by Shinde et al.,¹⁵ with slight modification. The reaction mixtures contained 0.5mL trypsin and chymotrypsin (8.000 Armour units of enzyme activity), 1.0mL 25mM tris HCl buffer (pH 7.4) and 1.0mL extracts of *S alata* (20–200µg/mL) and standard diclofenac sodium (20–200µg/mL). The mixtures were incubated for 5minutes at 37°C. At that point 1.0mL of 0.8% (w/v) casein was added. The mixtures were incubated for another 20minutes. 2.0mL of 70% perchloric acid was added to terminate

the reaction. Cloudy suspension was centrifuged. Absorbance of the supernatant was measured at 280nm against buffer as a blank. Protein inhibitory activity (in %) is calculated as follows:

$$\text{Protein inhibitory activity (\%)} = \left[\frac{100 (\text{O.D. of control} - \text{O.D. of test})}{\text{O.D. of control}} \right] \times 100 \text{ where O.D. is optical density.}$$

In-vitro antidiabetic activity

Alpha-amylase inhibition assay

Alpha amylase inhibitory activity was established by starch iodine method that was originally developed by with slight alterations.¹⁶ In alpha amylase inhibition method 1mL substrate–potato starch (1%w/v), 1ml of drug solution [Acarbose (std drug) / aqueous extract (AQSA)] of five different concentration such as 20, 40, 60, 80, 100 and 200 µg/mL, 1mL of alpha amylase enzyme (1%w/v) and 2mL of acetate buffer (0.1M, 7.2pH) was added. NOTE–Potato starch solution, alpha amylase solution and drug solution was prepared in acetate buffer. The above mixture was incubated for 1h. Then 0.1ml Iodine–iodide indicator (635mg Iodine and 1gm potassium iodide in 250mL distilled water) was added in the mixture. Absorbance was taken at 565nm in UV–Visible spectroscopy. Percent inhibition (%) was calculated as previously published protocol. All the tests were performed in triplicate.

$$\text{Inhibition of alpha–Amylase (\%)} = \frac{(\text{Abs. control} - \text{Abs. sample})}{\text{Abs. Control}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

Statistical analysis

Data were expressed as MEAN±S.E.M. Statistical comparisons between groups were performed by one way analysis of variance (ANOVA) followed by Dunnett test ***p<0.0001, considered extremely significant, **p<0.001 and *P<0.01are considered significant; p<0.05, non significant.

Result

In-vitro anti-oxidant activity

Ferric reducing power assay (FRAP)

The reductive capabilities of extracts of *S alata* were observed and compared with Ascorbic acid. The mean absorbance at different concentration (20–200µg/ml) of ETSA, AQSA as well as standard Ascorbic acid (20–200µg/ml) were calculated and plotted using Graph Pad Prism. The reductive capabilities were found to increase with increasing of concentration and absorbance in various extract as well as standard ascorbic acid. The mean absorbance of AQSA are significantly lower while ETSA extract was comparable with absorbance of Ascorbic acid (p<0.05) and considered not significant. Results are given in Table 1.

Table 1 Absorbance for ferric reducing power assay

Conc.(µg/mL)	Ascorbic acid (nm)	ETSA (nm)	AQSA (nm)
20	2.85±0.04	1.96±0.03	1.16±0.04
40	2.92±0.05	2.13±0.02	1.37±0.06
60	3.20±0.03	2.25±0.01	1.55±0.04

Table Continued

Conc.(µg/mL)	Ascorbic acid (nm)	ETSA (nm)	AQSA (nm)
80	3.33±0.05	3.34±0.01	1.85±0.03
100	3.51±0.10	3.54±0.02	2.36±0.05
200	4.11±0.09	3.85±0.05ns	2.64±0.04***

Each value represents the mean±SD, N=3, when compared to Ascorbic acid and results were analyzed by one way ANOVA followed by Dunnet test. ***p<0.0001, considered extremely significant, **p<0.001 and *P<0.01 are considered significant; p>0.05, non significant

Result for *In-vitro* antiinflammatory activity

Result for antiproteinase activity

The *S alata* crude extracts exhibited significant antiproteinase activity from various extracts. The maximum inhibition was observed from ETSA extract 85.42% as compared to AQSA extract 61.13%. The standard Diclofenac 80.17% showed the maximum antiproteinase inhibitory action as per Table 2. Both the extracts showed significant inhibition in dose dependant manner. The IC₅₀ of ETSA (p<0.001) is considered non-significant as compared to Diclofenac. IC₅₀ are given in Figure 1.

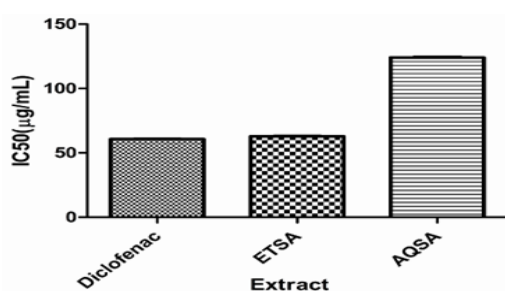


Figure 1 IC50 of S alata extracts.

Table 2 Result for antiproteinase activity

Conc. (µg/mL)	Diclofenac (%)	ETSA (%)	AQSA (%)
20	38.59±0.18	35.40±0.11	14.29±0.11
40	43.35±0.11	40.37±0.20	20.79±0.12
60	49.38±0.14	48.02±0.20	27.46±0.11
80	56.84±0.09	57.65±0.11	34.42±0.18
100	63.52±0.17	63.54±0.20	42.15±0.23
200	80.17±.12	85.42±0.14	61.13±0.14

Each value represents the mean±SD, N=3, when compared to Diclofenac and results were analyzed by one way ANOVA followed by Dunnet test. ***p<0.0001, considered extremely significant, **p<0.001 and *p<0.01, considered significant; ns p>0.05, non significant

Result for *In-vitro* antidiabetic activity

Result for alpha amylase assay

The digestive enzymes of intestine i.e alpha-amylase plays a fundamental role in the carbohydrate digestion. One of the therapeutic approach lessens the post prandial glucose level in blood by the inhibition of alpha-amylase enzyme. These can be an imperative strategy in management of blood glucose. The *in-vitro* α-amylase inhibitory studies demonstrated that *S alata* has well anti-diabetic

activity. The percentage inhibition at 20, 40, 60, 80, 200µg/ml concentration of crude plant extracts shown concentration dependent reduction in percentage inhibition. At a concentration of 20µg/ml of *S alata* extracts ETSA and AQSA showed a percentage inhibition 35.98% and 35.29% respectively and for 200µg/ml extracts showed inhibition of 87.88% and 97.34%. As the result shows ETSA and AQSA extract of *S alata* shows non-significant activity as compared to Acarbose standard drug. At 200µg/ml concentration of AQSA and ETSA extract shows almost similar activity as Acarbose. The IC₅₀ of AQSA is comparable with IC₅₀ of acarbose and found to be not significant and IC₅₀ of ETSA extract (P<0.01) was found to significant but comparable with IC₅₀ of Acarbose (P<0.05). Percentage inhibition are given in Table 3 and IC₅₀ are given in Figure 2.

Table 3 Alpha-amylase inhibition assay for S alata extracts

Conc. (µg/mL)	Acarbose (%)	ETSA (%)	AQSA (%)
20	33.91±0.69	35.98±0.69	35.29±0.34
40	44.98±0.91	43.25±1.24	42.44±1.05
60	52.82±0.72	50.86±0.34	47.52±0.39
80	62.62±0.91	57.90±0.39	56.63±1.39
100	71.28±1.58	63.89±0.52	74.74±0.91
200	87.54±1.03	87.88±1.24	97.34±0.52

Each value represents the mean±SD, N=3, when compared to acarbose, and results were analyzed by one way ANOVA followed by Dunnet test. ***p<0.0001, considered extremely significant, **p<0.001 and *P<0.01 are considered significant; p>0.05, non significant

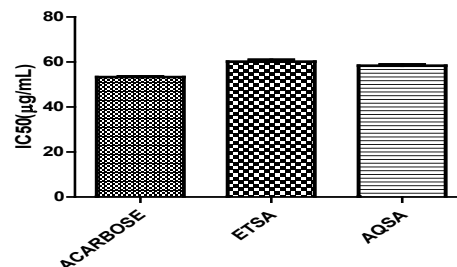


Figure 2 IC50 of S alata extracts.

Discussion

As beforehand announced the reducing power of bioactive compounds was related with antioxidant activity. Along these lines, for the explanation of the relationship between antioxidant effect and reducing power of the phenolics, the assurance of reducing power was a need.^{17,18} Our recognitions are in concurrence with the previous findings of Siddhuraju and Becker, where high content of phenolics in the ethanolic extracts of leaves contrasted with aqueous extract was reported. Our previously reported studies of standardization of extracts and phytochemical estimations reveals significant presence of tannins, saponins, terpenoids, flavanoids, phenols, steroids, carbohydrate and iridoids in *S alata* ETSA extract in contrast AQSA extract contains all the above phytochemicals with the exception of irridoids and terpenes. In addition, TPC (mg of GA/g) and TFC (mg of Quercetin/g) in the various extracts of *S alata* were also determined and found to be in the following decreasing order: ETSA>AQSA>CHSA> PESA.¹⁹⁻²¹

Antioxidant activities of both the extracts of *S alata* were assessed by reducing power assay, which relies upon their capacity to lessen ferric ion to ferrous form. As shown in Table 1, reducing power activity increased in a dose dependent manner. The mean absorbance of AQA is significantly lower while ETSA extract was tantamount with absorbance of Ascorbic acid. In ferric reducing antioxidant power assay (FRAP), the test solution of yellow shade changes to various shades of blue and green as it depends upon the reducing power of each compound. The presence of radicals (i.e antioxidant) causes the conversion of the Fe^{3+} /ferricyanide complex used in this method to the ferrous form. Consequently the development of Prussian blue colour is measured spectroscopically, the Fe^{2+} concentration can be monitored; a higher absorbance shows a higher reducing power.²² The reductive capabilities of ETSA and AQA extracts were detected and compared with Ascorbic acid. The highest reducing power was showed by ETSA extract i.e more antioxidant compounds converted to Fe^{2+} . The reducing power in the extracts were increased and showed that some components in the extract were electron donors that could respond with the free radicals to convert them into more stable products to terminate radical chain reaction. Antioxidants are strong reducing agents and this is principally based on the redox properties of their hydroxyl groups and the structural relationship between different parts of their chemical structure.²³⁻²⁵ The AQA extract gave off an impression of being less effective when compared with ETSA extract. By and large, the higher polyphenols extraction yield relates with the higher antioxidant activity, most likely because of the consolidated activity of the substances in variable concentrations and their high hydrogen atom donating ability. Our findings are all around related with the measure of phenolic constituents present in respective extract. The phenolics shows in ETSA extract are able to terminate the radical chain reaction by converting free radicals to more stable products.

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich wellspring of serine proteinase and are confined at lysosomes. It was already detailed that leukocytes proteinase assume an imperative part in the development of tissue damage during inflammatory responses and huge level of insurance was given by proteinase inhibitors.²⁵ Our examination uncovered eminent antiinflammatory effect of ETSA and AQA extracts. Flavonoids and steroidal terpenes are known to indicate antiinflammatory activity by hindering the COX and LOX frameworks.²⁶ The anti-inflammatory effect of extracts may be attributed to the presence of flavonoid entity inspite of flavanoids and steroids both are seen in both the extracts.²⁷ alpha-amylase plays a crucial role part in the carbohydrate digestion. One of the therapeutic approach of antidiabetics is to reduces the post prandial glucose level in blood by the inhibition of alpha-amylase enzyme. These can be an important strategy management of blood glucose.²⁸ The present finding reveals that both the extracts of *S alata* efficiently inhibits alpha-amylase enzyme *in vitro* in a dose dependent manner.^{29,30} It was proposed that inhibition of the activity of alpha-amylase would postpone the debasement of carbohydrate, which would in turn cause a lessening in the absorption of glucose, as a result the reduction of postprandial blood glucose level elevation.³¹ In alpha-Amylase inhibition method, alpha-Amylase hydrolyses alpha-bond of large alpha linked polysaccharides, for example, glycogen and starch to yield glucose and maltose. This assay is based on the formation of

starch – iodine complex due to inhibition of alpha-amylase. The presence of polyphenols in the aqueous extract from the aerial parts showed a decent outcome in alpha-amylase inhibition assay, suggesting that the extract might be effective in inactivation of this enzyme could be due to precipitation by formation of inactive enzyme-inhibitor complex or enzyme-inhibitor-substrate complex.³² The fundamental driving forces in the complex formation are hydrophobic interactions and hydrogen holding. In hydrophobic interaction, aromatic ring of phenolic compound and a hydrophobic part of proteins are included, while hydrogen holding occurs between hydroxyl groups of polyphenol and H-acceptor groups (NH, OH and COOH) of proteins.³³ The plant showed significant inhibition activity, so further the compound isolation, purification and characterization which is responsible for inhibiting activity, has to be done for the usage of various activities.

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Conflict of interest

Author declares that there is no conflict of interest.

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