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Article

In Vitro Hormone-Sensitive Lipase Inhibitory And Antioxidant Activities, Phytochemical Screening Of Methanolic Extract, And Its Fractions From *Solanum Nigrum*

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ABSTRACT

Solanum nigrum (SN) Linn. (Solanaceae) methanolic extract and its fractions were investigated for their health-benefit properties, in particular with respect to antioxidant activity and inhibitory ability toward key enzyme hormone-sensitive lipase (HSL) with impact in diabetes and obesity. The SN chloroform fraction was the most promising in regard to the antioxidant effects, namely in the scavenging capacity of the free radicals 2,2-diphenyl-1-picrylhydrazyl and 2,2-azinobis[3-ethylbenzthiazoline]-6-sulfonic, as well as the most effective inhibitor of HSL. In addition, the phytochemical screening of the active fraction was also carried out. These results revealed that chloroform fractions of the methanolic extract of SN bears strong HSL inhibitory and antioxidant properties; it can, therefore, be used as a potential antidiabetic herb.

Key words: Hormone sensitive lipase, *Solanum nigrum* Linn, Type 2 diabetes mellitus.

1. INTRODUCTION

Type 2 diabetes mellitus (T2D) is an age-related chronic multifactorial metabolic disorder typically characterized by hyperinsulinemia, dyslipidemia, insulin resistance, and hyperglycemia [1-3]. The disease is often associated with obesity, hypertension, and increased risk of cardiovascular disease. According to the World Health Organization (WHO), T2D affecting 10% population across the world and the situation is most vulnerable in India, China, and the United States with a large number of affected individuals [4]. Due to the forecasted epidemic in T2D, new therapies with few side effects and a robust effect and which address both the insulin resistance and dyslipidemic components of the disease are needed.

Elevated free fatty acids plasma levels (FFAs) are believed to be a major pathogenic factor of insulin resistance and T2D by inhibiting glucose uptake and utilization by muscles [5]. As a consequence, the blood glucose level will increase. Furthermore, chronic hyperglycemia is usually accompanied by abnormalities in lipid metabolism [6]. Thus, in recent years interest has grown in the area of lipid metabolism and its effect on the regulation of glucose control and, in turn, diabetes. Hormone-sensitive lipase (HSL) is a vital enzyme in lipid metabolism as it catalyzes the rate-limiting step in the hydrolysis of stored triacylglycerol, diacylglycerol, monoacylglycerol, and cholesterol esters as well as retinyl esters in adipose tissue [7]. The two products of HSL-mediated hydrolysis are glycerol and FFA; thus, HSL inhibition could potentially decrease plasma levels of FFA, implicated in a variety of pathological conditions [8]. There is an interest in finding compounds that modulate the activity of HSL, as this may shed light on the pathogenesis and potential treatment of human diseases such as diabetes and obesity.

The modern medicines approved by the US Federal Drug Administration to relieve T2D symptoms have severe side effects and drug resistance after prolonged treatment [9]. Consequently, it has become a necessity to develop the new agents that are pharmacologically safe, cost-effective, and immediately available with minimal side effects. Therefore, to avoid this, the WHO has paid greater attention to the development of improved and safer herbal medicines [10-12]. However, medicinal plants and their products that extensively prescribed for the treatment of T2D worldwide have no known scientific base of their activity. Hence, medicinal plants have to be evaluated methodically to test their potential to treat T2D. In this context, some years ago we embarked on a long-term research project aimed at the development of potential agents for T2D. As part of this project, we report herein the extraction and fractionation of *Solanum nigrum* (SN) Linn. and evaluation of their inhibitory activities against HSL target enzyme involved in T2D. Kinetic analysis of inhibition, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis[3-ethylbenzthiazoline]-6-sulfonic (ABTS) radical scavenging activities and cytotoxicity and phytochemical analysis were also described.

2. MATERIALS AND METHODS

2.1. Plant Collection and Sample Preparation

The whole plant of SN (Figure 1) was collected in October 2013 from

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Figure 1: *Solanum nigrum.*

Seshachalam hills of Kadapa and its surroundings a place situated in Andhra Pradesh, India. Dr. A. Madhusudana Reddy, Assistant Professor, Department of Botany, Yogi Vemana University identified the harvested material employed for the purpose. The herbarium specimen with voucher no. YVU-AGD-06 was deposited in the herbarium of Yogi Vemana University. The plant material was air-dried at 25–30°C for 2 weeks, weighed, ground, and sieved into a fine powder. The smooth powder was stored in air-tight container in darkness at −20°C until further use.

2.2. Plant Extraction and Fractionation

Ground plant material (100 g) was extracted thrice with 500 mL of 90% methanol in water by soaking for 2 days. The plant extracts obtained were filtered *in vacuo* through Whatman No.1 filter paper. The combined filtrates were concentrated using rotavapor (Heidolph, Germany) at 30°C. The obtained methanolic extract was fractionated sequentially by different solvents based on polarity, namely chloroform, n-butanol, and water for decreasing of the complexity of extract and for choosing active fraction.

2.3. Extraction of the HSL Enzyme

Isolated fat cells were extracted from rat epididymal adipose tissues as described earlier with minor modification [13]. Briefly, Wistar male rats were sacrificed by cervical dislocation, and their epididymal fat pads were removed quickly and rinsed 3 times in normal saline. The tissue was weighed, minced into small pieces and placed in liquid nitrogen and grinded well to get powder. The resulting powder was treated as follows for each 1.0 g of tissue; 10 mg of collagenase was added. Subsequently, fat cells were liberated from the tissue fragments by gentle stirring with a rod. To this, 3 mL of KRB (pH 7.4) supplemented with 4% BSA were added. The mixture was incubated and agitated in a metabolic shaker over 2 h at 37°C. The resulting suspension was centrifuged for 1 min at 400 g at 20°C. Fat cells floated to the surface while stromal-vascular cells settled at the bottom. Stromal-vascular cells were removed by aspiration. Fat cells were decanted and washed by suspending them in 10 mL of warm (37°C) KRB-BSA solution followed by centrifugation (for 1 min at 400 g at 20°C) and a second round of removing stromal-vascular cells by aspiration. This washing procedure was repeated 2 times.

HSL was extracted from epididymal fat cells as reported earlier with minor modifications [14,15]. Briefly, 1 mL of suspended fat cells (in KRB-BSA solution) was further diluted by 2.5 mL KRB-BSA and incubated at 37°C for 30 min. Subsequently, the suspension was centrifuged at 100 *g* for 1 min to separate the infranatant from the fat cells. For each 1.0 mL of suspended fat cells, a 1.125 mL homogenization buffer (each 100 mL prepared from 50 mM Tris-HCl, pH 7.0, 250 mM sucrose, and 10 µL protease inhibitor cocktails) was added, and the mixture was manually agitated 20 times. The homogenate was centrifuged at 4540 g and 4°C over 10 min. Subsequently, 250 µL of diethyl ether was added to the homogenate and abruptly shaken and centrifuged at 1200 g over 5 min at 4°C. The upper ether layer was aspirated. The subsequent supernatant was used as HSL extract. HSL extract aliquots (0.5 mL) were stored in Eppendorf tubes at −80°C for later use.

2.4. HSL Inhibition by Test Extracts

The inhibition of HSL activity by the prepared extract and fractions was measured using the spectrophotometric assay that measures the release of *p*-nitrophenol as previously described [16]. However, *p*-nitrophenyl butyrate was employed as an HSL substrate at 10 µM in the enzymatic assays instead of 5 mM. Extract of HSL enzyme (100 µL) was added to the reaction mixtures. The volume was completed to 1 mL using phosphate buffer and pre-incubated with each concentration of the tested extract/fraction for at least 10 min at 37°C before adding the substrate. The final concentration of dimethyl sulfoxide (DMSO) was fixed and did not exceed 2.0%; to rule out any potential biological activity by DMSO itself. The percentage of residual activity of HSL was determined for the tested extract/fraction by comparing the lipase activity of HSL with and without the tested extract/fraction by measuring the solution absorbance's spectrophotometrically at a wavelength of 400 nm at three time points: 1, 3, and 6 min. Inhibition of HSL by tested extracts was calculated using the following formula.

% of inhibition = 1-test inclination/Blank inclination×100

Where test inclination is the linear change in absorbance per minute of test material and blank inclination is the linear change in absorbance per minute of blank inclination. The percentage inhibition of HSL activity data was used to evaluate the IC_{50} values. The percent inhibition was plotted against the logarithmic transformation of the corresponding test extract/fraction concentrations for determining the IC_{50} values (concentration required to give 50% inhibition (IC_{50}). The concentration required to give 50% inhibition (IC_{50}) of tested extract/fraction was tabulated in Table 1.

2.5. Kinetic Study on Enzyme Inhibition

Kinetic characterization of enzyme inhibition was performed at different concentrations of the substrate using the above said assay method [16]. Assay mixture (1 mL) contains 200 mM phosphate buffer (pH 7.7), and 100 µL of enzyme extract. Inhibitor with various concentrations 20, 60, and 120 mg/mL was added and incubated for 5 min at 25°C. Then, added 15 µL of substrate at different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mM). Assay without inhibitor was conducted as a control.

The double reciprocal plots of 1/V versus 1/[S] were constructed. To get inhibition constants (Ki_1 and Ki_2), secondary plots were generated with slopes and intercepts versus inhibitor concentrations.

2.6. Antioxidant Activity Assay

2.6.1. ABTS free radical scavenging assay

The ABTS acid radical scavenging activity of chloroform fraction of methanolic extract of the titled plant was evaluated following a procedure developed by Re *et al*., with minor modifications [17]. Mixture of 2 mM ABTS and 2.45 mM potassium persulfate solutions was stored overnight in the dark at room temperature to produce free radicals. Finally, a mixture of 1 mL of ABTS radical solution and 3 mL of pyrogallol solution in ethanol in the concentration range of 10–30 mg/mL were incubated for 30 min in the dark. After the addition of 10 mL of test fraction, change of absorbance was recorded at 734 nm.

Table 1: *In vitro* HSL inhibitory activities and ABTS and DPPH radical scavenging capacities of 90% methanolic extract of SN and their derived fractions

Extract	Concentration	% of Inhibition	IC_{50} values of HSL (µg/mL)	ABTS µmol TE/g	DPPH µmol AAE/g
90% MeOH	5	24.28	36.95±1.08	-	-
	30	43.72			
	90	60.18			
	150	69.88			
	270	80.22			
$CHCl_3$	5	42.95	8.74±0.75	139.62±2.85	216.89±3.78
	30	64.33			
	90	70.22			
	150	87.67			
	270	93.98			
n-BuOH	5	4.28	349.06±15.76	-	-
	30	20.72			
	90	32.18			
	150	39.88			
	270	50.22			
H_2O	5	7.28	1441.79±92.94	-	-
	30	15.72			
	90	22.18			
	150	31.88			
	270	40.22			

HSL: Hormone-sensitive lipase, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2,2-Azinobis[3-ethylbenzthiazoline]-6-sulfonic, SN: *Solanum nigrum*

Trolox was used as a positive control and results were expressed in Trolox equivalents.

2.6.2. DPPH radical scavenging assay

Antioxidant activity of active chloroform fraction of methanolic extract of titled plant was estimated through DPPH radical scavenging capability according to the modified method of Sarikurkcu *et al*. Chloroform fraction (1.5 mL) of plant at concentrations of 50, 200, and 400 µg/mL was added to 9 mL of the DPPH solution (60 mM) [18]. The reaction mixtures were prepared under dim light. After vigorous shaking, the mixtures were incubated in the dark for 30 min. The decrease in the purple color was measured at 517 nm using 96-well microplate reader. Ascorbic acid and methanol were used as positive and negative controls, respectively. The DPPH radical scavenging capacity was expressed as ascorbic acid equivalents.

2.7. Cell Culture and Treatment

Human neuroblastoma SK N SH cells (National Centre for Cell Sciences, Pune, India) were cultured in minimum essential medium (MEM) supplemented with 1 mM non-essential amino acids, 0.5 mM L-glutamine, 0.1 mM sodium pyruvate, and 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere of 5% CO_2. When SK N SH cells reached 80% confluence, they were used in the following *in vitro* experiment.

2.7.1. Cell viability and MTT assay

Cell viability of SK N SH cells was measured by MTT assay as described previously by Jeelan *et al.* [19-21]. Cultures of SK N SH cells (0.2×10^6 cells per well) were seeded into 96-well plate containing in 200 mL of medium supplemented with 10% FBS. The fraction at various concentrations in DMSO was added to wells and plate was placed within a humidified CO_2 incubator with 5% CO_2 at 37°C for 24 h. 20 µL of MTT reagent at a final concentration of 5 mg/ mL were added each well and incubated for an additional 4 h period in a humidified atmosphere. The medium was removed, then insoluble formazan crystals were dissolved in 200 mL of 0.1 N acidic isopropyl alcohol. Calorimetric measurement of MTT reduction was recorded at 570 nm. The optical density of control cells treated with MEM was taken as 100% viability.

2.8. Phytochemical Screening

Phytochemical analysis of secondary metabolites such as alkaloids, flavonoids, saponins, tannins, and terpenoids was performed using standard protocols as described by Trease and Evans [21-24].

2.8.1. Test for alkaloids

For the confirmation of alkaloids in the titled plant, 5mL of methanolic test solution was taken into a test tube, 1.5 mL of 10% HCl was poured into it heated the test tube having the mixture for 20 min, cooled and filtered. The filtrate was divided into two portions. To the first portion, 1 mL of Dragendorff's reagent was added. Formation of a reddish or orange colored precipitate indicates the presence of alkaloids. To the second portion, Mayers reagent was added. Formation of turbid or white precipitate indicates the presence of alkaloids.

2.8.2. Test for steroids

A red color produced in the lower chloroform layer when 2 ml of plant extract was dissolved in 2 ml of chloroform and 2 ml con. H_2SO_4 sulfuric acid was added in it indicates the presence of steroids.

2.8.3. Test for flavonoids

A 5 mL of each test methanolic solution of plant extract was added in a test tube, and 5 mL of dilute ammonia solution was added followed by addition of 1 mL conc. H_2SO_4. A change in color to yellow indicates the presence of flavonoids in the sample.

2.8.4. Test for tannins

A 5 mL of test solution was added with few drops of 0.1% ferric chloride solution. An indication of a brownish-green coloration shows the presence of tannins.

2.8.5. Test for terpenoids

A 5 mL of methanolic test solution was taken in a test tube, then 2 mL of chloroform were mixed, and then 3 mL of sulfuric acid were added. Formation of reddish brown color indicates the presence of terpenoids in the selected plant.

2.8.6. Test for saponins

In a test tube, 5 mL of test solution was boiled with 10 ml water for few minutes and filtrated. The filtrate was vigorously shaken. The persistent froth was observed. Then, 3 drops of olive oil were mixed with the froth and shaken vigorously. The formation of the emulsion indicates the presence of saponins in the samples.

3. RESULTS AND DISCUSSION

Solanum with approximately 1400 species, is one of the largest genera of flowering plants [25]. SN Linn. (Solanaceae), commonly known as European Black Nightshade, is a medicinal plant native to Eurasia, and grows wildly and abundantly intemperate, subtropical, and tropical areas of China and India, as well as in Americas, Australasia, and South Africa [26]. Meanwhile, SN is also an edible plant, which is considered as a nutritious vegetable in several countries, such as India, Indonesia, Malaysia, Nigeria, and Congo as there are abundant nutrients in the plant such as minerals, vitamins, proteins, and certain hormone precursors. Conventionally, SN has long been used to treat pain, inflammation, fever, edema, cough, cold, asthma, skin diseases, dysentery, liver, heart problems, and cancer in many traditional systems of medicine worldwide, including traditional Chinese medicine [27]. Furthermore, the plant possesses a wide spectrum of pharmacological properties and acts as antioxidant, anticancer, antihyperglycemic, antiulcerogenic, hepatoprotective, hypotensive, neuroprotective, nephroprotective, sedative, diaphoretic, diuretic, antituberculosis, and hypolipidemic effects [28]. Recently, many chemicals have been isolated from SN, including steroidal alkaloids, flavonoids, tannins, saponins, glycosides, phenolic, anthocyanin, and polysaccharides [29].

The present study is aimed to assess the biological potentials including anti-HSL and antioxidant activities, kinetics of enzyme inhibition and the phytochemical screening of methanol extract and its derived fractions of SN to develop potent antidiabetic agent.

3.1. Extraction Yields (%)

The shade dried, powdered whole plants of SN were extracted with 90% methanol in water. The obtained crude methanolic extract (SNM) was suspended into the water and fractionated by successive solvent-solvent extraction with chloroform and n-butanol. Dried yield percentage of 90% methanol extract was found to be 18.23. The fractionation of methanolic extract produced the yield percent of chloroform (SNC), n-butanol (SNB), and residual aqueous (SNW) fractions as 5.2, 3.03, and 8.13, respectively. Initially, methanol showed the highest extractive capacity. As indicated by data, different extractive capacities of solvents used for fractionation were in descending order of residual aqueous > chloroform > n-butanol.

3.2. In Vitro Biological Activity

3.2.1. Inhibition of HSL enzyme

To assess the antidiabetic potency of the titled plant, the extract and derived fractions were tested for their HSL inhibitory activity by *in vitro* enzyme assay as per earlier reported methods [16]. The IC_{50} values of tested extract and fractions on HSL are showed in Table 1. Orlistat was used as reference drug and showed IC_{50} value of 2.1 µg/mL. The methanolic extract inhibited the HSL activity by 60.29% at 100 µg/ mL. The IC_{50} value of methanolic extract was determined to be 36.95 ± 1.08 µg/mL against HSL.

Among fractions, the most active was found to be SNC with an IC_{50} value of 8.74 ± 0.75 µg/mL on HSL. From these data, it is evident that the $CHCl_3$ fractions have excellent antidiabetic potency (activities of remaining fractions at various concentrations were showed in Table 1, and dose-dependent inhibitory activity against HSL was showed in Figure 2).

3.2.2. Kinetic study of inhibition

To explore the mode of inhibition of most active fraction enzyme kinetic study was conducted. The initial velocity was measured at different concentrations of the substrates (S) using three different concentrations of the fraction. The reciprocal of the initial velocity (1/v) was plotted against the reciprocal of concentrations of substrates (Lineweaver–Burk plot) to calculate the rate of the enzyme activity, in turn, the mode of inhibition (Figure 3a). At an increasing concentration of the inhibitor, increased slopes and intercepts were noticed in Lineweaver–Burk plots. The data points were intersected in the second quadrant (upper left quadrant) of double reciprocal plots.

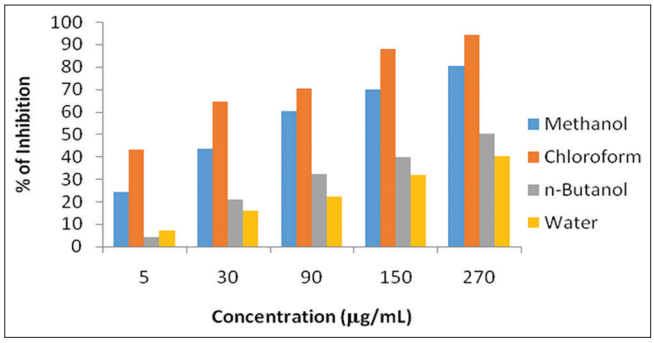

Figure 2: Dose-dependent percent inhibition by 90% methanolic extract and its derived fractions of SN on hormone-sensitive lipase.

Table 2: Qualitative phytochemical screening of the methanolic extracts of plants

Extract	Steroids	Alkaloids	Flavonoids	Tannins	Terpenoids	Saponins
90% MeOH	+++	+++	++	+	++	+++

+++: Rich in quantity, ++: Moderate present, +: Low in quantity, —: Absent

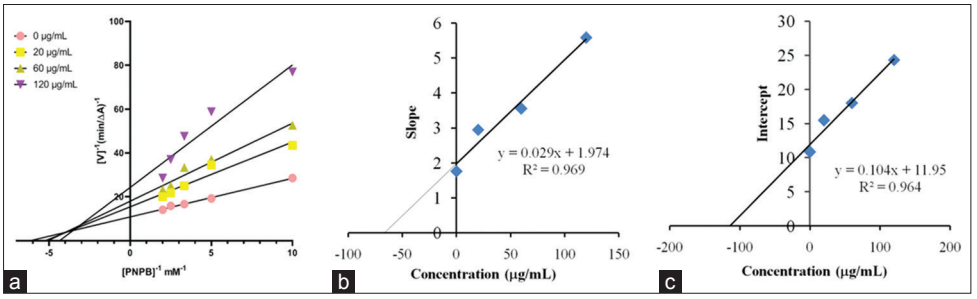

Figure 3: (a) Lineweaver–Burk plot of hormone-sensitive lipase with substrate *p*-nitrophenyl butyrate (PNPB) (0.1–0.5 mM of concentration) in the absence and the presence of different concentrations of the active fraction at 20, 60, and 120 µg/mL. (b) Secondary plots of the Lineweaver–Burk plot of the active fraction; slope versus various concentrations. (c) Intercept versus various concentrations.

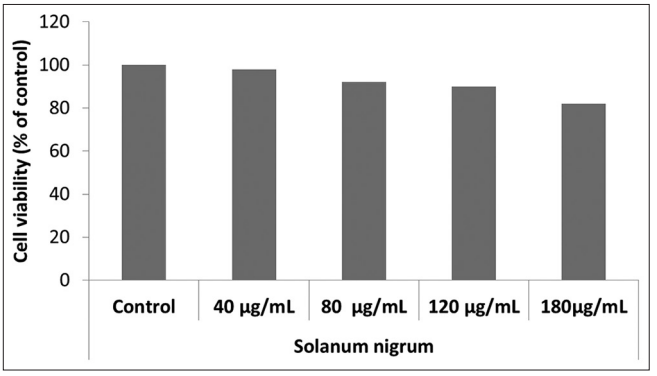

Figure 4: Neurotoxic effects of the active fraction on SK-N- SH cells (human neuroblastoma cell line). Bar chart shows the percentage of cell viability in the presence or absence (control) of indicated concentrations of active fraction.

The observations inferred a mixed type of inhibition for most active fractions. For mixed inhibitor, the inhibitory constants (Ki1 and Ki2) for most active fraction were calculated as 68.06 (binding to the free enzyme) and 114.9 µg/ mL (binding to the enzyme-substrate complex) using secondary plots (Figure 3b and c). The values of inhibition constants revealed that active fraction has a strong affinity to bind to HSL enzyme.

3.3. Antioxidant Activity

The antioxidant activity of active fractions was determined using the free radical DPPH and ABTS by the addition of various concentrations of active fraction. The total data are tabulated in Table 1.

Concerning DPPH assay, the degree of color change of purple-colored DPPH radical solution by electron donation ability of fractions was recorded. In DPPH assay, the active fraction SNC showed strong RSA with ascorbic acid equivalents of 216.89 ± 3.78 µg/mL. However, higher DPPH RSA activity indicates that the phytoconstituents of a tested fraction have capacity to donate hydrogen to a free radical and prevent the potential damage. With respect to ABTS assay, the reduction of ABTS radical cation with a hydrogen-donating capacity of the fraction was measured spectrophotometrically. Fraction SNC had remarkable RSA with Trolox Equivalents of 139.62 ± 2.85 µg/mL.

3.4. Cell Viability

The safety of the extract is absolutely crucial for a successful drug. To grab this, the possible toxicity effects of extract and fractions in the SK N SH cells (human neuroblastoma cell line) were measured using the MTT assay. The evaluation results were summarized in Figure 4. After 24 h incubation of most active fraction at various concentrations (40, 80, 120, and 180 µg/mL) displayed concentration-dependent cell viability.

In all, and based on the results obtained, under the above conditions, the most active fraction showed the cell viability in the range of 82-98%. Interestingly, the cell survival was almost equal to control level at low concentrations 40 µg/mL and was thus nontoxic to SK N SH cells.

3.5. Phytochemical Analyses

Phytochemicals such as polyphenols, flavonoids, alkaloids, steroids, terpenoids, and tannins that are obtained from plant sources have excellent potential to combat chronic diseases besides promising health-promoting properties while acting in combination. Thus, the phytochemical analysis reveals significant information about the medicinal value of plants [30].

Phytochemical screening in the present study has revealed the presence of steroids, alkaloids, flavonoids, saponins, tannins, and terpenoids. Among screened constituents, alkaloids, and steroids were found significantly higher in the active fraction of titled plant. The secondary metabolites such as flavonoids and terpenoids were found to be moderate, whereas low tannins.

Alkaloids have a wide range of pharmacological properties including antimalarial, antiasthma, and anticancer properties as reported (Table 2) [31].

4. CONCLUSION

In the light of our findings, it can be concluded that methanolic extract and its derived chloroform fraction of plant screened herein exhibited high inhibitory activity against HSL enzyme. The active chloroform fraction has also showed high antioxidant potential and cell viability in neuronal cells. To the best of our knowledge, we herein divulge the first report on HSL inhibitory activity of SN. In conclusion, the present study demonstrates the potential of SN as a therapeutic remedy for the

treatment of T2D. The study warrants further investigations to isolate and characterize the active substances in this plant and to explore its potential in combating T2D.

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